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**VPU-MEDIATED INTRACELLULAR TARGETING OF HIV-1 CORE
PROTEIN PRECURSOR PR55^{GAG} AND ASSOCIATION OF PR55^{GAG}
WITH LIPID MICRODOMAINS**

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ABSTRACT

HIV-1 has been suggested to use lipid rafts for assembly and budding. Lipid rafts can be separated from the bulk membranes by extraction with non-ionic detergents, such as Triton X-100 at cold, followed by a density gradient centrifugation. The analysis of intracellular Pr55^{gag} on iodixanol density gradients yielded intermediate density buoyant complexes while raft-associated proteins floated to the light-density fractions. Extracellular virus-like particles (VLPs) showed a similar intermediate flotation pattern after TX-100 extraction suggesting that Pr55^{gag} was not a genuine raft-associated protein. The lipid analysis of TX-100 extracted VLPs suggested that the intermediate buoyant complexes were largely devoid of membrane cholesterol and phospholipids. We also tested the extractability of the membranes with Brij98 which has been shown to detect rafts at physiological temperatures. Our analyses showed that intracellular Pr55^{gag} as well as VLPs were largely resistant to Brij98-extraction. We concluded that Pr55^{gag} associates with lipid microdomains distinct from the classical TX-100-resistant lipid rafts.

We also analyzed the intracellular targeting of Pr55^{gag}. It has been under an intense debate at which cellular membrane the productive assembly takes place, as Pr55^{gag} has been seen to accumulate at both the internal membranes and the plasma membrane depending on the cell type. To resolve this issue, we performed our analyses in HeLa H1-cells, where the Pr55^{gag} can be found both at the plasma membrane and the internal membranes. We employed pulse-chase studies and a subcellular fractionation assay by which an efficient separation of the plasma membrane from the internal membrane fraction is achieved. The kinetic analyses revealed that in the HeLa H1-cells newly synthesized Pr55^{gag} is exclusively targeted to the plasma membrane. To our surprise, the plasma membrane-associated Pr55^{gag} was subsequently endocytosed, if the viral accessory protein Vpu was defective in our proviral construct. Our work was the first to implicate that Vpu had an important role in the subcellular localization of Pr55^{gag}.

We probed by which mechanism the Pr55^{gag} is taken up into the cells in the absence of Vpu. Our analyses implicated that neither clathrin-mediated endocytosis nor macropinocytosis was involved in Pr55^{gag} uptake. In contrast, the cholesterol depletion affected the uptake of Gag thus suggesting the possibility that cholesterol-mediated uptake might be involved. However, cholesterol depletion had a more pronounced effect if it was applied before the maximal membrane insertion and at least some level of the higher-order multimerization of Pr55^{gag} were achieved. This implicated that the cholesterol-dependent uptake pathway was only indirectly associated with the endocytosis of Pr55^{gag} as cholesterol depletion most likely interfered with Gag multimerization and virus assembly.

LIST OF PUBLICATIONS

This thesis is based on following papers, which will be referred to in the text by their Roman numerals:

- I. Holm K, Weclawicz K, Hewson R, Suomalainen M. 2003. Human Immunodeficiency Virus Type 1 Assembly and Lipid rafts: Pr55^{gag} Associates with Membrane Domains That Are Largely Resistant to Brij98 but Sensitive to Triton X-100. *J. Virol.* 77(8): 4805-4817.
- II. Harila K, Prior I, Sjöberg M, Salminen A, Hinkula J, Suomalainen M. 2006. Vpu and Tsg101 Regulate Intracellular Targeting of the Human Immunodeficiency Virus Type 1 Core Protein Precursor Pr55^{gag}. *J. Virol.* 80(8):3765-3772.
- III. Harila K, Salminen A, Prior I, Hinkula J, Suomalainen M. The Vpu-regulated endocytosis of HIV-1 Gag is clathrin-independent. 2007. *Virology* 369(2):299-308.

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LIST OF ABBREVIATIONS

AIDS	acquired immunodeficiency syndrome
AP	adaptor protein
APOBEC3G	apolipoprotein B mRNA editing enzyme
	catalytic polypeptide-like 3G
β -TrCP	beta transducine-repeat containing protein
BHK	baby hamster kidney
BSA	bovine serum albumin
CA	capsid
CD	cluster of differentiation
CHMP	charged multivesicular body protein
CIE	clathrin-independent endocytosis
CLASP	clathrin-associated sorting protein
CME	clathrin-mediated endocytosis
CHC	clathrin heavy chain
DC-SIGN	dendritic cell-specific intracellular adhesion molecule 3-grabbing non-integrin
EE	early endosome
EIAV	equine infectious anemia virus
EM	electron microscopy
ER	endoplasmic reticulum
ESCRT	endosomal sorting complexes required for transport
Env	envelope protein
Gag	group specific antigen
GEEC	GPI-anchored protein-enriched early endosomal compartment
GPI-anchor	glycosylphosphatidylinositol anchor
HIV-1	human immunodeficiency virus type 1
HIV-2	human immunodeficiency virus type 2
HRS	hepatocyte growth factor-regulated tyrosine kinase substrate
IN	integrase
KDa	kilo Dalton
LTR	long terminal repeat
MA	matrix
MES	2-[N-morpholine]ethanesulfonic acid
MHC	major histocompatibility
MHR	major homology region
mRNA	messenger-RNA
MVB	multivesicular body
NC	nucleocapsid
Nef	negative factor
ORF	open reading frame
PBS	phosphate-buffered saline
PFU	plaque forming unit
PI(3)P	phosphatidylinositol-3-phosphate
PI(4,5)P2	phosphatidylinositol-4,5-bisphosphate
PM	plasma membrane

PNS	post nuclear supernatant
Pol	polymerase
POSH	plenty of SH3-domains
PR	protease
Pr55 ^{gag}	Gag precursor protein
Rev	regulator of virion expression
RNAi	RNA interference
RRE	rev responsive element
RT	reverse transcriptase
RTC	reverse transcription complex
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SFV	Semliki forest virus
siRNA	small interfering RNA
SIV	simian immunodeficiency virus
TASK-1	Twick-related acid-sensitive K ⁺ channel 1
Tat	transactivator protein
TAR	trans-activating response element
TEM	tetraspanin-enriched microdomain
TGN	trans Golgi network
TM	transmembrane
TR	transferrin receptor
Tsg101	tumor susceptibility gene 101
Ub	ubiquitin
TX-100	triton X-100
Ubp	Vpu binding protein
UNAIDS	Joint United Nations Programme on HIV/AIDS
Vif	viral infectivity factor
VLP	virus-like particle
Vpr	viral protein R
VSV	vesicular stomatitis virus

1 INTRODUCTION

1.1 MOLECULAR BIOLOGY OF HIV-1

1.1.1 The virus

Viruses are obligate parasites that are critically dependent on the functions provided by a living cell for their replication. Virus particle (the virion) is essentially a gene-transfer vehicle that is needed for the transportation of the viral genome from one cell to another and is thus the means of the spreading of infection within an individual and between individuals.

To fulfil their genome transport function viruses encode a set of structural proteins that are capable of forming new viral particles together with newly synthesized viral genomes. Structural proteins assemble into a shell-like capsid structure wherein the viral genome is enclosed. The capsid is supposed to protect the viral genome from harsh conditions in the extracellular environment and deliver the genome safely into a new cell. Also, for some viruses capsid structure provides a suitable environment to start a new replication cycle by concentrating essential viral components to the site of initial steps of multiplication. In addition to structural proteins, some viruses (e.g. retroviruses) encode enzymatic proteins and accessory proteins that are needed for successful replication. The viral envelope, a host cell derived lipid bilayer is an essential structural component of some virus families. Viruses that are not surrounded by a lipid envelope are referred to as naked viruses.

As viruses are intracellular parasites they have evolved to exploit host cell machinery for their multiplication and spreading. Virtually all the steps in the virus life cycle are dependent on the interactions with the host cell components in a way or another. Host cell surface proteins, carbohydrates and lipids are used by viruses to attach and enter into the susceptible cells. Then, the host cell nucleic acid and protein synthesis machineries are then taken over by viruses in order to direct the synthesis of viral genomes and proteins. After synthesis, the viral structural proteins must assemble into the appropriate capsid structures with all essential components included and nascent virus particles must be released from the host cells.

Although the viral structural proteins in general contain all the information needed for the assembly of new viral particles, it has become evidently clear that host cell components are as well needed to orchestrate the assembly process and the release of progeny of virions. During past few years, at least in the case of retroviruses, the knowledge of interactions between host cell components and assembling virus particles has expanded enormously. However, still much controversy has remained concerning exact molecular interactions required for successful particle assembly and productive release.

1.1.2 HIV-1 and AIDS

The first reports of a new clinical syndrome among previously healthy homosexual men in the United States were published 1981 (Gottlieb et al. 1981). Two years later the causative agent of this syndrome, an acquired immunodeficiency syndrome (AIDS), was isolated (Barre-Sinoussi et al. 1983, Gallo et al. 1984) and later named as Human Immunodeficiency Virus type-1 (HIV-1). In 1986 a new HIV type, now referred to as HIV-2 was isolated (Clavel et al. 1986).

HIV-1 comprises of at least of three, possibly four distinct virus groups, termed M (Main), O (Outlier), N (non-M-non-O) and P (designation pending) (Plantier et al. 2009) with the predominant M-group of viruses being responsible for over 90% of reported HIV/AIDS cases. The group M is further divided into distinct genetic subtypes named by the letters A to K. Subtypes are classified on the basis of the 20-30% interclade differences that are present in envelope (*env*) nucleotide sequences (Louwagie et al. 1993). In addition to subtypes mentioned, several intersubtype mosaic viruses generated by genetic recombination have been described (Laukkanen et al. 2000, Liitsola et al. 1998, Robertson et al. 1995).

Current evidence suggests that both HIV-1 and HIV-2 entered the human population as a result of multiple cross-species transmission events from simian immunodeficiency virus (SIV)-infected non-human primates (Hahn et al. 2000). HIV-1 is most closely related to SIVcpz isolated from the chimpanzee subspecies *Pan troglodytes troglodytes* (Gao et al. 1999) while it is believed that HIV-2 is a result of transmission of SIV from sooty mangabeys (SIVsm) to humans (Gao et al. 1994, Hirsch et al. 1989). The oldest confirmed HIV-1 positive human blood sample dates back to 1959 and to central Africa where HIV-1 has been thought to have originated (Zhu et al. 1998). Careful evolutionary analysis of this sample estimated that last common ancestor of M-group viruses arose near year 1930 (Korber et al. 2000). Based on another sample from Kinshasa 1960 it was found that different subtypes were already well-established at 1930's implicating that both viruses analysed from these samples evolved from a common ancestor circulating in Africa near the beginning of the 20th century (Worobey et al. 2008).

At the end of year 2009, it has been estimated that approximately 33.5 million people around the world are living with HIV (AIDS epidemic update 2009, UNAIDS) (http://data.unaids.org/pub/Report/2009/JC1700_Epi_Update_2009_en.pdf). Every year there are nearly 3 million new infections taking place which makes around 8000 newly infected persons each day. Approximately two million people lost their lives during the year 2008 because of AIDS. The most affected regions are Sub-Saharan Africa (representing 67% of HIV infections worldwide) and Caribbean, where 5.2 % and 1.0 % of the adult population carries HIV infection respectively. In western and central Europe there were approximately 850 000 infected individuals at the end of 2008 (UNAIDS). According to the Swedish Institute for Infectious Disease Control there were 8935 reported HIV-infections in Sweden by the end of 2009 (<http://www.smittskyddsinstitutet.se/statistik/hivinfektion/>). The corresponding figure in Finland was at end of March 2011 2809 reported HIV cases (www.thl.fi).

1.1.3 Retroviruses

Retroviruses are enveloped single-stranded RNA viruses that replicate through a DNA intermediate by virtue of a virus encoded RNA-dependent DNA polymerase, also called reverse transcriptase (RT). According to the International Committee on Taxonomy of Viruses (<http://www.ncbi.nlm.nih.gov/ICTVdb/Ictv/index.htm>) the family of *Retroviridae* is divided into seven distinct genera as shown in Table 1. The first three genera comprises of simple retroviruses as they possess only three open reading frames (ORFs), named *gag*, *pol* and *env*. These abbreviations stand for the group-specific antigen (*gag*), polymerase (*pol*) and envelope (*env*), respectively. The last four genera are considered as complex retroviruses as they carry additional ORFs along to ones carried by the simple retroviruses. These additional ORFs vary between different complex retroviruses. As seen in the examples of each genera of *retroviridae*, this family of viruses has a broad host range.

Genus	Example
Alpharetrovirus	Avian leucosis virus
Betaretrovirus	Mouse mammary tumour virus
Gammaretrovirus	Xenotropic murine leukemia-related virus
Deltaretrovirus	Human T-lymphotropic virus
Epsilonretrovirus	Walleye dermal sarcoma virus
Lentivirus	Human immunodeficiency virus type-1
Spumaretrovirus	Human foamy virus

Table 1. The family of *retroviridae*.

1.1.4 Virion architecture and gene products of HIV-1

As shown in table 1, HIV-1 is a member of the lentivirus genus of the *Retroviridae* family (Chiu et al. 1985) (Latin; *lentus*, slow). HIV-1 virions are spherical in shape and have a diameter of approximately 110 nm. An infectious virion contains two identical copies of single-stranded RNA, about 9500 bases in length each with 5' cap structure and 3' poly-A-tail. Genomic RNA has a positive polarity, i.e. it can directly function as a messenger-RNA (mRNA).

Viral genome is surrounded by a protein capsid structure which consists of *gag*-gene products. The dimeric RNA is incorporated into virus particles via interactions of zinc finger motifs in nucleocapsid (NC)-domain of the Gag protein and the cis-acting packaging sequence located in the untranslated region of the genomic RNA (Berkowitz et al. 1996). The outermost layer of a virus particle is composed of a host cell-derived lipid envelope. Viral surface glycoproteins (spike proteins) are embedded in the envelope together with proteins acquired from the host cell membranes.

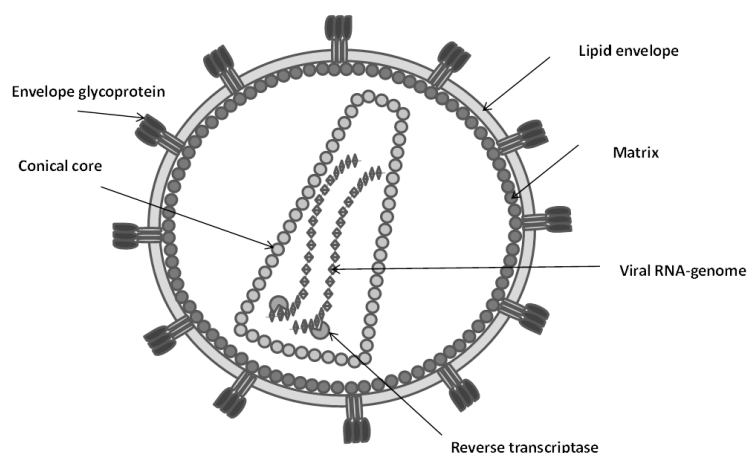


Fig.1. HIV-1 virion.

The genomic structure of HIV-1 is similar to that of other retroviruses, including genes *gag*, *pol* and *env* encoding for structural and enzymatic proteins. As distinct from simple retroviruses, HIV-1 encodes two regulatory proteins (Tat and Rev) and several accessory proteins (Nef, Vif, Vpr and Vpu). The *gag* gene encodes for the major structural protein precursor (Pr55^{gag}) which is later cleaved to form mature virion core proteins matrix (p17, MA), capsid (p24, CA), nucleocapsid (p7, NC) and p6-proteins. The *pol* gene encodes for the precursor of three enzymatic proteins, protease (PR), reverse transcriptase (RT) and integrase (IN). In common with other retroviruses *pol* is translated from the genomic length viral mRNA to produce a polyprotein Pr160^{Gag-Pol}. The *gag* and *pol* genes overlap and the *pol* gene is in -1 reading frame relative to that of *gag*. A ribosomal frameshift which occurs at a low frequency (~5%) allows expression of the *pol* gene (Jacks et al. 1988).

Viral membrane proteins are encoded by the *env* gene. Envelope proteins are translated as a precursor protein gp160 which is subsequently cleaved by cellular proteases into mature surface (gp120, SU) and transmembrane (gp41, TM) subunits. *Env* gene products are transported to the cellular membranes via the secretory pathway (Doms et al. 1993) and these proteins are heavily glycosylated (Freed et al. 1990, Mizuochi et al. 1990). The mature form of the envelope glycoprotein is critical for receptor recognition and fusion of the virion envelope with the host cell plasma membrane (Apte and Sanders 2010).

The major receptor for HIV-1 is a cellular transmembrane protein CD4 (Lifson et al. 1986). In addition to CD4, two additional co-receptors have been described for HIV-1, namely chemokine receptors CCR5 and CXCR4 (Doranz et al. 1997). In addition to receptor recognition and membrane fusion the *env* precursor gp160 also contributes to downregulation of the surface expression of CD4 by blocking the journey of newly synthesized CD4 at the endoplasmic reticulum (Crise et al. 1990, Willey et al. 1992b).

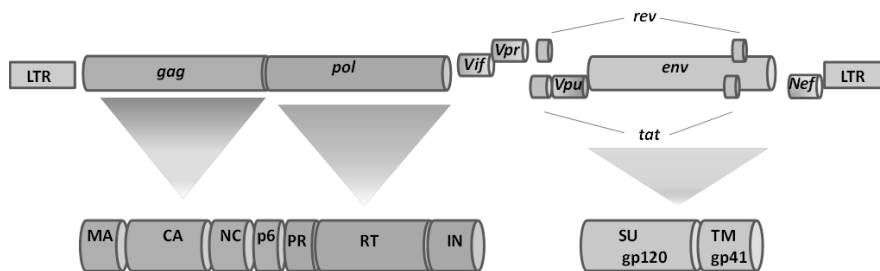


Fig.2. Genomic organization of HIV-1.

HIV-1 genome is flanked by 5' and 3' long terminal repeat (LTR) sequences. The major function of LTR sequences is to provide signals recognized by the cellular transcription machinery. LTR domains have adopted complex secondary structures including several stem loop-folded domains. In addition to recognition sequences for cellular transcription complexes, LTR also contains an enhancer sequence called transactivating response element (TAR), which is a binding site for a virally encoded transactivator protein Tat. Tat increases the production of full-length viral mRNAs from 5'LTR up to several hundred fold and is consequently essential for the viral replication (Pumfery et al. 2003, Romani et al. 2010). Besides Tat, another regulatory protein, Rev, is encoded by the HIV-1 genome. This protein directs the transport of incompletely spliced or unspliced viral transcripts from the nucleus to the cytoplasm for translation. Rev functions as a homo-oligomeric complex by binding to the Rev responsive element (RRE)-site located in the *env* coding region. The nuclear export signal in Rev interacts with host proteins, such as Exportin1/Crm1, that are essential cellular mediators of nuclear export (Fukuda et al. 1997).

Nef

Nef is a membrane-associated, N-terminally myristoylated 27-35 kDa protein that is critical for the high levels of viremia and the progression of HIV-infection to AIDS (Deacon et al. 1995, Kestler et al. 1991, Kirchhoff et al. 1995). Nef is abundantly produced at early stages of virus life cycle and the global effect of this protein is enhancement of viral replication and infectivity (Chowers et al. 1994, Miller et al. 1994). At molecular level, Nef has been hypothesized to function as a molecular adaptor that is capable of altering multiple cellular pathways, such as protein trafficking events, signal transduction and apoptotic cascades via various protein-protein interactions thus contributing to HIV-1 pathogenesis (Foster and Garcia 2008, Roeth and Collins 2006).

The first and comprehensively characterized function of Nef was the ability to downmodulate the surface expression of CD4, the primary receptor of HIV-1 (Anderson et al. 1993, Garcia and Miller 1991). Nef has been shown to induce an adaptor protein 2 (AP-2)-mediated capture of cell surface-associated CD4 to clathrin-coated pits leading to enhanced internalization of CD4 to an acidic compartment for degradation (Chaudhuri et al. 2007, Craig et al. 2000, Lindwasser et al. 2008, Piguet

et al. 1998, Piguet et al. 1999). The removal of CD4 from the cell surface facilitates the incorporation of the viral envelope proteins into the virions, enhances the release of particles and increases the virus infectivity (Lama et al. 1999, Ross et al. 1999).

In multiple studies, the surface expression of various other membrane-associated proteins, such as MHC-I, MHC-II, CD8, co-stimulatory molecules CD80 and CD86 has been also shown to be downregulated by Nef (Chaudhry et al. 2005, Le Gall et al. 2000, Schwartz et al. 1996, Stove et al. 2005, Stumptner-Cuvelette et al. 2001). The downmodulation of MHC-I molecules from the cell surface or early in the secretory pathway by Nef renders infected cells less susceptible for the attack by cytotoxic CD8+ T-lymphocytes by preventing the effective antigen presentation and thereby promoting the immune evasion of infected cells (Kasper et al. 2005, Yang et al. 2002). The withdrawal of MHC-II from the surface of professional antigen presenting cells, such as dendritic cells and macrophages have been implicated to have an adverse effect on the induction of antiviral immune responses (Stumptner-Cuvelette et al. 2001).

In contrast to downregulation of cell surface-associated molecules, some membrane proteins, such as lectin DC-SIGN (Dendritic Cell-Specific Intercellular adhesion molecule 3-Grabbing Nonintegrin) are specifically retained at the plasma membrane of an infected cell by Nef (Sol-Foulon et al. 2002). Early studies proposed that DC-SIGN-expressing dendritic cells bind glycans on HIV Env and carry viral particles as stowaways to the adjacent T-cells and even to peripheral lymphoid tissues that harbour populations of HIV susceptible cells without being productively infected themselves (*trans* infection) (Geijtenbeek et al. 2000, Kwon et al. 2002). However, recent data have expanded the consequences of HIV-DC-SIGN interaction. It has been suggested that DC-SIGN can promote the infection of dendritic cells themselves and progeny virions released can then infect nearby target cells (*cis* infection). Furthermore, binding of HIV to DC-SIGN has been suggested to trigger signal transduction events that promote the production of immunosuppressive cytokine IL-10 which compromises the maturation of dendritic cells (Shan et al. 2007, Tsegaye and Pohlmann 2010).

Nef is known to interfere with various intracellular trafficking pathways by interacting with a number of proteins associated with endocytic transport pathways, such as clathrin adaptor protein complexes (APs) (Craig et al. 2000, Janvier et al. 2003). These interactions have been shown to alter the morphology of early endosomal compartment and affect the multivesicular body (MVB) biogenesis (Costa et al. 2006, Madrid et al. 2005). Besides interactions with endocytic trafficking apparatus Nef has a complex role in modulating multiple signal transduction events and apoptosis pathways in HIV-1 infected as well as in bystander immune cells. By altering signal transduction cascades originating from T-cell surface Nef modulates the activity of CD4+ T-cells, their chemotactic properties via inhibiting actin remodelling (Fackler et al. 2000, Greenway et al. 2003, Pulkkinen et al. 2004, Stolp et al. 2009, Stolp et al. 2010) and controls for apoptotic events within infected cells and in nearby microenvironment (Greenway et al. 2002, Wolf et al. 2001, Xu et al. 1999). Taken together, by means of inducing alternations in intracellular milieu and

membrane trafficking Nef is capable of both directly and indirectly enhance the pathogenic effects of HIV-1 infection.

Some studies have proposed additional roles for Nef. It has been shown that Nef increases the transcription of cholesterologenic enzymes and the biosynthesis of cholesterol. Nef binds to the newly synthesized cholesterol and transports it to the site of virus assembly. Virions produced in the presence of Nef have a high content of cholesterol and are more infectious than virions released from cells infected with a *nef*-defective virus (Zheng et al. 2003). In another study, Nef was found to specifically interact with Gag-Pol. It was speculated that interaction of Nef with HIV-1 structural proteins leads to the entrapment of these proteins to the cholesterol-enriched assembly sites (Costa et al. 2004).

Vif

Viral infectivity factor (Vif) has been characterized as improving the infectivity of HIV-1 virions by overcoming a host cell restriction, mediated by apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G (APOBEC3G). APOBEC3G belongs to a family of cytidine deaminases that remove an amino group from cytosine bases on single-stranded DNA hence creating uracil bases (Yu et al. 2004). Activity of APOBEC3G was speculated to introduce hypermutations into the viral nucleic acid during the minus strand synthesis in order to block HIV-1 replication in target cells (Sheehy et al. 2002, Zhang et al. 2003). On the other hand it has been reported that APOBEC3G-induced deamination actually had little effect on viral cDNA synthesis, but promoted the degradation of newly synthesized viral cDNAs before the integration of the provirus into the target cell genome (Mariani et al. 2003). According to a general consensus, Vif physically interacts with APOBEC3G and prevents its incorporation into virions (Goila-Gaur and Strebel 2008). It has been also suggested that Vif accelerates the degradation of APOBEC3G by ubiquitin ligase complex and proteasomes thus reducing the intracellular pool of APOBEC3G available for packaging into virus particles (Mehle et al. 2004, Sheehy et al. 2003, Yu et al. 2003). However, these implications have been also recently challenged (Dang et al. 2008, Kao et al. 2007, Opi et al. 2007).

Vpr

As with other HIV-1 accessory proteins, the viral protein R (Vpr) has been assigned for many diverse functions. Vpr, a 14 kDa protein is readily packaged into assembling virus particles through interactions with LXXLF-motif located at the C-terminal part of Pr55^{gag} p6-domain (Bachand et al. 1999, Kondo and Gottlinger 1996). Vpr enhances the replication of HIV-1 in differentiated macrophages which is attributed to the activity of Vpr in facilitating the import of HIV-1 preintegration complex (PIC) from the cytoplasm to the nucleus through nuclear pores early in the virus life cycle (Popov et al. 1998, Vodicka et al. 1998). Vpr also enhances the replication of HIV-1 by transactivating the promoter region in the viral LTR and by arresting the cell cycle to the G2-phase (Goh et al. 1998, Kino and Pavlakis 2004). The latter function is executed by Vpr through inactivation of cell division cycle protein (Cdc25C) that controls the G2/M transition (Elder et al. 2001, Goh et al.

2004). Other major functions reported for Vpr include the induction of intrinsic apoptosis pathway in different cell types, suppression of immune responses, facilitation of reverse transcription, reduction of HIV-1 mutation rate and contribution to cytopathogenicity via an ability to form ion channels on the membranes (Romani and Engelbrecht 2009).

Vpu

Vpu is a 16 kDa (81-residue) constitutively phosphorylated protein that is expressed in the late stage of virus replication from a bicistronic *vpu-env* mRNA. Vpu is an integral membrane protein consisting of an N-terminal membrane anchor (residues 1-27) and C-terminal cytoplasmic domain. Vpu homo-oligomerizes at cellular membranes (Strebel et al. 1989) forming monovalent cation selective ion channels (Schubert et al. 1996b). Vpu is not incorporated into HIV-1 particles implicating that Vpu action must be executed within an infected cell. *Vpu* gene is found exclusively in HIV-1 and two strains of SIVs (SIV_{cpz} and SIV_{gsn}) but not in other lentiviruses such as close relative HIV-2 (Bour and Strebel 2003, Bour and Strebel 2003, Bour and Strebel 2003). In primate lentiviruses that do not encode for Vpu, the functions of this protein are conducted, at least partially, by viral proteins Env and Nef (Le Tortorec and Neil 2009, Zhang et al. 2009).

Initial studies suggested that Vpu catalyses at least two apparently independent functions, binding and degradation of intracellular viral receptor CD4 (Willey et al. 1992a) and enhancement of virus particle release from the cell surface (Klimkait et al. 1990). These two functions have been mapped to Vpu cytoplasmic and N-terminal transmembrane domains, respectively (Schubert et al. 1996a). According to one report, these two functions of Vpu may actually be interconnected, since the presence of CD4 at the cell surface was found to interfere with particle release (Bour et al. 1999). In addition to these two functions Vpu has been reported to induce apoptosis in infected cells by inhibiting the degradation of a cellular signal mediator protein IκB. The suppression of IκB degradation by Vpu leads to constitutive inactivation of a nuclear transcription factor NF-κB. When NF-κB remains inactive, the expression of anti-apoptotic genes, such as Bcl-2 family proteins is blocked (Akari et al. 2001).

Vpu exerts the downregulation of CD4 surface expression together with two other viral proteins Env precursor (gp160) and Nef. While Nef is engaged in the endocytosis of cell-surface associated CD4, Env precursor forms complexes with the newly synthesized CD4 in the ER and blocks the transport of CD4 to the plasma membrane (Crise et al. 1990, Jabbar and Nayak 1990). Vpu interacts simultaneously with the cytoplasmic domain of ER-trapped CD4 and beta transducine-repeat containing protein (β-TrCP) in SCF^{β-TrCP} E3 ubiquitin ligase complex. This interaction leads to ubiquitination and translocation of CD4 to the cytoplasm and subsequent degradation by the cytoplasmic proteosomes (Binette et al. 2007, Margottin et al. 1998, Meusser and Sommer 2004, Schubert et al. 1998). Vpu itself somehow escapes ubiquitination and degradation, thus serving only as an adaptor protein for proteosomal degradation of CD4. Consequently also, the transport of Env precursor then resumes towards the cell surface and after processing the mature envelope glycoprotein is incorporated into the viral particles. Vpu has also recently

shown to bind the invariant chain of MHC-II and reduce the expression of this protein on the cell surface. This apparently reduces the antigen presentation and contributes to attenuated immune responses and viral persistence (Hussain et al. 2008, Nomaguchi et al. 2008).

The enhancement of virus particle release by Vpu transmembrane domain remained poorly understood for years after it was first implicated (Klimkait et al. 1990) although several studies were made in order to understand the biology behind the Vpu action. For instance it became evident that the ability of Vpu to promote particle release was dependent on the cell confluency and growth conditions (Deora and Ratner 2001) and critically dependent on cell type (Geraghty et al. 1994, Neil et al. 2006, Varthakavi et al. 2003). Also, an intriguing correlation between the ability of Vpu to form ion conductive channels on the membranes and its ability to enhance virion release (Schubert et al. 1996b) evoked speculations over molecular mechanism behind the Vpu action. It was then implicated that Vpu, through forming ion channels could either globally modify the cellular environment to favour the particle assembly or modulate the local electric potential of the plasma membrane leading to the facilitated formation of budding structures and release of virus particles from the cell surface (Bour and Strebel 2003, Hsu et al. 2010). In accordance with these speculations were the findings that Vpu shares homology with a background potassium channel TASK-1 (Twik-related acid-sensitive K⁺-channel) (Hsu et al. 2004). It was suggested that Vpu, mechanistically through a molecular piracy intercalates with TASK-complexes and interferes with the inherent activity of the channel as well as induces an accelerated degradation of TASK-1 by β TrCP-mediated ubiquitin-proteasome pathway. A recent study was in agreement with the earlier speculations of Vpu modulating the membrane potential of the budding membrane (Hsu et al. 2010) while others have been shown that the abolishment of the ion channel activity of Vpu did not interfere with Vpu-mediated augmentation of virus release (Bolduan et al. 2011).

The suggestion that Vpu could operate through an intimate interaction with a host cell component was not novel *per se* as it was earlier implicated that Vpu action may involve a cellular 34 kDa protein Ubp (Vpu binding protein) (Handley et al. 2001). It was speculated that Ubp might function as a negative factor in viral particle assembly by inhibiting the proper transport of Gag to the site of assembly. The role of Vpu would be to relieve this restriction of Gag transport by binding Ubp and removing it from interfering the intracellular Gag trafficking. Although this study did not provide any conclusive data to support these implications, it was a step forward in understanding the mechanism of Vpu action.

It was soon suggested that Vpu counteracts a host cell restriction factor which impedes the particle production (Varthakavi et al. 2003). It was found that human, but not simian cells possess an activity that restricts efficient particle release in the absence of Vpu. This restrictive activity was dominant also in human-simian heterokaryons but could be alleviated by expression of Vpu. This study was the first to give an explanation why the ability of Vpu to enhance particle release was cell type-dependent but failed to give more detailed information about the nature or identity of this restrictive factor present in human cells. Comparable results

suggesting that Vpu acts through the interactions with host cell components rather than being responsible for enhancement of virion release alone were obtained from studies showing that Vpu, but not Pr55^{gag} co-localizes with recycling endosomes and disruption of trafficking through these structures abolished the ability of Vpu to augment the particle release (Varthakavi et al. 2006). Furthermore, expression of Vpu was shown to prevent the endocytosis of plasma membrane associated Pr55^{gag} and its accumulation into intracellular compartments without inducing a general block in endocytic trafficking. This data suggested that Vpu counteracts cellular activity that normally leads to the internalization of Pr55^{gag} or virions from the plasma membrane (Neil et al. 2006). Interestingly, the endocytosis of Pr55^{gag} was found to be dependent on functional late (L)-domain in Pr55^{gag}.

The conclusive evidence of the mechanism how Vpu enhances virion release from the cell surface came from recent studies suggesting that Vpu antagonizes an interferon- α induced host cell factor called tetherin/CD317/BST-2 (Neil et al. 2007, Neil et al. 2008). Tetherin is a type II single-pass glycosylated transmembrane protein with C-terminal GPI-anchor and homodimerisation sites in its ectodomain (Sauter et al. 2010). Tetherin traps nascent fully formed virions on the surface of the host cells and blocks their release as cell-free virus particles (Fitzpatrick et al. 2010, Hammonds et al. 2010, Perez-Caballero et al. 2009). Following surface tethering, trapped virions are subsequently endocytosed to CD63+ compartments and directed to lysosomal degradation (Miyakawa et al. 2009, Neil et al. 2006, Van Damme and Guatelli 2008). In addition to restricting HIV-1 release from the cells, tetherin has been shown to possess similar activity against broad spectrum of other enveloped viruses such as diverse retroviruses, filoviruses and herpesviruses (Jouvenet et al. 2009a, Sauter et al. 2010).

The molecular mechanism by which the particle entrapment at the plasma membrane is reversed by Vpu is currently under intense investigation. In some reports it has been implicated that Vpu counteracts the tetherin-mediated virus retention by blocking the insertion of the tetherin N-terminal transmembrane domain into virion envelopes thereby preventing the bridging of virus particles to the plasma membrane (McNatt et al. 2009, Perez-Caballero et al. 2009). This model of direct bridging of viral particles to the plasma membrane by tetherin dimers have been however questioned as it has been shown that virions cannot be released from the plasma membrane by cleaving the C-terminal GPI-anchor (Fitzpatrick et al. 2010). Furthermore, electron micrographs taken from the cells infected with Vpu-deleted virus suggest that particles are linked together by long filamentous structures that clearly cannot be formed by tetherin dimers alone (Hammonds et al. 2010). It has been also suggested that the function of Vpu is to relocate tetherin from the plasma membrane to the intracellular compartments hence sequestering it away from the site of productive particle assembly (Dube et al. 2009, Dube et al. 2010, Habermann et al. 2010, Mitchell et al. 2009, Van Damme et al. 2008). In addition, it has been reported that Vpu targets internalized tetherin for proteosomal or lysosomal degradation by β -TrCP-dependent mechanism (Andrew et al. 2011, Douglas et al. 2009, Goffinet et al. 2009, Mangeat et al. 2009, Mitchell et al. 2009) although opposing reports suggest that neither tetherin degradation nor translocation from the plasma membrane is required for the Vpu-mediated enhancement of particle release (Goffinet et al. 2010,

Miyagi et al. 2009, Tervo et al. 2011). A recent report implicated that instead of relocating tetherin from the cell surface Vpu acts in post-ER compartment and interferes with resupply of the newly synthesized tetherin from within the cell (Andrew et al. 2011).

In contrast to Vpu, tetherin is incorporated into viral particles both in the presence or absence of Vpu expression (Fitzpatrick et al. 2010, Habermann et al. 2010) suggesting that there may be alternative mechanisms behind the action of Vpu. While still highly speculative, a recent report suggested that tethered particles are precursors for extracellular VLPs and Vpu may execute its action after the budding has already been completed (Karetnikov and Suomalainen 2010).

1.1.5 An overview of the HIV-1 replication cycle

The replication cycle of HIV-1, which takes approximately 52 hours in productively infected CD4+ T-cells (Murray et al. 2011) begins with the attachment of a virus particle to target cell surface. The trimeric, surface gp120 protein (SU) on the virion surface engages CD4 on the host cell, including conformational changes that promote further binding to chemokine receptors, CCR5 or CXCR4 depending on virus isolates (Greene and Peterlin 2002). The sequential interactions between SU, CD4 and chemokine co-receptor eventually leads to a conformational change in gp41 which exposes the N-terminal hydrophobic, trimeric coiled-coil structure called fusion peptide. The fusion domain inserts itself into the target cell membrane and subsequent hairpin formation promotes the fusion of lipid bilayer of the viral envelope and the plasma membrane of a target cell. Following the membrane fusion, HIV viral core is released into the target cell interior (Chan and Kim 1998). Of note, a recent report suggested that instead of a direct fusion at the plasma membrane HIV-1 enters cells via clathrin- and dynamin-dependent endocytosis followed by fusion with the endosomal membrane (Miyauchi et al. 2009).

Once inside the cell, viral uncoating takes place. This step in retroviral replication cycle has remained poorly understood but refers to events that immediately follow the viral membrane fusion and penetration but precede the reverse transcription of the viral genome. During this process at least a part of the proteinacious viral core structure is released into the host cell cytoplasm (Freed 2001). An *in vitro* study of purified viral cores showed that uncoating is dependent on the activation of T-cells, suggesting that activated cell lysate harboured a host factor that was essential for uncoating (Auewarakul et al. 2005).

Successful uncoating generates the viral reverse transcription complex (RTC) and this high molecular weight assembly is liberated from the entry site. RTC docks with actin microfilaments (Bukrinskaya et al. 1998) and associates with other cellular components, such as cyclophilin A (Towers et al. 2003, Trkola 2004). During this step the viral RNA genome is converted to a double-stranded linear DNA. This process is catalysed by the RT-enzyme (Freed 2001).

Upon completion of reverse transcription, the preintegration (PIC) complex is formed and transported to the nucleus along the microtubules (McDonald et al. 2002).

Nuclear import is guided by viral determinants (e.g. MA, IN, Vpr, central DNA-flap) as well as host cell proteins, such as importin 7 (Fassati et al. 2003). Integration of the HIV genome is promoted by the viral integrase (IN). Host factors are also required, such as barrier to autointegration, but the precise mechanism of the host components has remained unknown (Greene and Peterlin 2002). Integration leads to either a transcriptionally latent or an active form of infection.

Integrated DNA, referred to as a provirus, behaves essentially like a cellular gene. In the host cell genome the 5' LTR functions like other eukaryotic transcriptional unit, containing promoter and a number of regulatory elements important for RNA polymerase II-driven transcription. The basal transcriptional activity from the HIV LTR is low. The viral mRNA synthesis is greatly increased in the presence of the viral transcriptional transactivator protein Tat. Tat binds to the transactivation response element TAR in the nascent viral transcript and functions as an adaptor protein for cellular protein complex called positive transcription-elongation factor b. RNA polymerase II is phosphorylated by transcription-elongation factor b and this is a signal for transition from transcription initiation to elongation. Thus, the function of Tat is to promote the formation of an elongation competent transcription complex and as a consequence the processivity of RNA polymerase II is enhanced (Bannwarth and Gatignol 2005).

The viral protein Rev is an essential regulator of HIV-1 mRNA and protein expression. Rev binds to Rev responsive element (RRE) in the viral RNA transcript and promotes the nuclear export of incompletely spliced mRNA and increases the association of these mRNAs with polyribosomes (D'Agostino et al. 1992). In the cytoplasm these mRNA-species are then employed in the translation of viral structural proteins and only the unspliced genome-length mRNA is packaged into progeny virus particles. A recent study implicated that Rev may also play a role in removing an inhibitory ribonucleoprotein complex from the viral transcript therefore allowing a more efficient translation of viral proteins and promoting encapsidation of unspliced viral transcripts (Blissenbach et al. 2010).

Following the synthesis of the full complement of viral proteins the assembly process can begin. Assembly of HIV-1 is driven by the viral core protein precursor Pr55^{gag} but is also influenced by Env and the viral genome (Freed 1998) as well as a series of host cell factors. Assembly of HIV-1, as other C-type retroviruses, takes place at cellular membranes. At the site of assembly Gag and a minor core component, Gag-Pol, attach to the membrane and oligomerize into immature viral cores. The nascent immature virions contain an electron dense, doughnut-shaped layer under the lipid envelope. In these particles, Pr55^{gag} forms hexameric ring-like arrays and individual molecules are radially arranged with N-terminus facing to the membrane while C-terminus is extended towards the center of the particle (Huseby et al. 2005, Wilk et al. 2001).

The terminal steps in replication cycle are budding of a nascent particle through the cellular membrane and virus maturation. The maturation takes place during or shortly after budding and is defined as an ordered array of cleavages of Pr55^{gag} executed by a virus-encoded protease. The cleavage is followed by subsequent rearrangements of

the cleavage products. NC and the dimeric RNA genome first condense into a complex at the center of the virion. CA assembles then into a conical shell that surrounds the NC/RNA complex whereas majority of MA remains associated with the viral membrane (Wieggers et al. 1998). The p6-protein appears to locate outside the conical core in the mature virus particle (Welker et al. 2000). In addition to four distinct Gag proteins, two spacer peptides p1 and p2 are also cleaved (Henderson et al. 1990). The precise role of these peptides and location in a virus particle have remained uncovered, but it has been suggested that the spacer peptide p2 is critical for correct assembly phenotype (Gross et al. 2000, Morikawa et al. 2000) while p1 confers to the ESCRT-dependence (Endosomal Sorting Complexes Required for Transport) of HIV-1 budding (Popova et al. 2010). Virus maturation is required for the infectivity of nascent virions.

1.2 CELLULAR PATHWAYS LINKED TO THE ASSEMBLY AND BUDDING OF HIV-1

1.2.1 The raft concept

An early work of Singer and Nichols proposed that the lipid bilayer of a cell membrane functions as a neutral two-dimensional solvent for membrane proteins having little influence on their function (Singer and Nicolson 1972). More recently, the cell membrane has been proposed to exist at least at three different chemical states that are critically dependent on the temperature and lipid content of the membrane. A membrane can possess “frozen” gel-like properties, but this state is thought to exist only below the melting temperature of the lipids. More relevant biological states are liquid-ordered, a crystalline phase of the membrane (L_c) and liquid-disordered (L_d) phase, which in turn describes membrane areas that are not specifically arranged to any higher-order, L_c -like structure (Brown and London 1998). It has been suggested that the liquid-ordered phase (L_c) acts as a concentration platform for certain proteins thus assigning these domains for specialized membrane functions (Simons and Ikonen 1997). This model of functional ordered domains floating in the less-ordered sea of bulk lipids has gained wide agreement among the scientist, even though the direct evidence for the existence and exact function of these domains are often uncritically evaluated (Lai 2003, Munro 2003, Shaw 2006).

The term lipid raft is coined to these ordered membrane domains that are rich in sphingolipids and cholesterol (Brown and London 1998, Simons and Ikonen 1997). These domains are insoluble in non-ionic detergents such as Triton X-100 at low temperatures and can be separated on the basis of this property from the bulk membrane by using density gradients (Brown and Rose 1992). The detergent resistance and low buoyant density of lipid rafts is based on relative tight packing of saturated sphingolipid hydrocarbon chains and cholesterol on these membrane patches compared to more loose and disordered packing of less saturated glycerophospholipids on the bulk membranes (Brown and London 2000).

The first direct suggestions of the existence of lipid rafts came from early studies of polarized epithelial cells (Simons and van Meer 1988, van Meer et al. 1987). In these

studies it was proposed that sphingolipids might aggregate into a distinct domain in the Golgi complex, from where they are subsequently transported to the apical side of an epithelial cell as a single unit, as a membrane patch. GPI-anchored proteins were found to be sorted to the apical surface of the epithelial cells by the same means as sphingolipids (Lisanti et al. 1988) and the first operational definition for these entities as detergent-resistant, cholesterol-dependent and glycolipid-enriched membrane subdomains came with an extensive study of GPI-anchored protein placental alkaline phosphatase (Brown and Rose 1992).

Lipid rafts are considered to be small (<50 nm), highly dynamic sterol enriched lipid-protein assemblies that float freely on the plane of the exoplasmic leaflet of the plasma membrane (Pralle et al. 2000, Simons and Ikonen 1997). The inner leaflet has been speculated to be coupled to the outer leaflet by interdigitation of long fatty acid tails present in outer leaflet sphingolipids (Simons and Toomre 2000). One report provided an indirect evidence of the existence of discrete microdomains also in the inner leaflet of the plasma membrane. It was observed that inner leaflet membrane proteins concomitantly clustered upon cross-linking the outer leaflet GPI-anchored raft proteins (Prior et al. 2003). However, a lipidomics study of detergent resistant membranes suggested, that lipid rafts are largely outer leaflet structures with substantially less rigorously selected inner leaflet lipids (Pike et al. 2005). On the other hand though a study using model lipid bilayers demonstrated that manipulation of one leaflet of the membrane induces changes in the other leaflet as well, suggesting strong interleaflet interactions (Collins and Keller 2008).

Lipid rafts have been suggested to participate in various cellular processes, such as numerous signal transduction events (Simons and Toomre 2000), T-cell activation and signalling (Simons and Gerl 2010) and intracellular trafficking and sorting of lipids and lipid-anchored proteins (Ikonen 2001, Simons and Gerl 2010). In addition to roles in normal cellular function, lipid rafts have also been suggested to act as entry platforms for a wide range of pathogens and toxins (Simons and Ehehalt 2002) as well as serve as assembly sites for various enveloped viruses, such as HIV-1, Influenza A and Ebola (Suomalainen 2002). In addition to exploitation of lipid rafts by infectious agents, membrane microdomains have been linked to various human diseases, such as neurological disorders, autoimmune diseases and lipid metabolism related problems (Simons and Ehehalt 2002).

Lipid rafts and associated components have been traditionally studied by using non-ionic detergent extractions at cold temperatures followed by density centrifugation. Triton X-100 has been most widely used, but others, such as Brij-series and Lubrol WX (Drevot et al. 2002, Roper et al. 2000, Schuck et al. 2003) have also been introduced. Another biochemical approach to probe raft involvement is the manipulation of lipid raft constituents. Cholesterol can be sequestered from the membranes by sterol-binding drugs, toxins or detergents or alternatively, rafts can be disrupted by loading cells with exogenous lipids (Simons and Toomre 2000). Any of these biochemical methods however, does not necessarily give reliable information about rafts or raft-associated proteins, since methods described above are very prone to artifacts caused by reagents or conditions used (Ikonen 2001, Lai 2003). For instance, cholesterol extraction may indeed increase the size of membrane

microdomains (Pike 2006) instead of dissolving them indicating the artificial nature of these methods. In addition, the usage of different detergents with differential solubilisation abilities yields easily variable data of lipid raft composition (Schuck et al. 2003).

Lipid rafts are not easy to visualize, since they are too small to be studied by standard light microscopy. Antibody-mediated patching of raft-associated components followed by immunofluorescence microscopy (Harder et al. 1998) has been used to detect coalesced raft domains. This method however, does not necessarily provide any direct information of what kind of assemblies rafts exists in the native membranes.

After reconciliation with biochemical methods yielding inconsistent data, more sophisticated techniques, such as Fluorescence Resonance Energy Transfer (Kenworthy et al. 2000), Single Particle Tracking (Kusumi et al. 2004, Pralle et al. 2000), Fluorescence Recovery After Photobleaching (Kenworthy et al. 2004) and immunoelectron microscopy of native membrane sheets (Prior et al. 2003, Wilson et al. 2004) have been applied to study rafts at molecular level in living cells. Results from these studies suggested that lipid rafts are small, probably in the low nanometer range and partitioning of molecules to raft domains is highly dynamic. However, depending on technique used, the estimations of exact size of rafts and their temporal stability yielded variable data, indicating that the raft field still lacked consensus over the basic aspects of the nature and function of these lipid microdomains. A report from Keystone Symposium on lipid rafts and cell function stated that “Membrane rafts are small (10-200 nm), heterogenous, highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes. Small rafts can sometimes be stabilized to form larger platforms through protein-protein and protein-lipid interactions” (Pike 2006).

Advanced high-resolution microscopy-based techniques have been recently applied to visualize lipid rafts in living cells (Simons and Gerl 2010). These studies have revealed the existence of dynamic cholesterol- and sphingolipid-dependent nanoscale clusters at the plasma membrane harbouring GPI-anchored proteins and other membrane proteins. These clusters have been seen to coalesce into functionally relevant ordered assemblies (Eggeling et al. 2009, Lingwood and Simons 2010, Meyer et al. 2006, Pinaud et al. 2009, Sharma et al. 2004, Simons and Gerl 2010). Moreover, analyses of lipid contents of these microdomains have supported the conclusion of selective inclusion of raft-associated lipid in these clusters as well as in intracellular raft carriers (Klemm et al. 2009, Zech et al. 2009). This new data strongly imply that ordered lipid domains can be formed in the context of native membranes. It remains however to be determined to what extent do the physico-chemical properties of transmembrane proteins such as attraction of certain lipid species or formation protein-protein interactions and networks actually rule the composition of these nanoassemblies (Douglass and Vale 2005, Lingwood and Simons 2010).

1.2.2 Endocytosis and MVB biogenesis

Endocytosis is a complex process in which a portion of the plasma membrane buds inwards towards the cytoplasm and forms vesicles that contain cellular membranes and proteins as well as extracellular fluid. The endocytic membrane trafficking has an essential role in delivering membrane components, receptor-bound ligands and solute molecules to various intracellular destinations. Given that endocytosis also plays a central role in regulation of cellular signalling and downregulation of cell surface receptors, it can be extremely selective: a subset of lipids and proteins are internalized whereas others seem to be actively retained in the plasma membrane (Gong et al. 2008). The endocytic pathway basically consists of a complex network of repeated cycles of membrane deformation with or without coat protein assembly and disassembly, vesicular transport and membrane fusion to the target compartment.

Eucaryotic cells exhibit at least three main endocytic pathways that are clathrin-mediated endocytosis (CME), macropinocytosis/phagocytosis and clathrin-independent endocytosis (CIE). The latter can be further divided either into dynamin-dependent and dynamin-independent uptake pathways or according to the small GTPases that regulate the given uptake route (Mayor and Pagano 2007). Regardless of the specific terminology assigned, it has become clearly evident that CIE consists of several distinct uptake pathways of which each mediates the endocytosis of different set of cargo molecules and carries their contents into different intracellular destinations (Howes et al. 2010, Mayor and Pagano 2007).

Endocytosis begins at the cell surface with the deformation and vesiculation of a region of the plasma membrane into which membrane associated cargo is sequestered (Johannes and Mayor 2010). The newly formed vesicle is then transported to and fuses with a membranous intracellular organelle called early endosomal compartment (EE), although some pathways may use intermediate itineraries in the way to the EE, such as GPI-anchored protein-enriched early endosomal compartments (GEECs) (Howes et al. 2010, Mayor and Pagano 2007). The main function of early endosomal compartment is to redirect the incoming cargo either via tubular recycling compartment back to the plasma membrane or along the endosomal pathway for degradation (Saftig and Klumperman 2009, Traub 2010). Cargo destined for degradation is subsequently delivered to the late endosomal compartment which matures to form a multivesicular body (MVB) upon budding of vesicles from the limiting membrane to the intraluminal region (Traub 2010). Ultimately, the late endosomal compartment/MVB fuses with lysosomes where intraluminal vesicles of MVBs are degraded by lysosomal hydrolases (Felder et al. 1990, Saftig and Klumperman 2009).

Sorting signals determine the location and fate of endocytosed proteins. These signals can be carried within the amino acid sequence of a protein (e.g. tyrosine-based sorting signal YXX ϕ /NPXY or LL-(di-leucine)-motif) or they can be postrtranslationally appended to the proteins in a regulated manner (e.g. ubiquitination, phosphorylation) (Bonifacino and Traub 2003, Traub 2009).

Ubiquitin is a 76 amino acid protein that becomes conjugated to the proteins through the sequential action of three enzymes: ubiquitin-activating enzyme (E1), ubiquitin conjugating enzyme (E2) and ubiquitin ligase (E3). The ubiquitin attachment to a target peptide results in generation of an isopeptide bond between the C-terminus of ubiquitin and lysine residues in substrates (Weissman 2001). Substrates can be modified either with monoubiquitin or with a polyubiquitin chain that is an array of ubiquitin moieties linked through lysines present in ubiquitin itself (Hicke 2001).

It is now well-established that different types of ubiquitin conjugates are involved in the regulation of different cellular processes. Monoubiquitination is implicated in the endocytosis of plasma membrane-associated proteins and sorting of these proteins to the MVB and subsequently to the lysosomes. In contrast, polyubiquitin chains have a well-characterized role in targeting proteins for degradation by the 26S proteasome (Hicke 2001). Ubiquitin signals can be removed and trimmed by various cellular deubiquitinating enzymes (Weissman 2001), suggesting that ubiquitination is a crucial regulatory element in cells.

Biogenesis of MVBs is extensively studied in yeast (Babst et al. 1997, Babst et al. 1998, Bilodeau et al. 2002, Odorizzi et al. 1998, Piper et al. 1995, Raymond et al. 1992) and great deal of current understanding of this cellular pathway originates from these studies (Katzmann et al. 2002). Yeast cells rely on a single pathway in MVB formation which occurs at the late endosomal compartment when the limiting membrane of this organelle invaginates and buds into its lumen. This gives a characteristic morphology of numerous intraluminal vesicles within a larger membrane-enclosed endosome. Mammalian cells have been described to contain more than one kind of MVB pathway, one similar to that of yeast, and at least a two of pathways that involve different kinds of membrane lipid derivatives such as lysobisphosphatidic acid (LBPA) or ceramide (Hurley et al. 2010, Matsuo et al. 2004, Trajkovic et al. 2008).

Cargoes destined to the intraluminal vesicles of MVBs, such as internalized surface receptors seems to be selected on the basis of monoubiquitin signal on their lysine moieties, although alternative sorting signals may exist (Katzmann et al. 2001, Reggiori and Pelham 2001, Urbanowski and Piper 2001). The vesicle formation and budding towards the lumen of MVB is mediated by a complex set of proteins called ESCRTs (Endosomal Sorting Complexes Required for Transport). Three distinct ESCRT-complexes have been described, termed ESCRT-I, II and III (Babst et al. 2002a, Babst et al. 2002b, Bache et al. 2003, Katzmann et al. 2001, Williams and Urbe 2007).

ESCRT-complex initiation, sometimes referred to as ESCRT-0 (Hurley and Hanson 2010) consists of two different proteins, hepatocyte growth factor-regulated tyrosine kinase substrate (HRS) and signal transducing adaptor molecule and it is essential for initial recognition and clustering of ubiquitinated cargo at the endosomal membrane (Wollert and Hurley 2010). HRS is recruited to the endosomal membrane through the binding to the endosome-enriched lipid phosphatidylinositol-3-phosphate (PI(3)P) (Raiborg et al. 2001). In addition to cargo selection, HRS also recruits flat clathrin coats into the ESCRT-initiation complex as well as it is responsible for the

recruitment of downstream ESCRT-complexes to the site of MVB formation (Bache et al. 2003, Katzmann et al. 2003, Raiborg et al. 2002, Sachse et al. 2002). The function of clathrin in ESCRT-assembly is to stabilize HRS-complex to distinct microdomains at the MVB limiting membrane (Raiborg et al. 2006, Wollert and Hurley 2010).

ESCRT-I consists of Tsg101 (tumour susceptibility gene 101, also known as VPS23), VPS28, VPS37 (VPS stands for Vacuolar Protein Sorting) (Babst et al. 2000, Bishop and Woodman 2001, Katzmann et al. 2001) and possibly also protein called Mvb12 (Curtiss et al. 2007, Oestreich et al. 2007). Tsg101 binds HRS, ubiquitin and ESCRT-associated protein ALIX (also known as AIP1) (Bache et al. 2003, Katzmann et al. 2001, Matsuo et al. 2004) and it functions in cargo recognition at the MVB limiting membrane. The role of ALIX/AIP1 is probably to regulate MVB biogenesis and inward budding through a conical lipid LBPA present at the endosomal membranes of mammalian cells (Matsuo et al. 2004). In addition to MVB budding, ALIX/AIP1 has linked to other cellular processes involving membrane fusion and fission, such as apoptosis, endocytosis and cytokinesis (Chatellard-Causse et al. 2002, Morita et al. 2007, Odorizzi 2006).

ESCRT-II is a heterotetramer consisting of one copy of each VPS22 and VPS36 and two molecules of VPS25 (Babst et al. 2002b). This complex has been shown to bind both ubiquitin and PI(3)P (Slagsvold et al. 2005, Teo et al. 2006). At least in yeast, it also recruits the ESCRT-III complex to the MVB limiting membrane (Babst et al. 2002a). The interaction between ESCRT-II and -III in mammalian context may not be strictly required, as ESCRT-II independent degradation of cellular receptors has been reported (Bowers et al. 2006). Indeed, it has been suggested that ALIX/AIP1 or some yet unknown factor may provide an alternative link between ESCRT-I and ESCRT-III (Langelier et al. 2006, Williams and Urbe 2007).

The last multisubunit complex to be recruited to the site of MVB biogenesis is an ESCRT-III complex that consists of at least 10 CHMP-proteins (Charged Multivesicular body Proteins) (Babst et al. 2002a, Martin-Serrano et al. 2003, Morita and Sundquist 2004). ESCRT-III makes multiple contacts with other MVB sorting pathway components and related proteins, such as ESCRT-II, ALIX/AIP1 and deubiquitinating enzyme AMSH (Agromayor and Martin-Serrano 2006, Katoh et al. 2003, Martin-Serrano et al. 2003, McCullough et al. 2006, Teo et al. 2004). A recent *in vitro* reconstitution of MVB biogenesis has provided new insight of the precise role of ESCRT-complexes. It was suggested that ESCRT-I and ESCRT-II localize at the neck of the MVB-bud and together confine ESCRT-0 stabilized cargo in the inward budding regions as well as to recruit ESCRT-III for to carry out the final scission reaction in order to release the budding vesicle into the lumen of a MVB (Wollert and Hurley 2010).

Given their crucial role in MVB biogenesis, ESCRT-complexes and clathrin coat are removed from the limiting membrane of the MVB just prior the inwards budding has completed (Babst et al. 1998, Sachse et al. 2004). The dissociation of at least ESCRT-III components from the membrane is catalysed by VPS4, an ATPase (Lata et al. 2008, Wollert et al. 2009) while there is no direct evidence of earlier ESCRT-

components of being substrates for VPS4. These may instead require for other factors such as Did2, Vta1 and VPS60 for their disassembly from the limiting membrane of an MVB (Nickerson et al. 2010). Abolishment of VPS4 function as well as that of Did2 and Vta1 and VPS60 in mammalian cells produces enlarged endosomal compartments (called class E phenotype in yeast) and alters the size of internal vesicles in MVBs (Fujita et al. 2003, Nickerson et al. 2010).

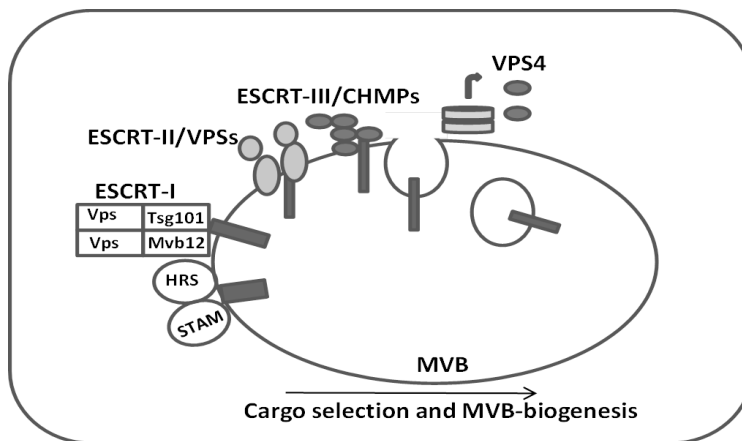


Fig. 3. A schematic drawing depicting the ESCRT-mediated cargo selection at the MVB limiting membrane (simplified from Williams and Urbe 2007)

1.2.3 The main three endocytic pathways dissected

Endocytosis occurs in most mammalian cell types and it can be categorized at least into three major pathways as mentioned before: (i) the clathrin-mediated endocytic pathway (CME), to multiple distinct (ii) clathrin-independent endocytic routes (CIE) and (iii) macropinocytosis/phagocytosis-driven entry pathways. Given that endocytosis is in most parts a constitutive process intended to fulfil the nutritional requirements of the cell and regulate multiple signalling events, many intracellular pathogens have adapted to use endocytosis to gain access into the host cells. For instance, in the view of viruses, indeed a majority of them are dependent on endocytic processes for their entry to the cells (Ghigo 2010, Marsh and Helenius 2006). Hijacking of endocytic vesicles provide a convenient means for the incoming viruses to reach their replicative compartments without a need of confronting cellular obstacles such as cortical actin or cytosolic sensors of pathogen associated molecular patterns. Furthermore, by entering cells by endocytosis, no viral components are left at the plasma membrane for the detection by the host immune surveillance (Marsh and Helenius 2006).

Clathrin-mediated endocytosis (CME)

The best characterized endocytic process, the receptor-mediated endocytosis, involves the internalization of receptors and their ligands by clathrin coated pits.

CME follows the general scheme of the coat-assisted endocytic process, i.e. nucleation of the pit and assembly of the coat at the confined regions of the plasma membrane, cargo sequestering to the pit and subsequent membrane deformation, scission of the vesicle into the cell interior, uncoating the basket-like clathrin lattice and finally the fusion of the vesicle with the acceptor compartment (Mousavi et al. 2004, Traub 2009).

The clathrin coated pit is indeed a multi-component endocytic unit that in addition to clathrin and sequestered cargo proteins is composed of several different adaptor proteins and assisting factors, such as regulators of the adaptors (Maldonado-Baez and Wendland 2006). The assembly unit of clathrin itself, called a triskelion, is a three-legged structure consisting of three heavy and light chains (Kirchhausen and Harrison 1981). Clathrin triskelions can form structures of variable curvature, allowing these protein scaffoldings engulf cargoes of different sizes and shapes (Fotin et al. 2004). According to the current view, the role of the clathrin coat is likely to fix and stabilize the budding vesicle (Ford et al. 2002, Nossal 2001) rather than induce membrane deformation as it was suggested earlier (Maldonado-Baez and Wendland 2006).

Clathrin itself is unable to bind to either phospholipids or integral membrane protein components. Therefore, recruitment of vesicle cargo occurs through intermediary proteins known as nucleators, scaffolds and adaptors (Henne et al. 2010). The function of these proteins is to initiate the pit formation, determine the selective inclusion of membrane anchored proteins into the vesicles, form an interface between the clathrin coat, incorporated cargo proteins and accompanying membrane bilayer as well as participate in membrane deformation (Johannes and Mayor 2010, Traub and Wendland 2010).

The most abundant and best studied adaptor protein (AP) found in clathrin coated pits originating from the plasma membrane is AP-2. This heterotetrameric protein complex, by using separate domains, can simultaneously bind to membranes through interaction with phosphatidylinositol-4,5-bisphosphate (PI(4,5)P₂), to the sorting signals in the cytosolic tails of cargoes, to clathrin itself as well as to many other clathrin-associated sorting proteins (CLASPs), such as AP180/CALM, Eps15, epsin and Dab2 (Collins et al. 2002, Honing et al. 2005, Maldonado-Baez and Wendland 2006, Mishra et al. 2002, Owen et al. 2004, Rohde et al. 2002, Traub 2009, Traub and Wendland 2010). Over 20 different binding partners have been described for AP-2 (Owen et al. 2004), thus it has been speculated that AP-2 functions as a central scaffold organizer in clathrin coated pits (Traub and Wendland 2010). Given the central role of AP-2, the coats can still be formed with diminished levels of AP-2 (Motley et al. 2003), suggesting that other adaptors may substitute the function of AP-2 in a redundant manner (Traub 2003) or alternatively, remaining AP-2 levels are enough to direct the pit formation without being itself involved in cargo sorting (Motley et al. 2006).

In addition of AP-2, AP-1 has been found to be enriched in clathrin coated vesicles. These vesicles however are found in the trans-Golgi network (TGN) and endosomes while AP-2 is found on vesicles originating from the plasma membrane. Two

additional adaptor complexes have been also characterized, namely AP-3 and AP-4. Both of these adaptors are found on TGN/endosomal membranes, with AP-3 merely localizing on tubular endosomal compartment (Peden et al. 2004) and AP-4 on TGN. AP-3 and AP-4 appear to function independently of clathrin, suggesting that internal membranes may harbour an alternative scaffolding protein other than clathrin (Robinson 2004).

Vesicle fission of a newly formed clathrin coated pit is mediated by dynamin, a GTPase that self-assembles into collar-like structures around the neck region that connects the invaginated clathrin coated vesicle and the donor membrane (Ramachandran 2010). The exact molecular mechanism of dynamin-mediated vesicle scission remains to be resolved, but several models have been suggested. Classically, dynamin has been viewed as a mechano-chemical enzyme where GTP-hydrolysis induces a conformational change that provides the mechanical force needed for the vesicle release (Chen et al. 2004, Stowell et al. 1999). A more recent study has challenged this classical view by showing that conformational change on dynamin collar is not sufficient for vesicle scission and suggests that other factors are required in order to execute the vesicle fission (Ramachandran and Schmid 2008). According to another model, interaction of dynamin with the plasma membrane (PI(4,5)P₂) and conformational change in dynamin helices upon GTP-hydrolysis together drive the local increase of membrane PI(4,5)P₂ density and clustering that leads to PI(4,5)P₂ phase separation from the bulk membrane and to the spontaneous vesicle release (Bethoney et al. 2009, Liu et al. 2006, Liu et al. 2009). The third model proposes that dynamin is just a classical GTPase that in biologically active GTP-form regulates the downstream interactions of the effector molecules at the vesicle neck that are required for vesicle fission (Merrifield et al. 2005, Ramachandran and Schmid 2008). Dynamin has been shown to have multiple interacting partners, such as sorting nexin-9, syndapin, amphiphysin and endophilin that have been suggested to mold and stabilize membrane curvature and link actin cytoskeleton to the endocytic events (Johannes and Mayor 2010, Ramachandran 2010).

Clathrin-independent carriers

In comparison to the clathrin-mediated endocytic pathway, alternative clathrin-independent internalization routes have remained relatively poorly understood. Accumulating data suggest that CIE can be categorized into two major pathways according to the dependence of the internalization mode on dynamin (Mayor and Pagano 2007). However, both of these two pathways consist of several morphologically distinct carriers that are each regulated by different proteins such as coat protein caveolin or different small GTPases (Gong et al. 2008, Howes et al. 2010, Mayor and Pagano 2007).

Of dynamin-dependent internalization pathways the caveolae-mediated endocytosis refers to the internalization of material via flask-shaped plasma membrane invaginations which are associated with structural coat proteins caveolin-1 and -2 (Parton and Simons 2007). Caveolins are integral membrane proteins that associate with lipid bilayers enriched in cholesterol and sphingolipids (Ortengren et al. 2004). Recent data have demonstrated that another set of proteins called cavins associate

with caveolin coats in a tissue specific manner (Bastiani et al. 2009). Cavins have been implicated in stabilizing the caveolin scaffolds at the site of bud formation, promote membrane curvature and regulate caveolae budding to the cytoplasm (Hansen et al. 2009, Hill et al. 2008, McMahon et al. 2009). Similar to CME, dynamin is required for the fission of a caveola from the donor membrane (Oh et al. 1998), but in contrast to other coated vesicular carriers, caveolae retain their coats associated with the vesicle membrane after the release of the vesicle into the cell interior (Pelkmans et al. 2004, Tagawa et al. 2005). Caveolae bring their contents into early endosomal compartment in the Rab5-dependent manner from where coat units can be recycled back to the plasma membrane or destined for degradation via MVB/late endosomal pathway (Gong et al. 2008, Hayer et al. 2010, Parton and Simons 2007). Many types of cargo can be carried by caveolae, such as lipids, proteins and lipid-anchored proteins, but the definite role of these carriers has still remained obscure as same cargo molecules can be also internalized by a caveolin-independent manner (Mayor and Pagano 2007, Parton and Simons 2007).

The second clathrin-independent internalization pathway that relies on dynamin for endocytic vesicle scission is regulated by small GTPase RhoA. The knowledge of this pathway is very limited, but it has been shown at least to mediate the constitutive internalization of lipid raft resident interleukin-2-receptor (Lamaze et al. 2001). It has been implicated that actin dynamics may be crucial for this pathway, since RhoA is a known regulator of actin fibers (Mayor and Pagano 2007).

Rho-family member GTPase CDC42 and Arf-family member Arf6 have been identified as regulators for dynamin-independent CIE-pathways (Mayor and Pagano 2007, Naslavsky et al. 2004, Sabharanjak et al. 2002). Among other cargoes such as fluid-phase uptake, both of these pathways mediate internalization of GPI-anchored proteins and are therefore commonly referred to as CLIC/GEEC-pathways (Clathrin-Independent Carrier/GPI-Enriched Endosomal Compartment) (Gong et al. 2008, Mayor and Pagano 2007). CDC42-mediated uptake is dependent on cholesterol and actin dynamics (Chadda et al. 2007) and forms long tubular invaginations at the site of carrier formation at the plasma membrane (Kirkham et al. 2005, Sabharanjak et al. 2002). It is not clear at the moment whether Arf6-regulated pathway is distinct of CDC42-pathway since these two pathways seem to share common features. It has been speculated that these two internalization mechanisms may be different variations of the same uptake modes or alternatively, it may as well be possible that CLIC/GEEC-pathway used is dependent on the cell type (Kalia et al. 2006, Mayor and Pagano 2007, Naslavsky et al. 2004).

Macropinocytic and phagocytic entry routes

Macropinocytosis refers to a process used by cells to internalize large amounts of fluid, solutes and membranes. Although macropinocytosis can occur constitutively in some tumor cell lines and immature dendritic cells (Norbury 2006), external stimulation usually by growth factors or other mitogenic signals, such as phorbol esters causes the actin-driven formation of membrane protrusions that fuse with the plasma membrane forming large endocytic vesicles known as macropinosomes (Ghigo 2010). Three types of membrane ruffles have been described in

macropinocytosis (i) planar lamellipodia-like membrane folds, (ii) cup-shaped circular ruffles and (iii) large balloon-like blebbings of the plasma membrane (Mercer and Helenius 2009). Macropinosomes are irregular in size and shape, have no discernible coat and do not concentrate receptor molecules or specific particulate cargo (Johannes and Lamaze 2002, Mercer and Helenius 2009).

The induction of macropinocytic activity leads to the activation of a complex signaling cascade that is initiated by receptor tyrosine kinase activated Ras superfamily GTPases. Ras in turn activate another set of small GTPases, such as Rac1, Rab5 and Arf6 as well as various kinases, including Pak1 and protein kinase C (Liberali et al. 2008, Swanson 2008). These all together activate the effectors of actin polymerization that subsequently leads to the actin remodeling and ruffle formation (Mercer and Helenius 2009). Other factors implicated in regulation of membrane ruffling include Na^+/H^+ -exchangers and membrane cholesterol as inhibition of cation exchanger by amiloride or depletion of membrane cholesterol block the induction of membrane ruffling (Grimmer et al. 2002, West et al. 1989).

Final steps in macropinosome formation include membrane ruffle extension, cup formation and macropinosome closure (Swanson 2008). Internalized macropinosomes can either fuse with lysosomes (e.g. in macrophages) or recycle back to the cell surface release their contents to the extracellular space. The final fate of macropinosomes seems to be induction as well as cell type-dependent (Ghigo 2010, Mercer and Helenius 2009).

Besides growth factor induced activation of macropinocytic pathway, a variety of particles, such as viruses and apoptotic cells can induce macropinocytosis in many cell types and can be internalized together with ingested fluid. A growing evidence suggest that many viruses, including both enveloped and non-enveloped viruses enter into the cells via macropinocytosis (Karjalainen et al. 2008, Marechal et al. 2001, Mercer and Helenius 2008, Mercer and Helenius 2009). Intriguingly, it has been demonstrated that enveloped viruses can mimic apoptotic bodies by modifying the lipid contents of their envelopes in order to facilitate their entry into the host cells by inducing phosphatidylserine-mediated macropinocytosis (Hoffmann et al. 2001, Mercer and Helenius 2008, Mercer and Helenius 2009). Other functions assigned to macropinocytic pathway include the aforementioned uptake of apoptotic remnants, uptake of nutrients from the cell exterior, enhancement of cell motility, antigen presentation and immune surveillance as well as regulation of activated signaling cascades (Amyere et al. 2002, Ghigo 2010, Norbury 2006).

Phagocytosis refers to the uptake of large particles by professional phagocytes such as macrophages and dendritic cells. In contrast to macropinosomes which carry cargo and fluid in an unspecific manner, phagocytosis do not involve massive fluid uptake and is strictly dependent of receptor mediated recognition of the particles to be internalized. Phagocytosis is actin-driven uptake mechanism and relies on dynamin in phagosome fission. Phagocytic cargo, such as bacteria and fungi is degraded by endo-lysosomal pathway (Ghigo 2010, Haas 2007, Mercer and Helenius 2009).

1.3 HOST AND VIRUS INTERACTIONS IN THE ASSEMBLY AND BUDDING OF HIV-1

1.3.1 Pr55^{gag} and assembly

The major structural protein of HIV-1, Pr55^{gag} can assemble and bud from cells in the absence of any other viral factors (Gheysen et al. 1989), implicating that this precursor protein has an intrinsic property to multimerize into spherical particles at the host cell membranes. In numerous mutational studies three distinct domains of Pr55^{gag} have been mapped to be essential for successful particle production. M-domain, that is located at the N-terminal part of p17 (MA) mediates the membrane association of Pr55^{gag} while I-domain at the C-terminus of p24 (CA) is required for efficient Pr55^{gag} multimerization. Lastly, L-domain, also referred to as a late domain is located within the p6-protein and is essential for release of the viral particles from the host cell membrane (Parent et al. 1995).

M-domain and myristic switch are critical for the membrane binding of Pr55^{gag}

The M-domain of Pr55^{gag} consists of a bipartite membrane-binding signal composed of N-terminally attached myristic acid moiety and a stretch of basic amino acids (17-31) located at the N-terminus of the matrix domain (Zhou et al. 1994). The covalently attached 14-carbon saturated fatty acid myristate is a crucial determinant for membrane association of Gag and thus also for particle production (Bryant and Ratner 1990, Gottlinger et al. 1989, Pal et al. 1990, Spearman et al. 1994). The role of myristic acid is regulated by a myristic switch mechanism which implies that myristic acid first adopts a sequestered conformation within the matrix domain. Upon encountering an appropriate stimulus at the host cell membranes it becomes exposed and is inserted into the bilayer (Ono and Freed 1999, Spearman et al. 1997, Zhou and Resh 1996). Recent studies have identified multiple triggers for myristic switch, including Pr55^{gag} multimerization and membrane-associated phosphoinositides (Saad et al. 2006, Shkriabai et al. 2006, Tang et al. 2004).

The acyl modification at the N-terminus of the matrix domain alone does not provide sufficient hydrophobic energy for stable membrane association (McLaughlin and Aderem 1995). Therefore, it has been proposed that basic residues within the N-terminal part of MA are also required for efficient membrane association of Pr55^{gag}. These positively charged amino acids are expected to strengthen the membrane associations via ionic interactions with acidic phospholipids present at the cytoplasmic leaflet of the plasma membrane (Hill et al. 1996, Zhou et al. 1994). Furthermore, residues in near vicinity of myristoylation signal as well as several downstream sequences of M-domain have been shown to confer in membrane affinity of Pr55^{gag} (Ono and Freed 1999).

In addition to role of M-domain in membrane binding, sequences within p17 (MA) region have been implicated in targeting of virus assembly. Some amino acid substitutions in MA seem to redirect assembly from the plasma membrane to the internal membranes (Freed et al. 1994, Hermida-Matsumoto and Resh 2000, Ono et

al. 2000, Yuan et al. 1993) suggesting that amino acids conferring membrane specificity lies within the matrix domain. Several reports claim that large deletions on MA have no significant impact on particle production (Accola et al. 2000, Borsetti et al. 1998, Lee and Linial 1994, Reil et al. 1998) implying that HIV-1 Gag could harbour membrane association determinants also in domains other than MA. Indeed, one study has shown that substitution mutations in a very C-terminal part of CA led to impairment in particle production because these mutants had a lowered affinity to the membranes (Liang et al. 2003). Furthermore, the sequences within interaction domain on NC and the major homology region (MHR) at the C-terminal part of CA have been suggested to contribute in membrane affinity (Ebbets-Reed et al. 1996, Sandefur et al. 1998).

I-domain mediates the oligomerization of Pr55^{gag}

Following targeting to the site of assembly, the membrane-associated Pr55^{gag} oligomerizes into an immature core and concomitantly induces membrane curvature and formation of a membrane bud. Extensive mutational analyses have revealed that C-terminal CA (residues 282-363) with adjoining p2 spacer peptide and N-terminal NC-domains are required for efficient Gag multimerization and VLP production (Accola et al. 1998, Accola et al. 2000, Borsetti et al. 1998, Ono et al. 2000, Sandefur et al. 2000).

The C-terminal part of CA contains a region that is important for assembly and is called the major homology region. This region consists of a stretch of amino acids that show considerable sequence homology among lentiviral CA-proteins. Substitutions or deletions in residues within or immediate upstream of MHR severely impair particle formation (Borsetti et al. 1998, Mammano et al. 1994), implicating that MHR is an evolutionally conserved subdomain that is essential for Gag-Gag interactions. In addition, to MHR the N-terminal part of NC-domain is also involved in Gag multimerization (Ono et al. 2000, Sandefur et al. 2000). This domain is called the interaction domain (I-domain) and it minimally consists of seven first N-terminal amino acids of the p7 (NC)-domain. Two basic arginine residues within the I-domain are essential for its function, suggesting that I-domain contributes to electrostatic interactions, most likely with RNA. The sequences downstream of minimal I-domain, including two zinc finger structures have been shown to enhance I-domain function (Sandefur et al. 2000), further implicating that RNA based interactions promote Gag-Gag interactions (Cimarelli et al. 2000).

Taken together, assembly is predominantly driven by protein-protein interactions established by the C-terminal region of CA within the Pr55^{gag} polyprotein. These initial interactions are enhanced upon stabilization of oligomers by membrane association through the N-terminal MA-domain. The formation of Gag oligomers at the membranes directed with these domains may create new functional surfaces that further increase affinity of Gag-multimers to membranes (Ebbets-Reed et al. 1996, Provitera et al. 2001, Sandefur et al. 1998, Sandefur et al. 2000). In addition, to protein-protein interactions assembly intermediates are stabilized by binding to RNA which has been speculated to function as a structural scaffold for assembling particles (Jouvenet et al. 2009b, Muriaux et al. 2001, Sandefur et al. 2000).

p6-late domain (L) function

The last stage of budding requires a final pinching off-step to release the assembled virus particle from the host cell membrane. This step is mediated by the p6 domain at C-terminal part of precursor Gag protein which contains conserved PTAP-motif also called late domain. Disruption of this motif results in a distinct phenotype where nascent immature particles remain attached to the plasma membrane via a membranous tether resulting in defect in particle release and accumulation of virus particles at a near vicinity of the host cell membrane (Demirov et al. 2002b, Freed 2002). The late phenotype of PTAP-mutations seems to be dependent on cell type and experimental conditions used (Morikawa 2003). For instance, while p6-domain mutations impaired virus release in adherent cell lines (e.g. HeLa) and primary macrophages, the mutations had only minor effects on particle release in T-cells lines and primary T-cells (Demirov et al. 2002b). In addition, expression of Gag alone in high level of expression systems seems to overcome the late domain defect compared to systems where the full length virus is used (Gheysen et al. 1989).

In addition to HIV-1, late domains have been also identified in Gag proteins of other retroviruses (Bouamr et al. 2003, Gottwein et al. 2003) as well as in unrelated enveloped viruses, such as Ebola virus (Licata et al. 2003). Viral late domains have been found to be functionally interchangeable between different viruses and they can exert their activity when positioned at an unnatural location within Gag or provided in *trans* (Martin-Serrano et al. 2001, Parent et al. 1995). This functional and positional flexibility suggests that late domains are not structural elements of particle assembly and are thus not involved in direct Gag-Gag interactions. Accumulating evidence instead implies that viral late domains function as docking sites for cellular factors that assist in viral egress through the cellular membrane (Freed 2002).

1.3.2 Pr55^{gag} trafficking in the cells

As other myristoylated proteins, Pr55^{gag} is expected to be synthesized on soluble ribosomes in the cytoplasm. It has been however remained unclear how does Pr55^{gag} reach its final destination at the cellular membranes. Pr55^{gag} can be actively transported to the site of assembly or alternatively the initial intracellular trafficking can occur by passive diffusion. The involvement of actin cytoskeleton, microtubules and vesicular transport pathways in Pr55^{gag} intracellular trafficking have been suggested (Martinez et al. 2008, Rey et al. 1996), but the evidence has been conflicting since no apparent co-localization of Pr55^{gag} with actin or tubulin has been detected (Perrin-Tricaud et al. 1999, Poole et al. 2005). Furthermore, the disruption of microtubules or late endosomal trafficking by pharmacological agents seem not to interfere Pr55^{gag} localization to the site of assembly at the plasma membrane suggesting that these cellular pathways do not mediate the initial targeting of the newly synthesized Pr55^{gag} in the cells (Jouvenet et al. 2006). One intriguing mechanism for Pr55^{gag} localization to the site of assembly would be the targeting of Pr55^{gag} encoding mRNA to the near vicinity of the assembly site. The localized synthesis would thus alleviate the need of specialized transport machinery for Pr55^{gag}

targeting to the plasma membrane. In addition, full length mRNAs specifically transported to the site of assembly would also conveniently serve as genomes for assembling virus particles. Recent evidence indeed suggests that Pr55^{Gag} trafficking to the site of assembly may be regulated already at the level of mRNA transport (Sherer et al. 2009), since Pr55^{Gag} encoded from transcripts mutated at RRE-sequence failed to assemble into extracellular particles and associate with the plasma membrane (Jin et al. 2009).

In order to build an immature virus particle, multiple Gag monomers must interact to form a lattice that ultimately becomes the doughnut-shaped shell of the complete, but immature virus particle. The identification of sequential assembly intermediates suggests that assembly of HIV-1 virus particles follows an ordered series of events and requires several host components, such as ATP and HP68 (Dooher and Lingappa 2004, Morikawa et al. 2000, Tritel and Resh 2001, Zimmerman et al. 2002). The traditional view of assembly however suggests that monomeric Gag is first transported to the plasma membrane where it becomes directly recruited into a developing particle. According to an alternative view, some level of multimerization may occur already in the cytoplasm and these cytosolic oligomers are then targeted to the site of assembly at the plasma membrane (Adamson and Jones 2004). There is some evidence to support the latter model of the assembly since small oligomeric Gag complexes (e.g. trimers) have been detected in the cytoplasm (Morikawa et al. 1998, Nermut et al. 2003, Spearman et al. 1997) as well as existence of larger discrete oligomeric structures have been described (Lee et al. 1999, Morikawa et al. 2000). It is however unclear whether these oligomeric Gag structures represent true assembly intermediates as the majority of newly synthesized cytosolic complexes have been shown to be rapidly degraded while only a minority reaches the plasma membrane (Nermut et al. 2003, Tritel and Resh 2001). However, the membrane association of these putative cytosolic assembly intermediates seems to be the trigger for further assembly to higher molecular weight assemblies and eventually incorporation of these complexes into particles (Lee et al. 1999, Lingappa et al. 1997, Morikawa et al. 2000, Tritel and Resh 2001). Although the precise steps in early assembly are still unclear, the evidence so far implicates a mixed assembly model in which small Gag assemblies form already in the cytosol and these are then rapidly relocated to the membranes where the higher order oligomerization is expected to proceed.

Two recent high resolution microscopy studies observing virus assembly in living cells have shed new light on the dynamics of Gag recruitment to the nascent virus particles. These reports show that Gag forms discrete clusters at the plasma membrane which are precursors for single virus particles suggesting that no large assembly platforms are required for successive virus production. Particle nucleation is driven by oligomerization of Gag recently attached to the membrane and subsequent incorporation of Gag molecules into growing virus particles occurs directly from the cytosolic pool of newly synthesized Gag (Ivanchenko et al. 2009, Jouvenet et al. 2008). No evidence was found to support the transport of preassembled multimeric Gag complexes to the plasma membrane (Ivanchenko et al. 2009). The overall assembly process of Gag core complex was found to be rapid,

while the egress of the nascent particle through the plasma membrane and completion of the budding event was speculated to be rate limiting (Ivanchenko et al. 2009).

Targeting to the assembly site

The identity of the cellular membrane which provides the site for particle assembly has been highly controversial in recent years and the debate seems still be ongoing. Initially, it was taken as a proof that Pr55^{gag} assembles at the plasma membrane of an infected host cell but this view has been challenged by numerous studies demonstrating considerable amounts of Pr55^{gag} or mature virus particles in late endosomal compartment (Nydegger et al. 2003, Pelchen-Matthews et al. 2003, Raposo et al. 2002, Sherer et al. 2003). These findings suggested that Pr55^{gag} is first targeted to the limiting membrane of late endosomes/MVBs and virions are formed by budding into the lumen of this compartment. Virion release to the extracellular milieu was thought to occur via an exosome-based secretory pathway when endosomes fused with the plasma membrane (Chertova et al. 2006, Gould et al. 2003, Nguyen et al. 2003). This endosomal assembly model was further supported by finding that ESCRT-machinery which normally operates at the limiting membrane of an MVB was essential for successful budding (Morita and Sundquist 2004). Although the intracellular accumulation of Pr55^{gag} and virus particles was most prominent in macrophages (Jouve et al. 2007, Kramer et al. 2005, Pelchen-Matthews et al. 2003, Raposo et al. 2002) accumulation of Pr55^{gag} was also observed in other cell types, such as T-cells and epithelial cell lines suggesting that HIV-1 assembly universally initiated at the endosomal compartment (Grigorov et al. 2006, Nydegger et al. 2003, Perlman and Resh 2006, Sherer et al. 2003).

Opposing studies however suggested that assembly of HIV-1 occurs at the plasma membrane (Finzi et al. 2007, Gomez and Hope 2006, Jouvenet et al. 2006, Jouvenet et al. 2008, Neil et al. 2006, Rudner et al. 2005). Biochemical analyses revealed that newly synthesized Pr55^{gag} is specifically targeted to the plasma membrane (Finzi et al. 2007, Jouvenet et al. 2006, Neil et al. 2006). An appearance of Pr55^{gag} or virions later in endosomes is due to endocytosis of the plasma membrane-associated assembly intermediates or fully formed particles. Furthermore, when using fluorescently labeled Pr55^{gag} derivatives it was also concluded that soon after synthesis Gag molecules appeared at specific areas at the plasma membrane and assembled there for individual virions (Gomez and Hope 2006, Jouvenet et al. 2008, Rudner et al. 2005). Interestingly, these results were also soon extrapolated to include macrophages as it was shown by using a membrane-impermeant dye that apparent intracellular compartments were actually connected to the plasma membrane by narrow membranous channels (Deneka et al. 2007, Welsch et al. 2007). Recently however, the specificity of this staining method has been questioned (Benaroch et al. 2010).

To further complicate this apparently contradictory data of the primary assembly site of HIV-1 virus particles it has been proposed that in macrophages Pr55^{gag} assembles both at the plasma membrane and in an internal compartment as no obvious internalization of the plasma membrane associated Pr55^{gag} was observed (Gousset et al. 2008). On the other hand, T-cells were reported to harbour domains of endosomal

origin at the plasma membrane which served as specific budding sites for both cellular exosomes and HIV-1 (Booth et al. 2006). Recently, it was also shown that the late endosomal compartment in T-cells can support for productive virus particle production (Joshi et al. 2009). Taken together, highly controversial data have been obtained so far from studies exploring the the membrane targeting of HIV-1. Differential data most likely reflect the different settings and method used during experimentation and the interpretation of the data. Alternatively, as HIV-1 can infect multiple cell types, the virus may have evolved to exploit different cellular pathways for ensuring efficient particle production. The choice of assembly pathway in a given experimental setting may be determined for instance by the temporal availability of cellular factors.

Membrane selection

Despite of ongoing controversy about exact assembly site of HIV-1, the initial data suggested that assembly takes place at the plasma membrane of an infected cell. As plasma membrane represents only a minor fraction of total cellular membranes and Pr55^{gag} potentially encounters a variety of intracellular membranes before arriving at the plasma membrane it has been long intriguing what is the basis of Pr55^{gag} being specifically localized at the plasma membrane for assembly. Initial clues what is conferring to this specificity came from in vitro studies showing that authentic assembly of VLPs required the presence of mammalian cell lysates in the assembly reaction (Campbell and Rein 1999). Further studies by the same group implicated that inositol phosphate derivatives present in the cell lysates were responsible for correcting the aberrant assembly phenotype observed in the absence of these lipid species (Campbell et al. 2001). More light was shed on the membrane specificity when it was reported that targeting of Pr55^{gag} to the plasma membrane is regulated by phosphatidylinositol-4,5-bisphosphate (PI(4,5)P2), an inositol derivative primarily resident in the cytoplasmic leaflet of the plasma membrane (McLaughlin and Murray 2005, Ono et al. 2004).

Recent studies have provided knowledge over more detailed mechanism behind the specificity of the plasma membrane targeting. (PI(4,5)P2) has been shown to bind directly the basic residues of the MA-domain, trigger the exposure of myristic acid and serving as an membrane anchor for Pr55^{gag} via its unsaturated 2' acyl chain and inducing membrane curvature to facilitate budding (Chukkapalli et al. 2008, Saad et al. 2008, Shkriabai et al. 2006). Consistent with these data, (PI(4,5)P2) is enriched in viral particles and this enrichment is dependent on the presence of globular MA-domain in the Pr55^{gag} polyprotein (Chan et al. 2008). As myristic switch and membrane binding affinity of MA-domain have been reported to increase upon trimerization of MA (Tang et al. 2004), it has been suggested that in addition to protein-lipid interactions also protein-protein interactions in the context of growing Pr55^{gag} shell at the inner leaflet of the cell surface may confer to membrane specificity of HIV-1 assembly. Furthermore, the interaction of MA-domain with RNA has been recently implicated in protecting Pr55^{gag} from promiscuous localization at the internal membranes. RNA was found to bind basic amino acids at the MA-domain and PI(4,5)P2 containing liposomes could outcompete RNA from interacting with MA. This data together suggest that there are multiple overlapping

mechanisms ensuring that Pr55^{gag} localizes to the correct membrane domain for assembly (Alfadhli et al. 2009, Chukkapalli et al. 2010).

1.3.3 HIV-1 assembly and lipid rafts

In many reports it has been suggested that assembly and budding of HIV-1 is critically dependent on lipid rafts (Graham et al. 2003, Lindwasser and Resh 2001, Nguyen and Hildreth 2000, Ono and Freed 2001, Rousso et al. 2000, Zheng et al. 2001). Rafts have been speculated to provide a platform for assembly, by allowing a selective enrichment of the viral proteins in discrete domains at the plasma membrane. This hypothesis has been encouraged by confocal microscopy studies, which have shown that Pr55^{gag} localizes to the discrete, punctate regions beneath the plasma membrane of infected cells (Hermida-Matsumoto and Resh 2000, Sandefur et al. 1998). The punctate appearance of Pr55^{gag} localization suggests that there are cellular factors present at distinct locations at the cell periphery and these factors may contribute to the virus assembly.

The strongest evidence that HIV-1 may assemble in lipid rafts has become from studies where Pr55^{gag} was found to associate with detergent resistant membranes (Lindwasser and Resh 2001, Nguyen and Hildreth 2000, Ono and Freed 2001). Also, HIV-1 spike protein, Env, was found to associate in detergent resistant membranes and this property was found to be dependent on Env palmitoylation (Rousso et al. 2000). Further evidence of possible budding of HIV-1 from rafts has become from lipid analysis of virus particles, where it was found that virion envelope possesses a lipid profile similar to that of rafts with a marked enrichment in sphingolipids and cholesterol compared to the plasma membrane of the host cells (Aloia et al. 1993, Brugger et al. 2006). Accordingly, removal of cholesterol, an essential component of lipid rafts, renders virions non-infectious (Campbell et al. 2004, Graham et al. 2003, Ono and Freed 2001, Zheng et al. 2001) further supporting the role of rafts in HIV-1 assembly. In a recent report it was shown that Env associates with distinct membrane patches that exclude other viral spike proteins expressed in same cells. Only one kind of spike protein containing VLPs were produced from co-transfected cells suggesting that a virion buds from an individual lipid raft that harbours only one kind of glycoproteins (Leung et al. 2008).

However, despite extensive studies of HIV-1 budding from lipid rafts, the evidence of HIV-1 being a genuine raft-associated virus mostly remains circumstantial. For instance, it was concluded that HIV-1 particles bud from raft domains because raft-associated GPI-anchored proteins (CD59 and Thy-1) as well as GM1 were incorporated into virions while non-raft associated CD45, a tyrosine phosphatase, was excluded from virus particles (Nguyen and Hildreth 2000). This piece of data may reflect of HIV-1 being associated with rafts, but exclusion of CD45 from HIV-1 virions can as well be due to steric hindrance between long cytoplasmic tail of CD45 and assembling Gag, rather than its non-raft localization in the site of assembly. Accordingly, incorporation of raft-associated components to virus particles may be a consequence of the fact that at the site of assembly the host components are rather indiscriminately included into particles along the Gag precursor (Chertova et al.

2006, Hammarstedt and Garoff 2004). Due to this rather sloppy assembly process, neither incorporation of raft-associated proteins into virions nor exclusion of non-raft-associated proteins cannot taken as a proof for HIV-1 utilizing raft membranes as assembly platforms.

Gradient centrifugation analyses after cold Triton X-100 extraction from several groups have shown that HIV Gag seems to associate with detergent resistant membranes at least for some extent but the interpretation of these results lack critical evaluation of the identity of the buoyant Gag complexes (Ding et al. 2003, Lindwasser and Resh 2001, Nguyen and Hildreth 2000, Ono and Freed 2001). In two of aforementioned studies HIV Gag was found to associate with detergent resistant membranes denser than classical lipid rafts and a term of barges instead of rafts was coined to these heavier structures (Lindwasser and Resh 2001). The electron microscopy analysis of these floating Pr55^{gag} complexes revealed that these high density structures represent merely multimerized Pr55^{gag} containing sheets formed by detergent extraction rather than cores assembling at lipid rafts (Ding et al. 2003). These studies clearly point out that one clear obstacle in obtaining reliable and consistent data seems to be a lack of standardized conditions used in detergent extraction studies. For instance the usage of dissimilar flotation gradients in parallel studies is one source of incoherent data and has kept creating ground for shady conclusions.

The analyses of VLPs have also spoken on the behalf of the assembly of HIV at lipid rafts based on comparison of virion envelope to that of detergent resistant membranes (Aloia et al. 1993, Brugger et al. 2006). Both have been found to be enriched with cholesterol and sphingolipids and this have been taken as a proof of HIV budding from lipid rafts. However, in these studies the composition of the virion envelope has been compared to the isolated total membranes which include both the plasma membrane and internal membranes. The presence of internal membranes in this fraction obscures the analysis of the actual lipid composition at the site of virus budding at the plasma membrane and therefore it is questionable whether these data provide solid evidence on HIV utilizing rafts for assembly.

In recent report plasma membranes of infected cells were isolated by using cationic silica beads that can give rise to relatively pure plasma membrane fraction (Chan et al. 2008, Spector et al. 1998). When this fraction was compared to the viral envelope it was found that the composition of the two membranes was strikingly similar except the enrichment of phosphoinositol phosphates at the viral membrane. In this particular study the authors did not find any evidence of HIV budding from lipid raft-like membranes.

In many reports it has been shown that virus production ceases or viral infectivity is lost upon treatment of virus producing cells or virions by cholesterol depleting agents such as β -methyl-cyclodextrin. These data have also led to the conclusion that virus egress occurs through raft domains (Campbell et al. 2004, Graham et al. 2003, Liao et al. 2003, Ono and Freed 2001, Zheng et al. 2001). However, the loss of infectivity may as well reflect the defect in viral entry upon cholesterol depletion or inactivation of viral particles rather than direct problems in egress. On the other hand, it has been

shown that Pr55^{gag} binding to the membranes is less efficient in the absence of membrane cholesterol explaining the reduced virus production from cholesterol depleted cells (Ono et al. 2007). Furthermore, cholesterol depletion is known to cause global effects such as loss of plasma membrane (PI(4,5)P₂) and reorganisation of cellular actin (Kwik et al. 2003), so the perturbation of virus production may also be an indirect consequence caused by cholesterol level modulating drugs rather than unbeatably proving the importance of lipid rafts in HIV assembly.

Taken together, the issue of HIV utilizing lipid rafts for its egress from the host cells is still controversial. Many reports show evidence on the behalf of the importance of lipid rafts in HIV assembly and budding, but the lack of consistent methodology in studying raft association apparently complicates the interpretation of the data.

1.3.4 Host cell factors that mediate the late stages of virus budding

To date, it is well established that HIV-1 Gag late domain PTAP as well as a downstream sequence LYPXnL (that resembles the late domain of EIAV) recruit a vast array of cellular proteins that are needed for effective release of viral particles (Morita and Sundquist 2004, von Schwedler et al. 2003). These proteins, termed ESCRT or class E Vps proteins are normally found to act at the site of MVB formation. The idea that retroviruses may utilize MVB-associated budding machinery for their escape from the host cells is supported by a fact that both reactions include an analogous budding mode, that is, away from the cytoplasm (Pornillos et al. 2002). Numerous studies have now linked cellular MVB formation machinery and retrovirus budding (Morita and Sundquist 2004).

HIV-1 PTAP-domain specifically binds a cellular protein Tsg101 (Demirov et al. 2002a, Garrus et al. 2001, Martin-Serrano et al. 2001, VerPlank et al. 2001) which is a part of a multiprotein complex ESCRT-I (Katzmann et al. 2001). HIV-1 Pr55^{gag} mimics a cellular protein HRS that normally is a binding partner of Tsg101 (Pornillos et al. 2003). Pr55^{gag} recruits Tsg101 to the site of budding by interacting with the UEV-domain of Tsg101. The recruitment of Tsg101 to the membrane is a first step in formation of an away from cytosol budding complex. The role of Tsg101 is then to recruit Vps28 and Vps37 which then together form an ESCRT-I complex. Analogous to events in MVB formation, the next complex recruited to the site of budding is ESCRT-II which is composed of another set of Vps proteins (Babst et al. 2002b). The third and the final complex to be recruited at the site of a bud is called ESCRT-III (Babst et al. 2002a). The components of this complex (the CHMP proteins) co-assemble into a lattice once recruited to the endosomal membrane (von Schwedler et al. 2003).

After ESCRT-III associated proteins have performed their function in formation of an away-from-cytosol budding, the AAA-type ATPase Vps4 catalyses the dissociation of the protein complex in an ATP-dependent manner. Class E Vps proteins are released from the membrane and recycled back to the cytoplasm for further rounds of membrane sorting (Babst et al. 1998, Babst et al. 2002a). The studies with mutant Vps4 ATPase have indicated that non-functional Vps4 induces a late-like phenotype

in HIV-1 transfected cells, i.e. budding viral particles remain tethered to each other on the cell surface (Garrus et al. 2001, von Schwedler et al. 2003). Of note however, in Vps4 mutant expressing cells the budding seems to proceed somewhat further than in cells transfected with HIV-construct lacking the L-domain (von Schwedler et al. 2003), suggesting that ESCRT-complexes are important either directly or indirectly in inducing membrane curvature and in formation a membrane stalk.

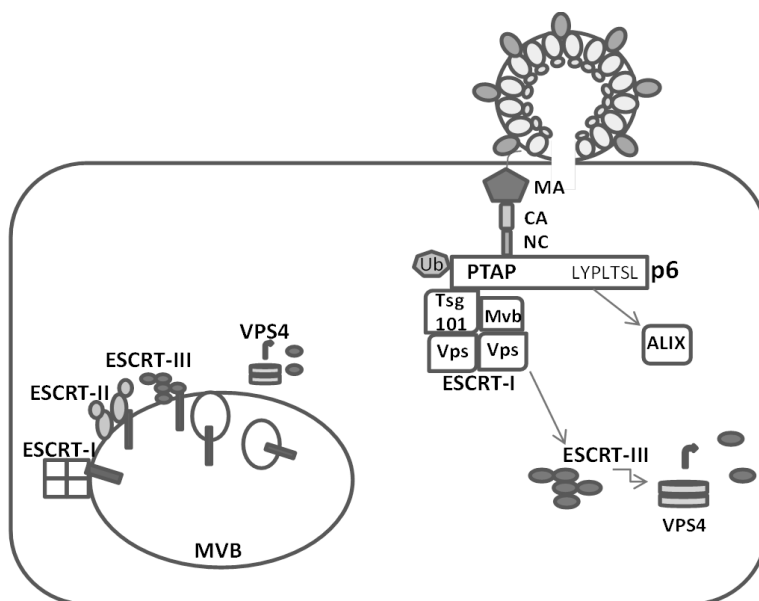


Fig. 4. ESCRT-recruitment by HIV-1 p6-late domain.

The ESCRT-II complex is omitted as its role in HIV-1 release has been questioned (Langelier et al. 2006). Simplified from Pornillos et al. 2007).

The role of downstream sequence LYPXnL has been shown to bind a protein called ALIX/AIP1 (Munshi et al. 2007, Strack et al. 2003). ALIX/AIP1 in turn binds Tsg101/ESCRT-I and CHMP4/ESCRT-III suggesting that ALIX/AIP1 may functionally link different ESCRT-complexes on the limiting membrane of MVB (Odorizzi et al. 2003, von Schwedler et al. 2003). Recent data shows that ALIX/AIP1 has also a binding site on NC-domain of HIV-1 Pr55^{gag}, perhaps providing alternative links between Pr55^{gag} and ESCRT-machinery (Popov et al. 2008, Popova et al. 2010).

ALIX/AIP1 has been shown to be a crucial facilitator of virus particle release in the context of retroviruses that rely only on single, LYPXnL-type late domain, such as EIAV (Fisher et al. 2007, Martin-Serrano et al. 2003). The role of ALIX/AIP1 in HIV-1 budding, which mainly rely on interaction of PTAP-motif with Tsg101 was shown to be auxiliary rather than indispensable if PTAP-motif was functional (Fisher et al. 2007, Fujii et al. 2009). Another study suggested that the role of ALIX/AIP1 is to recruit Nedd4-1 ubiquitin ligase through LYPXnL-motif to facilitate HIV-1 release in PTAP/ESCRT-independent manner (Sette et al. 2010). This alternative pathway

could provide a means to ensure an effective particle release for instance in cell types where the availability of Tsg101 is limiting (Usami et al. 2008).

1.3.5 Endocytic pathway in retrovirus assembly

The first clues that the host cell endocytic process may be involved in the late stages of retrovirus cycle become evident from a study where it was shown that EIAV late domain (YDDL) interacted with an adaptor protein complex AP-2 (Puffer et al. 1998). The functional relevance for this interaction was not determined, but the host cell endocytic membrane trafficking was for the first time connected to the late domain function of viral Gag.

The components of the endocytic machinery have also been shown to interact with HIV-1 Gag and play a role in the intracellular trafficking of Pr55^{gag}. Clathrin adaptor protein AP-2 was shown to bind Pr55^{gag} through a putative YXX ϕ -motif at MA-CA junction (Batonick et al. 2005), while AP-1 and AP-3 have binding sites within the MA-domain (Camus et al. 2007, Dong et al. 2005). Depletion of AP-1 or AP-3 from the virus producing cells was shown to reduce particle release while disruption of Gag-AP-2 binding site surprisingly had an opposing effect. EM data suggested that AP-1 and AP-3 could function upstream of ESCRT-complexes as depletion of these adaptor proteins did not induce late phenotype but diminished the formation of budding profiles at the cell surface (Camus et al. 2007, Dong et al. 2005). Furthermore, these studies suggested that AP-1 and AP-3 are probably involved in sequential steps of the same pathway since they inhibited virion release to the similar extent and could not functionally compensate each other. AP-1 was shown to interact with Tsg101 and Nedd4-1 implicating that AP-1 may facilitate virus release by recruiting ESCRT-machinery to the site of virus assembly (Camus et al. 2007). However, the precise role of these adaptor proteins in Pr55^{gag} trafficking remains obscure since AP-1 and AP-3 normally mediate cargo selection and incorporation into transport vesicles at intracellular compartments (Ihrke et al. 2004, Peden et al. 2004, Reusch et al. 2002). Based on current data it is difficult to establish why an effective particle release is dependent on these mediators because they do not normally operate at the site of HIV-1 budding at the plasma membrane. On the other hand the dependence of virus release on AP-1 and AP-3 may speak on the behalf of Pr55^{gag} routing through intracellular membranes prior to assembly at the plasma membrane (Grigorov et al. 2006, Perlman and Resh 2006).

As mentioned above, the interaction of Pr55^{gag} with AP-2 had negative effect on HIV-1 virus release (Batonick et al. 2005). The normal function of AP-2 is to sort cargo to clathrin coated pits at the plasma membrane (Traub 2003) and apparently AP-2 directs Pr55^{gag} to the endocytosis via binding YXX ϕ -recognition signal at MA-CA junction (Batonick et al. 2005). It was speculated that AP-2-mediated endocytosis may prevent excess budding from the plasma membrane which could be hazardous for producer cells or alternatively, endocytosis of the plasma membrane-bound Gag has a yet unidentified functional role in the productive infectious cycle (Batonick et al. 2005).

1.3.6 Pr55^{gag} ubiquitination

The implications of the potential role of ubiquitin in the HIV-1 assembly came from a study showing that virus particles contained free ubiquitin and a small percentage of Pr55^{gag} was monoubiquitinated at p6-domain (Ott et al. 1998). More evidence suggesting the role of ubiquitination in HIV-1 budding was obtained when it was shown that proteasome inhibitors, by abolishing the cellular pool of free ubiquitin (Mimnaugh et al. 1997) interfered with the particle release and produced a phenotype reminiscent of a late domain defective virus (Patnaik et al. 2000, Schubert et al. 2000, Strack et al. 2000). Furthermore, overexpression of ubiquitin variants mutated in residues important for ubiquitin function was shown to inhibit HIV-1 particle release (Strack et al. 2002).

Later studies indicated that Pr55^{gag} can be monoubiquitinated, in addition to p6-domain in all domains including MA, CA and NC and that PTAP- and LYPXnL-sequences in p6 were not required for Pr55^{gag} ubiquitination (Gottwein and Krausslich 2005). Further studies implicated that lysine residues downstream CA-domain were important for budding, as cumulative mutations of these residues delayed virus release kinetics suggesting that Pr55^{gag} ubiquitination was functionally involved in transient protein interaction network at the budding site (Gottwein et al. 2006). Despite of evidence above, it is not still clear whether Pr55^{gag} ubiquitination is required for efficient release or whether ubiquitination of transacting factors contribute to ubiquitin-dependent particle release (Zhadina et al. 2007).

1.3.7 Role of ubiquitin ligases in HIV-1 budding

It is now well established that PPXY-type late domains recruit Nedd4-like ubiquitin ligases to link viral late domain and ESCRT-machinery (Martin-Serrano et al. 2005), whereas LYPXnL-motifs recruit ALIX/AIP1 to promote virus release (Fisher et al. 2007, Strack et al. 2003). As HIV-1 lacks PPXY-late motif, and mainly rely on PTAP-Tsg101 interaction in recruiting ESCRT-machinery to complete budding (Garrus et al. 2001, VerPlank et al. 2001) the role of ubiquitin ligases in HIV-1 budding have remained relatively scarce. Interestingly however, Nedd4-type ubiquitin ligases can rescue budding of HIV-1 in case of defective PTAP- and LYPXnL-motifs (Chung et al. 2008, Usami et al. 2008).

Two studies have implicated that two distinct ubiquitin ligases, namely Tsg101-associated ligase and Mahoquinin (Amit et al. 2004, Kim et al. 2007) may regulate the activity and stability of Tsg101 instead of that of Pr55^{gag} and mediate capability of Tsg101 to recruit ESCRT-I components to the site of budding (Martin-Serrano 2007). Furthermore, a TGN-associated E3-ubiquitin ligase POSH has been shown to be essential for targeting Pr55^{gag} to the plasma membrane and virus release (Alroy et al. 2005) suggesting that ubiquitination of Pr55^{gag} or other cellular substrates may regulate productive assembly at multiple levels. In accordance with this, a recent report suggested that ALIX/AIP1 is a substrate for POSH and ubiquitination of ALIX/AIP1 by POSH may play an auxiliary role in facilitating HIV-1 release (Votteler et al. 2009).

1.3.8 Tetraspanins

Tetraspanins comprise a large group (33 members) of membrane proteins with four membrane spanning domains. Tetraspanins share little sequence homology, but are conserved in secondary and tertiary structures. They are present in high copy numbers in every cell type although the repertoire varies from a cell type to another (Hemler 2005). Tetraspanins, with their ability to associate with themselves and other receptor molecules are implicated to act as membrane organizers forming functional units called tetraspanin-enriched microdomains (TEMs) at cellular membranes. These domains have been assigned to increasing number of cellular functions such as cell adhesion, fusion and motility, intracellular signaling, actin organization as well as regulation of immune cell functions (Hemler 2005, Yanez-Mo et al. 2009). TEM are also exploited by various pathogens for their cellular entry, for instance HIV, hepatitis C virus (HCV), human papilloma virus (HPV) and *Plasmodium* (Gordon-Alonso et al. 2006, Harris et al. 2008, Silvie et al. 2006, Spoden et al. 2008).

The role of TEMs in assembly, budding and cell-to-cell spread of HIV-1 has been lately under intense investigation. Tetraspanins CD9, CD53, CD63, CD81 and CD82 have been found to form TEMs at the plasma membrane of various cell types and HIV-1 structural proteins along with ESCRT-I components were shown to co-localize to these discrete domains. At least some of these tetraspanins are also incorporated into virions also suggesting that TEMs are gateway for HIV-1 egress (Booth et al. 2006, Deneka et al. 2007, Nydegger et al. 2006). Furthermore, tetraspanins CD63 and CD81 were shown to be recruited to the specific, actin-dependent, Env-induced structures called virological synapses (VS) (Jolly et al. 2004, Jolly and Sattentau 2007) implicating in their role in cell-to-cell transmission of the virus. In addition, it has been suggested that CD81 in T-cells and CD63 in macrophages are of particular importance in organizing assembly platform for HIV-1 as downmodulation of these molecules impairs the particle production by several fold (Chen et al. 2008, Grigorov et al. 2009) In contrast, however, some studies have rendered tetraspanins dispensable for budding (Krementsov et al. 2009, Ruiz-Mateos et al. 2008).

Some evidence suggest that rather than serving as assembly facilitators or budding co-factors, tetraspanins actually negatively regulate cell-to-cell transmission and at least when overexpressed, reduce particle infectivity (Krementsov et al. 2009). In accordance with the latter observation, knockdown of tetraspanins in virus producing cells failed to increase cell-free infectivity (Ruiz-Mateos et al. 2008, Sato et al. 2008). In contrast though, opposing conclusions were drawn from knockdown studies of CD81 (Grigorov et al. 2009). Evidently, some studies speak on behalf of tetraspanins and TEMs owing an importance in the late steps of HIV-1 replication cycle, while critical data suggests merely a restrictive function for these membrane domains. Clearly more studies are required to resolve these apparent controversies.

1.3.9 Actin cytoskeleton and assembly

The role of actin and cytoskeletal components in virus release has been relatively poorly studied. It have been known for years that both actin and various actin binding proteins are found inside the virus particles and that actin-associated with the NC-domain of HIV-1 (Ott et al. 2000, Wilk et al. 1999). Also, virus release is impaired upon application of actin modulating agents, such as cytochalasin D or

latrunculin B (Sasaki et al. 1995). These studies however omit the fact that pharmacological agents may have global effects on the cellular functions. In principle though, actin could have either positive role in promoting the formation of a budding structure or negative, acting as a barrier for an assembling virion (Fackler and Krausslich 2006). Studies exploiting microscopic techniques have shown that HIV-1 remodels actin cytoskeleton of the host. Particularly, actin bundles were seen to assemble at the beginning of the virus budding process and while these disappeared immediately after a nascent virion left the host cell (Carlson et al. 2010, Gladnikoff et al. 2009). Although actin dynamics may not be critical for budding, this conceptually shows that retroviruses can rely on actin-driven mechanism when releasing particles.

2 AIMS OF THIS THESIS

1. To critically evaluate the possible role of lipid rafts in HIV-1 assembly and budding.
2. To identify viral and cellular factors involved in HIV-1 Pr55^{gag} assembly.
3. To probe for possible mechanism of endocytosis involved in Vpu-independent uptake of Pr55^{gag}-complexes.

3 MATERIALS AND METHODS

3.1 SEMLIKI FOREST VIRUS (SFV) EXPRESSION SYSTEM OF HIV-1 GAG

For studies investigating the association of HIV-1 Gag with lipid rafts we used Semliki forest virus (SFV)-driven expression of Pr55^{gag} (Liljestrom and Garoff 1991). Briefly, SFV structural genes have been replaced with the coding region of Pr55^{gag} fused with the capsid (C)-protein of SFV. SFV-C region works as a translational enhancer which ensures high production of the gene of interest. The recombinant SFV particles were produced by co-electroporation of Baby Hamster Kidney 21fibroblast cells with *in vitro* transcribed RNA from the expression plasmid encoding C/HIV-Pr55^{gag} and containing the SFV RNA packaging signal and with helper RNA. The helper RNA provides the SFV structural proteins but lacks the packaging signal. Recombinant SFV particles, capable for a single-round infection were made carrying the sequence of C/HIV-Pr55^{gag}. C-region of the translated fusion protein is cleaved by an autoprotease activity contained in the C-region (Aliperti and Schlesinger 1978). The recombinant SFV particles were used for expression of HIV-1 (strain BH10) Pr55^{gag} in Jurkat T-cells. Also, control proteins CD55, Tr2Δ (Suomalainen and Garoff 1994) and influenza NP (Zhao et al. 1998) were expressed in Jurkat T-cells by using SFV-expression system.

3.2 CYTOMEGALOVIRUS (CMV) PROMOTER-DRIVEN EXPRESSION OF HIV-1 GAG

To obtain more sustained level of Pr55^{gag} expression, nuclear cytomegalovirus (CMV) promoter-driven gene expression was used. pCMV-HIVgag was transfected to 293T-cells by Ca₂PO₄-precipitation technique.

3.3 METABOLIC LABELING OF JURKAT-CELLS WITH ³⁵S-METHIONINE

Recombinant SFV-infected Jurkat-cells were starved in methionine-free minimum essential medium for 30 min and subsequently metabolically labeled in methionine-free modified Eagle's medium containing 100 µCi ³⁵S-methionine per ml for 15 min and chased for various times in RPMI-BSA medium containing 10-fold excess of cold methionine (0-180 min).

3.4 CELLULAR FRACTIONATION FOR ANALYSIS OF PR55^{GAG} RAFT ASSOCIATION

- (i) Total cell lysates.

Pulse-chased Jurkat cells were taken up into lysis buffer (10 mM Tris-HCl, pH 7.4, 150mM NaCl, 1 mM EDTA, 1% Triton X-100, protease inhibitor cocktail) and lysed at 0°C for 30 min. Lysate was mixed with 56% iodixanol (Optiprep) and applied to the iodixanol flotation gradient (see Fig.5).

(ii) Total membrane fraction from crude cell homogenates.

Pulse-chased Jurkat cells were taken up into homogenization buffer at cold (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, protease inhibitor cocktail) and homogenized with a tight-fitting Dounce homogenizer. Immediately after homogenization NaCl was added to 150 mM and homogenate was mixed with 100% Nycodenz to give final 60% Nycodenz concentration. The sample was overlaid with 50% Nycodenz and a layer of homogenization buffer supplied with 150 mM NaCl. The gradient was centrifuged in an SW50.1 rotor at 63 000 x g for 45 min at +4°C. The total membrane fraction at the buffer/50% Nycodenz interphase was collected, solubilized with 1% Triton X-100 for 30 min either at 0°C or at +37°C and applied to the iodixanol flotation gradient.

(iii) Total membrane fraction from post-nuclear supernatants.

Pulse-chased Jurkat cells were homogenized (in buffer containing 25 mM Hepes-KOH pH 7.4, 0.25 M sucrose, 4 mM MgCl₂ and protease inhibitor cocktail) by using a tight-fitting Dounce homogenizer. The crude cell homogenates were centrifuged at 1400 x g for 4 min at +4°C to pellet nuclei and cellular debris. The post nuclear supernatant (PNS) was fractionated on the Nycodenz step gradient described above to separate membranes from cytosolic material.

The total membrane fraction from buffer/50% Nycodenz interphase was mixed with 1% Triton X-100 and after 30 min of incubation either at 0°C or +37°C the extracted membranes were analyzed on iodixanol flotation gradients. In the case of Brij98 extraction, the total membranes were mixed with 0.5% Brij98 and incubated for 5 min at +37°C. The extract was applied to a slightly modified density gradient in order to get a more precise separation of detergent resistant membranes from other floating complexes (see Fig. 5b).

3.5 PURIFICATION OF VLPS FOR THE ANALYSIS OF PR55^{GAG} RAFT ASSOCIATION

Recombinant SFV-infected Jurkat cells were metabolically labeled with ³⁵S-methionine for 3 h. Culture media were collected and cellular debris was removed by centrifugation at 200 x g for 4 min. VLPs were purified from the culture media by a two consecutive gradient centrifugations. The culture supernatant was first applied onto a linear 5-20% iodixanol gradient (Hammarstedt et al. 2000) and centrifuged in an SW40 rotor at 160 000 x g for 1 h at cold. VLP-containing fractions were pooled and mixed with dense iodixanol (56%). This was overlaid by layers of 27%, 20% and 5% iodixanol respectively. After centrifugation in a SW41 rotor at 77 000 x g for 14 h at +4°C VLPs were collected from the 20%/27% iodixanol interphase, extracted

with Triton X-100 or Brij98 under conditions described above and analyzed on iodixanol flotation gradients.

3.6 IODIXANOL FLOTATION GRADIENTS

The figure below depicts the compositions of Optiprep-gradients used to assess the possible association of Pr55^{gag}, virus-like particles (VLPs) and control proteins with raft-like membranes. Gradients were centrifuged in an SW41 rotor at 77 000 X g for 14 h at +4°C. One-milliliter fractions were collected from the top and equal aliquots of each fraction were analyzed by SDS-PAGE. Radioactivity in Pr55^{gag}, NP or TrΔ2-bands were analyzed by using BAS-III phosphoimager system. The amount of protein in each fraction was expressed as a percentage of the total (the sum of all fractions). SFV1-CD55 infected cells were prepared 5 h post infection and proteins in gradient fractions were precipitated with 10% trichloroacetic acid and analyzed by SDS-PAGE followed by Western blotting with anti-CD55 antibody. Endogenous Lck was analyzed from gradient fractions by Western blotting with anti-Lck antibody.

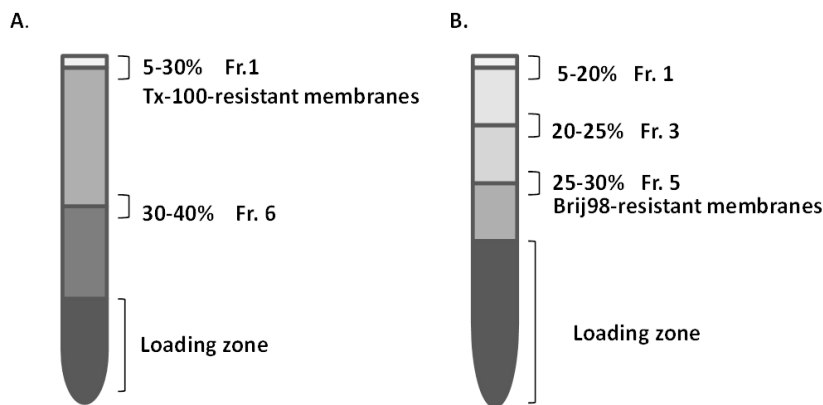


Fig. 5a) Triton X-100 gradient b) Brij98 gradient

3.7 ANALYSIS OF ³²P-ORTHOPHOSPHATE AND ³⁵S-METHIONINE LABELED VLPs

Jurkat T-cells were prelabeled with ³²P-orthophosphate for 40 h (Hammarstedt et al. 2000) and infected with SFV-C/HIVgag. Four hours post infection cells were metabolically labeled with ³⁵S-methionine and VLPs were collected for 3 h and purified as before. Culture medium from SFV-C/NP-infected Jurkat cells was prepared in similar way and used as a control to estimate the contamination of VLP fraction by cell-shedded microvesicles. Triton X-100 or Brij98-extracted VLPs and NP control were fractionated on iodixanol flotation gradients as described above, except the centrifugation used was significantly shorter (4,5 h). Buoyant Pr55^{gag} complexes were collected from 30%/40%-iodixanol interphase (or the 25% zone in

the case of Brij98 extraction), diluted with homogenization buffer and pelleted by centrifugation in a SW41 rotor at 160 000 x g for 1h at +4°C. The pelleted Pr55^{gag} complexes and non-extracted VLPs were taken up into 3% SDS and analyzed on 20% SDS-polyacrylamide gels. The ³²P-signal was determined by BAS-III phosphoimager system. ³⁵S-methionine label in extracted and non-extracted VLP samples was used to ensure that equal amounts of Pr55^{gag} complexes were loaded on 20% gels for phospholipid analysis.

3.8 PRODUCTION AND ANALYSIS OF ³H-CHOLESTEROL LABELED VLPS

Jurkat T-cells were infected with SFV-C/HIVgag or /NP for 30 min. The virus inoculum was removed and cells were metabolically labeled with ³H-cholesterol (10 µCi/ml) for 4 h in RPMI-BSA medium. The radioactive cholesterol was removed and VLPs were collected for 3 h. VLPs were harvested from the culture medium by a two-step procedure as before. VLPs collected from the second purification gradient were diluted and pelleted by a 2-h centrifugation at 173 000 x g in an SW50.1 rotor (+4°C). Pellets were solubilized with Triton X-100 or Brij98 in small volume under conditions used before and extracts were analyzed on short iodixanol flotation gradients. These consisted of iodixanol solutions similar to described before, but the volumes were significantly smaller. The gradients were centrifuged in TLS-55 rotor at 166 000 x g for 4h at 4°C. Fractions were collected from the top and analyzed by scintillation counting. The counts from the NP control were subtracted from the Pr55^{gag} VLP values and the amount of ³H-cholesterol radioactivity in different fractions was expressed as a percentage of the total.

3.9 CONFOCAL FLUORESCENCE MICROSCOPY

Subconfluent 293T-cells seeded onto glass coverslips were transfected with 3 µg of pCMV-HIVgag. After 20-22 h, cells were placed on ice and incubated for 20 min with fluorescein isothiocyanate-conjugated cholera toxin B subunit (10 mg/ml). After extensive washes cells were fixed with 4% formaldehyde (unpatched cells) or incubated on ice in PBS containing anti cholera toxin antibodies for 30 min and subsequently transferred to 37°C for 30 min for patching. Cells were fixed with 4% formaldehyde, permeabilized with 0,3% Triton X-100 and stained with mouse anti-Pr55^{gag} antibodies (EF-7 or 38:9, gifts from J. Hinkula) and tetramethyl rhodamine isocyanate-conjugated anti-mouse IgG. Preparations were mounted and analyzed with Carl Zeiss LSM 510 confocal system by using lasers with a wavelength of 488 nm and 514-543 nm.

3.10 PROVIRAL PLASMID CONSTRUCTS AND PRODUCTION OF VSV-G PSEUDOTYPED VLPS

In studies addressing the membrane targeting of the newly synthesized Pr55^{gag} we turned into expression of Pr55^{gag} and Gag/Pol from the proviral genomes. The viral proteins expressed from the context of virus genome mimic more closely the natural

infection than does the expression of single viral proteins from the expression constructs. For safety reasons certain areas were deleted from the proviral genomes in order to minimize the theoretical possibility of recombination events that could lead to production of a virus capable of productive infection during the course of the experiment. Table 2 summarizes the proviral plasmid constructs used in this study.

Proviral plasmid construct	Phenotype	Gag-expression profile
pHXB2D	<i>vpu-, vpr-, nef-</i>	Processed (MA, CA, NC, p6, p1, p2)
pHXB2D-Gag	<i>vpu-, vpr-, nef-, pol-</i>	Precursor Pr55 ^{Gag}
pHXB2D-GagHA	<i>vpu-, vpr-, nef-, pol-</i>	Pr55 ^{Gag} with C-terminal HA-tag
pHXB2D-Gagflag	<i>vpu-, vpr-, nef-, pol-</i>	Pr55 ^{Gag} with C-terminal flag-tag
pHXB2D-(ΔVpu/ΔEnv/ΔNef)	<i>vpu-, vpr-, nef-, env-</i>	Processed (MA, CA, NC, p6, p1, p2)
pNL4-3(Gag)	<i>pol-</i>	Precursor Pr55 ^{Gag}
pNL4-3(Gag/ΔVpu)	<i>pol-, vpu-</i>	Precursor Pr55 ^{Gag}
pNL4-3(GagΔp6/ΔVpu)	<i>pol-, vpu-, gag/p6-</i>	Precursor Pr55 ^{Gag} w/o p6-domain
pNL4-3(ΔEnv/ΔNef)	<i>env-, nef-</i>	Processed (MA, CA, NC, p6, p1, p2)

Table 2. The proviral constructs and the phenotype of lentiviral vectors.

Vesicular stomatitis virus (VSV) G-protein pseudotyped infectious lentivirus vectors carrying modified HIV-1 genomes were produced by co-transfecting pCMVΔR8.91 and pMD2.VSV-G (gifts from D. Trono) together with a plasmid directing the synthesis of a recombinant HIV-1 genome into 293T cells by a Ca₂PO₄ precipitation method. Next day the transfection medium was changed to fresh culture medium and viruses were harvested 24 h later. HeLa H1-cells were infected with these virus stocks (~1-5 PFU/cell) for ~24 h.

3.11 SUBCELLULAR FRACTIONATIONS BY SILICA COATING

VSV-G-pseudotyped lentivirus vector-infected, metabolically labeled (³⁵S-methionine, 50 μCi for 30 min) and chased HeLa H1-cells were detached from culture plates by a 2-min incubation in PBS- 0.02% EDTA at 37°C. Suspension cell were coated at +4°C with 1% cationic silica beads in coating buffer (20 mM MES, 150 mM NaCl, pH 6.66-6.68). Coated cells were treated with 1 mg/ml sodium polyacrylic acid to give the net negative charge to coated plasma membranes. Cells were taken up into homogenization buffer (25 mM Hepes-KOH, 150 mM NaCl, 2 mM MgCl₂, protease inhibitor cocktail, 2,5 mg/ml heparin) and homogenized with a tight-fitting Dounce homogenizer.

The crude cell homogenates were mixed with 100% Nycodenz solution to give a final 60% Nycodenz concentration. The sample was placed on cushion of 70% Nycodenz and overlaid with 50% Nycodenz and 25 mM Hepes-150 mM NaCl-1 mM EDTA-solutions. The gradient was centrifuged in an SW55 Ti rotor at 63 000 x g for 45 min at +4°C.

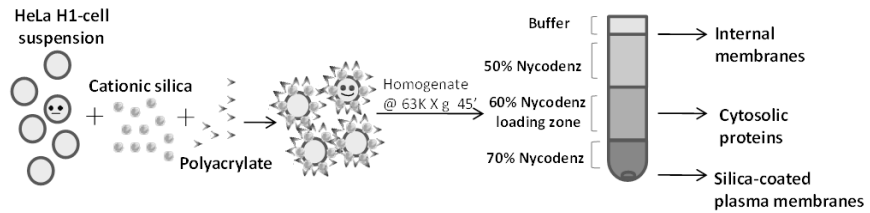


Fig.6. A schematic drawing of silica coating procedure.

The pelleted plasma membrane fraction was resuspended in hot 0.5% SDS-0.5M NaCl-buffer, boiled for 5 min and silica beads were removed from the sample by a brief centrifugation (1 min) full speed in a tabletop Eppendorf centrifuge. The internal membrane fraction from the buffer/50% Nycodenz interphase was collected, diluted fivefold with 25 mM Hepes-150 mM NaCl-1 mM EDTA-buffer and membranes were pelleted by centrifugation at 100 000 x g for 1 h at +4°C. The membrane pellet was solubilized as described above for the plasma membrane. Both resuspended membrane fractions were diluted with a 10-fold excess of lysis buffer (1% Triton X-100, 25 mM Hepes, 150 mM NaCl, 1 mM EDTA, protease inhibitor cocktail) and Pr55^{ag} proteins were immunoprecipitated from the lysates with mouse anti-HIV-1 CA-antibodies. HA-tagged Pr55^{ag} was immunoprecipitated by using rabbit polyclonal anti-HA and FLAG-tagged Pr55^{ag} by using mouse monoclonal anti-FLAG-antibody.

3.12 BIOTINYLATION OF CELL SURFACE PROTEINS

HeLa H1-cells were brought into suspension by a brief treatment with PBS-0.02% EDTA at 37°C and incubated in PBS containing 0.5 mg/ml of EZ-Link Sulfo-NHS-SS-biotin for 30 min at 0°C. The sample was quenched with 50 mM NH₄Cl, coated with 1% silica beads and fractionated as described above. Biotin-tagged proteins at the plasma membrane and the internal membrane fractions were visualized by dot blots using streptavidin-peroxidase polymer.

3.13 CONTROLS FOR SUBCELLULAR FRACTIONATION

Biotinylated transferrin proteins (15 µg/ml) bound to HeLa H1 cells by incubation at 0°C or internalized at 37°C for 20 min in a serum-free medium were used as markers for the plasma membrane and recycling endosomes respectively. In the case of internalized transferrin cells were incubated in buffer (pH 4,5) containing 25 mM

citric acid, 24 mM trisodiumcitrate, 280 mM sucrose and 10 μ M deferoxamine mesylate for 2 min to remove the cell surface-bound transferrin prior to silica coating and fractionation. Transferrin proteins in the plasma membrane and internal membrane fractions were visualized by Western blotting with streptavidin-peroxidase polymer.

The amount of endoplasmic reticulum (ER) marker protein calnexin and the late endosome/lysosome marker protein Lamp-1 in the plasma membrane and internal membrane fractions were determined by Western blotting with rabbit polyclonal anti-calnexin and goat polyclonal anti-lamp-1, respectively.

3.14 ELECTRON MICROSCOPY

HeLa H1-cells infected with recombinant lentiviral vectors were fixed at 24 h post infection. Fixed samples were first stained with 1% osmium tetroxide, followed by staining with 0.5% alcoholic uranyl acetate, dehydration in ethanol and embedding in Agar 100 resin. Sections (60 nm) were cut on an LKB-microtome and imaged by electron microscopy.

3.15 FILIPIN AND AMILORIDE TREATMENTS

Testing of cholesterol-dependence of Pr55^{gag} endocytosis HeLa H1-cells were treated with filipin complex (3 μ g/ml). Filipin was added to the chase medium either at the beginning of the chase or after 30 min of the chase and continued for further 180 min.

To test whether Pr55^{gag} occurred by macropinocytosis-like activity, 100 μ M amiloride, a potent inhibitor of induced macropinocytosis, was added to the chase medium. To measure macropinocytosis, cells were preincubated in minimum essential medium containing 0.2% BSA (MEM-BSA) for 30 min and then shifted to MEM-BSA containing 1 mg/ml of lysine fixable fluorescein isothiocyanate-conjugated dextran for 10 min. After washing cells were further incubated at MEM-BSA for 5 min. To strip the plasma membrane-associated dextran cells were placed on ice and incubated for 5 min in buffer (pH 5.5) containing 100 mM sodium acetate and 50 mM NaCl. Cells were detached from culture plates by incubation in PBS-0.02% EDTA on ice for 20 min, fixed with 2% paraformaldehyde and analyzed by a fluorescence-activated cell sorter.

3.16 VLP RESCUE FROM VPU Δ -VIRUS INFECTED HELA H1 CELLS

Infected HeLa H1-cells were metabolically labeled with ³⁵S-methionine and VLPs were collected for 180-240 min. Culture supernatants were filtered to remove the cellular debris, layered onto 20% sucrose cushion and centrifuged at 100 000 x g for 1 h. These pellets representing constitutively released extracellular particles were analyzed by SDS-PAGE.

To analyze the amount of VLPs tethered at the cell surface cells were incubated with trypsin (0.5 mg/ml) for 10 min at 37°C. Reaction was stopped by adding culture medium containing 7% fetal bovine serum. Cells were pelleted and culture supernatants were filtered and released VLPs were pelleted through a sucrose layer as described for constitutively released VLPs.

3.17 RNA-INTERFERENCE

Short interfering RNAs (siRNA) against Tsg101 or scrambled sequence control siRNA were transfected to HeLa H1-cells by using Oligofectamine. 20 pmol siRNA was used for each 10 cm plate (Garrus et al. 2001). Mock cells were treated with transfection reagent alone. After an overnight incubation cells were infected with recombinant lentivirus vectors and analysis of Pr55^{gag} was carried out 24 h post infection. The cellular Tsg101 levels were monitored by Western blotting using anti-Tsg101 antibody. Intracellular actin level was used to control the specificity of Tsg101 siRNAs.

Clathrin heavy chain (CHC) knockdown was achieved by using siRNAs against CHC. 10 cm HeLa H1 plates were transfected by using 40 pmol siRNA (Hinrichsen et al. 2003) and siLentFect according to manufacturer's instructions. Mock cells were treated with siLentFect alone. About 22 h post transfection cells were infected with recombinant lentivirus vectors and analyzed 24 h post infection. Intracellular level of CHC was determined by Western blotting with polyclonal goat anti-CHC antibody. The polyclonal anti-glyceraldehyde-3-phosphate dehydrogenase antibody was used as a control.

To analyze the effect of CHC knockdown on Pr55^{gag} endocytosis siRNA treated and infected HeLa H1 cells were incubated on ice for 30 min in PBS containing 0.2% bovine serum albumin and 500 ng/ml human biotin-tagged transferrin. After removal of unbound transferrin, cells were shifted to MEM-BSA and incubated at +37°C for 0-15 min to allow the internalization of cell-associated transferrin. Cells were then shifted back on ice and treated with an acid wash (0.2 M Na-citrate pH 4.5, 0.5 M NaCl) and rinsed extensively. Cell surface-bound transferrin was removed by a trypsin treatment and the reaction was stopped by inactivating trypsin by culture medium containing 7% fetal bovine serum. Cells were homogenized and PNS was prepared. The amount of trypsin-resistant biotinylated transferrin in PNS was analyzed by Western blotting using streptavidin-peroxidase polymer.

4 RESULTS

4.1 HIV-1 ASSEMBLY AND LIPID RAFTS: PR55^{GAG} ASSOCIATES WITH MEMBRANE DOMAINS THAT ARE LARGELY RESISTANT TO BRIJ98 BUT SENSITIVE TO TRITON X-100 (PAPER I)

4.1.1 Sample preparation strongly affects the outcome of flotation pattern after Triton X-100 extraction

Jurkat T-cells were infected with recombinant SFV-C -particles carrying HIV-1 Pr55^{GAG} coding region. High level of Pr55^{GAG} synthesis was obtained when using SFV-driven expression system (data not shown). Cells were metabolically labelled with ³⁵S-methionine and chased for various times. Cell homogenates were either directly solubilized with Triton X-100 or fractionated prior to the detergent treatment. Solubilized cell extracts were loaded on iodixanol gradients. During ultracentrifugation the detergent resistant membrane domains floated to a light-density fraction while detergent soluble material stayed in the loading zone.

In the first experiments a crude cell homogenate was used as a starting material for detergent extraction at cold. Our control for a raft associated protein, GPI-anchored CD55 (Solomon et al. 1998) efficiently floated to fraction 1 (5%/30% interphase) on an iodixanol density gradient as expected. In contrast to raft-resident CD55, only a minority of HIV-1 Gag was found in fraction 1 and the most of the protein floated to 30%/40% iodixanol interphase (fractions 5 and 6) instead. Less than a half of the total amount of Gag was found in the soluble pool (fractions 9 to 12), this fraction most likely presenting cytosolic, non-membrane associated Gag or detergent soluble Gag of membranous origin.

In parallel to HIV-1 Gag and raft-associated CD55, various other control proteins were tested for their flotation phenotype. Influenza virus nucleoprotein (NP), primarily a nucleus-associated protein and the cytosolic nucleocapsid protein C of SFV both floated to the 30%/40% iodixanol interphase after Triton X-100 extraction. Likewise, a non-raft membrane transferrin receptor (TR2Δ) ended up to the 30%/40% iodixanol interphase as well. In order to obtain an insight into the flotation behaviour of an analogously membrane-associated protein as Pr55^{GAG}, the distribution of an endogenously expressed Src family kinase Lck on iodixanol gradient was analyzed. Lck was mainly detergent soluble as had been previously reported (Hawash et al. 2002), but a fraction of this protein also concentrated at the 30%/40% iodixanol interphase as well as some Lck was also found in fraction 1.

The results from above suggested, that many different types of proteins from the crude cell homogenates tended to float to the 30%/40% iodixanol interphase and therefore it was difficult to assess whether this flotation phenotype correlated with Pr55^{GAG} being a raft-associated protein. Furthermore, an extreme viscosity of SDS-

solubilized fraction 5 and 6 samples implicated that chromatin contained in the crude cell homogenates might have ended up to this region of the gradient during the ultracentrifugation. The unspecific trapping of Pr55^{gag} or other marker proteins to the nuclear remnants may therefore explain the observed flotation phenotype of the proteins studied. In order to circumvent this potential source of artifacts, in next experiments an extra step was added to the extraction protocol.

Recombinant SFV-infected Jurkat cells were treated as before but total membranes from these crude cell homogenates were first isolated by a Nycodenz flotation gradient. The total membrane fraction contained both the plasma membrane and intracellular membranes.

When using total membranes as a starting material for the extraction, raft-associated CD55 still floated to fraction 1, suggesting that total membrane isolation by a Nycodenz gradient had no adverse effect on detergent extraction assay. Some of CD55 though remained in the bottom of the gradient this phenomenon most likely reflects the transient nature of lipid rafts or limited temporal association of raft proteins in these structures. The flotation phenotype of non-raft protein TRΔ2 remained largely unchanged compared to previous set of experiments, except the buoyant complexes now shifted from fraction 6 to fraction 5. In the case of Lck, the relative amount of the protein in the middle densities clearly reduced and the buoyant complexes shifted now from fraction 5 to fraction 6. We could not offer an explanation for these minor shifts occurring in this assay.

The analysis of influenza NP suggested that the modified extraction scheme was still prone to artifacts caused by unspecified nuclear material. As NP is a nucleus-resident protein our analysis clearly indicated that chromatinous material still contaminated our total membrane fraction as about 64% of intracellular NP co-fractionated with the total membranes in a Nycodenz gradient. After Triton X-100 solubilisation at cold and a run in an iodixanol gradient most of the membrane-associated NP floated to fractions 2 to 5 as did a small amount of co-purified cytosolic nucleocapsid protein (C) of SFV. When total membranes from NP expressing cells were solubilised at 37°C, in conditions that should lead to the complete solubilisation of Triton X-100-resistant membranes, NP was nearly exclusively found at the bottom of the density gradient. As it can be argued that floating NP-complexes could be associated with detergent resistant microdomains, we still favour the explanation of these less dense complexes originate from unspecific floating of chromatin-associated material to the intermediate densities.

The analysis of HIV-1 Pr55^{gag} by fractionation scheme above yielded both light density and intermediate density complexes. When the total membranes from Pr55^{gag} expressing cells were solubilised at physiological temperatures prior to the iodixanol density gradient, membrane-associated Gag was now equally distributed to the soluble pool and at the 30%/40% iodixanol interphase. According to these results, it would have been conceivable to suggest that newly synthesized Pr55^{gag} is at least partially associated with Triton X-100-resistant lipid rafts. But when the flotation profiles of NP and Pr55^{gag} were compared, a strikingly similar pattern of the behaviour upon Triton X-100 solubilisation at +37°C was observed, i.e. low density

complexes shifted to the soluble pool upon the extraction procedure. As both proteins are nucleic acid binding proteins these results led to a mere conclusion that these heterogeneous Triton X-100 insoluble (at 0°C) light density complexes most likely still represented structures that associated with unspecified nuclear remnants rather than represented Triton X-100-resistant lipid rafts.

In order to reduce potential artifacts caused by chromatin in our samples we further modified the flotation scheme. Infected Jurkat cells were taken up into a hypotonic homogenization buffer supplemented with Mg^{2+} , which promotes the integrity of the nuclear envelope. Prior to isolating the total membranes by a flotation gradient, the nuclei were removed from the crude lysate by a brief centrifugation. Post nuclear supernatant was then applied to the Nycodenz gradient and the total membrane fraction was then analyzed after Triton X-100 solubilisation on an iodixanol step gradient as before. When using PNS as a starting material for Triton X-100 extraction, the influenza NP did not co-purify with the cellular membranes suggesting nuclear remnants were now qualitatively removed.

At 0 min chase point Pr55^{gag} complexes were partially concentrated at the intermediate densities, while some of the protein scattered over fractions 9 to 12. After 2 hours chase the soluble pool had disappeared with Pr55^{gag} now exclusively concentrating at fractions 5 and 6. This shift most likely reflected the increased affinity of newly synthesized Pr55^{gag} to the membranes due to multimerization of Pr55^{gag} precursors (Lindwasser and Resh 2001, Ono and Freed 2001). Detergent extraction of these complexes at +37°C did not yield any changes in buoyant density. The analysis of Triton X-100-extracted extracellular VLPs yielded same buoyant complexes that ended up to the 30%/40% iodixanol interphase. This flotation profile of VLPs remained unchanged even if Triton X-100 treatment was performed at the physiological temperature. These results strongly suggested that Pr55^{gag} does not necessarily localise to the Triton X-100 insoluble lipid rafts during the assembly.

4.1.2 Virus-like particles are largely devoid of raft-like membranes

As the results from above suggested that Pr55^{gag} may not be a raft-associated protein we decided to further assess the nature of Pr55^{gag} complexes floating to 30%-40% iodixanol interphase. If these complexes were genuine Triton X-100-resistant lipid rafts then the lipid composition of these floating complexes should resemble those of naturally found in raft microdomains, i.e. they should be enriched in cholesterol and sphingomyelin. As VLP preparations can be obtained relatively free of contaminating cellular material it was a rational choice to use VLPs as a starting material for isolating floating Pr55^{gag} complexes.

To assess the cholesterol content of these complexes we labelled SFV/C-HIVgag infected Jurkat cells with 3H -cholesterol and ^{35}S -methionine. VLPs were recovered from the culture media by a two-step gradient centrifugation. Triton X-100 solubilised VLPs were run in an iodixanol gradient and the distribution of 3H -cholesterol and Pr55^{gag} in gradient fractions was analyzed. Our analysis showed that Pr55^{gag} signal was mainly found at the 30%/40% iodixanol interphase as before,

while the signal produced by ^3H -cholesterol was almost exclusively distributed to the bottom of the gradient representing the Triton X-100 soluble pool. This data clearly indicated that flotation of Pr55^{gag} is not due to these complexes being physically associated with the membrane cholesterol.

To determine whether membrane phospholipids (including sphingomyelin) instead of cholesterol are retained on the virion envelope upon Triton X-100 extraction and could explain the floating phenotype of Pr55^{gag} complexes. We labelled SFV-C/HIVgag infected Jurkat cells with both ^{35}S -methionine and ^{32}P -orthophosphate. VLPs were purified as described earlier and analyzed on iodixanol step gradient. The material was recovered from the 30%/40% iodixanol interphase and concentrated by pelleting. SDS-PAGE analysis of Triton X-100-extracted and non-triton-treated sample indicated that Triton X-100-extracted VLPs are largely devoid of phospholipids. After careful analysis of possible contamination by microvesicles of cellular origin and subtraction of background values we estimated that only at maximum of 12-17% of virion-associated phospholipids remained attached to buoyant Pr55^{gag} complexes after Triton X-100 treatment. These data together suggested that conventional Triton X-100 extraction at cold readily solubilized the bulk phospholipids and cholesterol from the envelope of the VLPs thus leaving the explanation for observed intermediate buoyancy unresolved at this point.

4.1.3 Confocal fluorescence microscopy reveals the co-localization of Pr55^{gag} with raft-like domains

The data from previous experiments suggested that Pr55^{gag} does not associate with Triton X-100-resistant lipid rafts. This does not however by any means exclude the possibility of Pr55^{gag} being associated with membrane microdomains other than classical Triton X-100-resistant lipid rafts as it is well established that cellular membrane do contain raft domains that are Triton X-100 sensitive (Hao et al. 2001, Pike et al. 2005, Roper et al. 2000, Schuck et al. 2003). To further study the possible association of Pr55^{gag} with lipid rafts we probed the raft association of Pr55^{gag} by confocal fluorescence microscopy. Individual raft domains are smaller than resolution of a light microscopy as such, but they can be induced to form larger patches by antibody cross-linking of raft-resident proteins.

GM1, a lipid raft associated glycolipid was chosen for antibody cross-linking since this ganglioside has also been reported to be incorporated into HIV-1 envelopes (Nguyen and Hildreth 2000). 293T-cells transfected with CMV-HIVgag were incubated with fluorescein isothiocyanate-conjugated cholera toxin B-subunit which specifically binds to GM1. Patching of lipid rafts were induced by an antibody against the toxin. Pr55^{gag} was visualized with an antibody against unprocessed HIVgag and tetramethyl rhodamine isocyanate-conjugated secondary antibody.

The confocal image analysis showed that Pr55^{gag} seems to co-localize with GM1-positive membrane patches. This apparent co-localization does not necessarily reflect the association of Pr55^{gag} with Triton X-100-resistant microdomains, since in this experimental setting the formation of raft patches was artificially induced.

4.1.4 Pr55^{gag} associates with Brij98-resistant but Triton X-100 sensitive membrane domains

To obtain further insight whether Pr55^{gag} localizes to rafts distinct from the Triton X-100 sensitive lipid microdomains we tested the solubility of the newly synthesized Pr55^{gag} with various detergents. Pr55^{gag} was found to be partially resistant for several non-ionic detergents of Brij-series as well as for CHAPS and Lubrol. We decided to focus on Brij98 as lipid rafts can be recovered with this detergent under physiological conditions (+ 37°C) (Drevot et al. 2002). In order to maximize the resolution capacity of the iodixanol gradient we slightly modified the final step of the assay. Instead of using 40%-30%-5% iodixanol solutions in the gradient, we added layers of 25% and 20% iodixanol while omitting the 40% solution.

The analysis of the newly synthesized Pr55^{gag} indicated that at 0 min chase point Pr55^{gag} is partially sensitive to Brij98 extraction but localizes to the Brij98-resistant microdomains in a time-dependent manner. This phenomenon most likely reflected the multimerisation of Pr55^{gag} molecules at the plane of membranes since a mutant lacking domains required for multimerisation (Δ NC) (Burniston et al. 1999, Sandefur et al. 1998) did not associate with Brij98-resistant domains even after a longer chase (120 min) but remained in the Brij98 soluble pool.

We next tested whether these floating Brij98-resistant complexes could be precursors for extracellular VLPs. Gradient-purified, ³⁵S-methionine labelled VLPs were extracted with Brij98 for 5 min at + 37°C and run on modified iodixanol gradients (described above). SDS-PAGE analysis of the gradient fractions showed that Brij98 solubilised VLPs readily ended up to the overlapping region at fractions 3-6 where did intracellular Pr55^{gag} complexes float on a similar gradient. This data strongly suggested that Brij98-resistant membrane domains could represent precursors for extracellular VLPs and therefore function as assembly platforms for HIV-1 Pr55^{gag}.

To further assess the nature of Brij98-resistant complexes the cholesterol content of these floating membrane domains was analyzed. SFV/C-HIVgag infected Jurkat cells were labelled with ³⁵S-methionine and ³H-cholesterol and VLPs were collected and purified as before. After Brij98 extraction VLPs were run on a shortened iodixanol gradient and fractions were analyzed by SDS-PAGE for ³⁵S-methionine signal and by scintillation counting for ³H-cholesterol, respectively. The gradient analysis indicated that majority of the signal for Pr55^{gag} and for cholesterol peaked at the 20%/30% iodixanol interphase suggesting that Brij98 extraction did retain majority of the envelope cholesterol on these floating complexes.

Analogously, the extractability of envelope phospholipids on floating Brij98-treated VLPs was assessed. SVF/C-HIVgag infected Jurkat cells were labelled by ³²P-orthophosphate and ³⁵S-methionine. VLPs were collected and purified from cellular contaminants as before and extracted with Brij98. Influenza NP-expressing Jurkat cells were used as controls for the microvesicular contamination of the VLP fraction. SDS-PAGE analysis of Brij98-extracted VLPs indicated that approximately a half of

the phospholipids remained attached to the floating Pr55^{gag} complexes suggesting that virion envelope is largely resistant to Brij98 extraction.

In conclusion, our data suggested that Pr55^{gag} may associate with lipid domains that are largely resistant to Brij98 extraction but sensitive for Triton X-100. These Brij98-resistant domains could possibly serve as platforms for the Pr55^{gag} assembly. Further studies are required in order to characterise these membrane domains and evaluate their biological relevance.

4.2 VPU AND TSG101 REGULATE INTRACELLULAR TARGETING OF THE HIV-1 CORE PROTEIN PRECURSOR PR55^{GAG} (PAPER II)

4.2.1 Experimental approach

In order to distinguish whether Pr55^{gag} assembles at the plasma membrane or at the internal membranes we employed a method of silica coating to assess the distribution of the newly synthesized Pr55^{gag} between these two membrane compartments. In this method, prior to homogenization and subcellular fractionation HeLa H1-cells were coated with cationic silica beads that give the plasma membrane increased density compared to the internal membranes (Spector et al. 1998). During centrifugation on a Nycodenz step gradient the less dense internal membranes float to the top of the gradient while the plasma membrane fraction is found in the pellet. Amounts of the newly synthesized Pr55^{gag} in the internal membrane fraction (I) and in the PM-fraction (PM) were assessed by immunoprecipitation and by SDS-PAGE analysis of these fractions. Our control experiments with both the plasma membrane-associated proteins and proteins resident in the intracellular compartment indicated that coating of HeLa H1-cells with cationic silica beads enabled an efficient separation of the plasma membrane from the internal membranes.

4.2.2 Newly synthesized Pr55^{gag} is first targeted to the plasma membrane in HeLa H1-cells

HeLa H1-cells were infected with VSV-G pseudotyped lentiviral vectors carrying modified HIV-1 proviral genomes and cells were metabolically labelled with ³⁵S-methionine followed by a chase of various lengths. After employing the silica coating and subsequent analysis, our initial experiments revealed that in HeLa H1-cells Pr55^{gag} can accumulate either at the plasma membrane or at the internal membranes so this cell line was ideal for distinguishing at which membranes the newly synthesized Pr55^{gag} is first targeted.

Our analysis indicated that after a short period of chase (e.g. 5 min) Pr55^{gag} accumulated at the plasma membrane with essentially no labelled Pr55^{gag} in the internal membrane fraction. After a longer chase (e.g. 60 min or 180 min) we observed a slight shift of newly synthesized Pr55^{gag} to the internal membranes, but still the majority of the protein was present at the plasma membrane. An efficient VLP production was observed (pNL4-3/Gag) already after 2 h of chase, determined

by appearance of labelled Pr55^{gag} in the culture medium. Furthermore, in an EM analysis numerous budding profiles were observed at the plasma membrane of pNL4-3/Gag infected cells while the internal membranes were essentially devoid of these profiles. These data together suggested that when expressed from the pNL4-3-proviral construct, the newly synthesized Pr55^{gag} is exclusively targeted to the plasma membrane and is subsequently incorporated into VLPs that bud from the plasma membrane.

4.2.3 Vpu regulates the intracellular localization of Pr55^{gag}

In the next experiments we tested whether Pr55^{gag} had an intrinsic affinity to the plasma membrane or whether other viral proteins had a role in directing Pr55^{gag} assembly to the cell surface in HeLa H1-cells. After having deleted various genes from the original proviral Gag-expressing construct, we found out that deletion of the *vpu* gene caused a dramatic change in the intracellular localization of the newly synthesized Pr55^{gag}.

After a period of a short chase the vast majority of labelled Pr55^{gag} was found at the plasma membrane as before, but during a longer chase Pr55^{gag} was shifted to the intracellular membranes. Upon deletion of *vpu*, the VLP release was also severely affected since the amount of virions found in the culture medium dropped significantly (~18-fold). Similar results were obtained in the context of Gag/Pol co-expression.

When cells infected with Vpu-deleted proviral construct were analyzed by the EM, a dual distribution of the budding profiles was observed. In addition to budding profiles present at the plasma membrane, an endosome-like intracellular compartment loaded with VLP-sized vesicles was detected. Furthermore, occasionally clear budding profiles at intracellular locations were recorded, although they were far more rare than those observed at the plasma membrane.

This data strongly suggested that Vpu regulates the intracellular localization of Pr55^{gag}. Deletion of this viral accessory protein retargets Pr55^{gag} from the plasma membrane to the intracellular compartments by endocytosis with a concomitant decrease in release of extracellular VLPs.

Interestingly, when Pr55^{gag} was fused to carboxyterminal HA-tag, no redistribution of Pr55^{gag}/HA to the internal membranes was observed in the absence of Vpu, suggesting that C-terminus of Pr55^{gag} is mechanistically important for correct trafficking of Pr55^{gag} in the cells. In striking contrast, C-terminal FLAG-tag fused to Pr55^{gag} had no similar effect.

4.2.4 Host protein Tsg101 is required for endocytosis of Pr55^{gag}

It is well documented that PTAP-motif in p6 -domain of Pr55^{gag} binds to a host factor Tsg101 and this interaction is indispensable for the productive release of HIV-1 viral particles from the cell surface (Garrus et al. 2001, Martin-Serrano et al. 2001,

VerPlank et al. 2001, von Schwedler et al. 2003). To test whether Tsg101 is involved in the endocytosis of Pr55^{gag}/ΔVpu a siRNA-mediated downregulation of Tsg101 was employed (Elbashir et al. 2001, Garrus et al. 2001).

In cells expressing reduced levels of Tsg101, the uptake of newly synthesized Pr55^{gag} construct was also reduced compared to control cells transfected with scrambled sequence siRNA. This implicated that endocytic uptake of Pr55^{gag} is mediated by Tsg101. As Tsg101 is a part of ESCRT-machinery (Katzmann et al. 2001) this data further implied that the endosomal sorting complex may be involved in the endocytosis of Pr55^{gag} from the plasma membrane.

According to some speculations the siRNA-mediated knockdown of proteins is not necessarily as specific for their targets as have been thought before since this method has been shown to induce alterations in the levels of non-related proteins and also potentially activate interferon-mediated antiviral pathways (Persengiev et al. 2004, Scacheri et al. 2004, Sledz et al. 2003). Therefore, to assure that inhibition of Pr55^{gag} endocytosis caused by Tsg101 knockdown was not due to potential off-target effect produced by siRNAs, the p6-domain of Pr55^{gag} was deleted. This deletion abolished the Tsg101 interaction site in Pr55^{gag}. Our results indicated that when Pr55^{gag} was truncated just prior to p6 coding sequence and the proviral construct did not express Vpu, the majority of the newly synthesized Pr55^{gag}/Δp6 remained at the plasma membrane. This data confirm that Tsg101/p6-interaction is required for Pr55^{gag} endocytosis in the context of Vpu-negative virus in HeLa H1-cells.

4.3 THE VPU-REGULATED ENDOCYTOSIS OF HIV-1 GAG IS CLATHRIN-INDEPENDENT (PAPER III)

As in previous work we reported that the newly synthesized, plasma membrane-associated Pr55^{gag} is endocytosed in the absence of viral accessory protein Vpu we here further investigate the endocytic phenotype of HIV-1 Gag in HeLa H1-cells.

4.3.1 The Vpu-regulated endocytosis of HIV-1 Gag is independent of clathrin but clathrin knockout leads to a slight assembly defect in the context of Gag/Pol coexpression

RNAi-mediated knockdown of clathrin heavy chain (CHC) was used to probe whether the endocytosis of Pr55^{Gag} is clathrin-mediated. HeLa H1-cells were treated with CHC-siRNAs prior to infection with Pr55^{gag}/ΔVpu and clathrin-mediated endocytosis was probed by measuring the uptake of biotin-tagged transferrin. Our results indicated that clathrin-mediated endocytosis was severely compromised in CHC-siRNA-treated cells but knockdown of CHC did not inhibit the uptake of the plasma membrane-associated newly synthesized Pr55^{gag}. This suggested that endocytosis of Pr55^{gag} is clathrin-independent. In the case of Gag/Pol co-expression, a partial retention of processed Pr55^{gag} (i.e. CA) at the plasma membrane fraction was observed in CHC-knockdown cells. This finding implicated that a subpopulation of virions may be internalized in a clathrin-dependent manner (Neil et al. 2006) or

alternatively, CHC-knockdown had an interfering impact on the late stages of virus assembly in Gag/Pol-expressing cells.

To distinguish between these two alternatives, the production of VLPs from Pr55^{gag} and Gag/Pol-expressing cells was analyzed both in the context of normal levels of CHC and in CHC-siRNA-treated cells. It was suggested first by others (Neil et al. 2006) that in the absence of Vpu HIV-1 Gag may be endocytosed from the plasma membrane as fully formed virions and membrane-bound particles can be released by a brief protease treatment. According to this finding we analyzed both virions produced directly to the culture medium and those that were released by a trypsin treatment. Our results showed that virtually no particles were produced to the culture medium from Pr55^{gag}/ΔVpu-expressing cells, but equal amounts of virions had remained tethered on the surface of both control and CHC-siRNA-treated cells and these could be released by a protease treatment. In the case of Gag/Pol-expressing cells the amount of tethered virions remained about three times lower in CHC-knockdown cells in repeated experiments albeit there was no difference in the levels of protein expression or membrane-associated Gag. This suggested that CHC-knockdown produced a moderate late assembly defect, by an unidentified mechanism in Gag/Pol-expressing HeLa H1-cells.

4.3.2 The Vpu-regulated Pr55^{gag} endocytosis is independent of macropinocytosis-like activity

Previous results suggested that Pr55^{gag} internalization was independent of clathrin-mediated endocytosis, so it was tested whether macropinocytosis played a role in Pr55^{gag} uptake. The flow cytometric analysis measuring the uptake of fluorescein-conjugated dextran into both noninfected and Pr55^{gag} or Gag/Pol-expressing HeLa H1-cells showed that the basal level of macropinocytosis activity was low and virus infection did not upregulate this activity. The effect of amiloride, an inhibitor of induced macropinocytosis on Pr55^{gag} endocytosis was tested in the absence of Vpu. Amiloride failed to block the shift of the newly synthesized Pr55^{gag} to the internal membranes indicating that uptake of Pr55^{gag} unlikely occurred by a macropinocytosis-like activity.

4.3.3 Interfering with plasma membrane cholesterol reduces uptake of cell surface-associated Pr55^{gag}

The possible dependence of Pr55^{gag} endocytosis on cholesterol was tested by filipin, an antibiotic commonly used to disrupt cholesterol-dependent raft-like microdomains at the cell surface. Filipin treatment reduced the amount of Pr55^{gag} at internal membranes when added at the beginning of the chase suggesting that endocytic uptake of Pr55^{gag} in ΔVpu background could occur by a cholesterol-dependent mechanism. However, the effect of filipin on Pr55^{gag} internalization was less pronounced if it was added after 30 min of chase when the maximal membrane insertion of newly synthesized Pr55^{gag} has already been achieved.

Since cholesterol is required for efficient membrane binding and higher-order multimerization of Pr55^{gag} (Ono et al. 2007), the observed effect could be due to an inability of plasma membrane-associated Gag proteins to efficiently multimerize in the presence of filipin. To probe this possibility the impact of filipin in the context of Gag/Pol co-expression was tested, since the processing of Pr55^{gag} offers a convenient indirect marker for efficient multimerization of Gag (Kaplan et al. 1994). Filipin was added to Gag/Pol-expressing cells after 30 min of chase and the chase was continued for further 180 min.

These analyses indicated that filipin-treated cells contained significant amounts of the plasma membrane-associated, unprocessed Pr55^{gag} even after a long chase suggesting that filipin affected the proper higher-order multimerization of Pr55^{gag}. In other words, filipin most likely reduced the overall virus assembly, since the processing of Pr55^{gag} was impaired in Gag/Pol-expressing cells. This led us to conclude that Pr55^{gag} endocytosis is only indirectly dependent on membrane cholesterol as cholesterol sequestration interferes with a step upstream of Pr55^{gag} endocytosis.

4.3.4 Pr55^{gag} trafficking is dependent on cell confluency

Our results have convincingly indicated that the newly synthesized Pr55^{gag} is initially targeted to the plasma membrane of an infected cell and endocytosed unless the viral accessory protein Vpu is present. These data have been produced by using confluent HeLa H1-cultures. Interestingly, when subconfluent cultures (cell density ~30-40%) were used for the corresponding experimentation it was found that in the absence of Vpu the newly synthesized Pr55^{gag} was targeted both to the plasma membrane and to the internal membranes and during the chase there was only a minor shift of the initially plasma membrane associated Pr55^{gag} to the internal compartment. This suggested that the targeting of Pr55^{gag} in subconfluent cells could occur in a random manner or the uptake in these cells could be more rapid than in confluent cells. To test between these two hypotheses the subconfluent cultures were infected with Pr55^{gag}/HA, a tagged variant that we have previously shown to remain at the plasma membrane even in the absence of Vpu. Our results indicated that Pr55^{gag}/HA in subconfluent cells was targeted almost exclusively to the plasma membrane and remained there even after a long chase as in confluent cells. Taken together, these data implicated that cell confluency can have an impact on the intracellular targeting of Pr55^{gag} when the protein is expressed in the absence of Vpu.

5 DISCUSSION

In the first paper (paper I) we have critically evaluated whether the detergent extraction followed by an iodixanol step-gradient is a suitable method for separation of raft membranes from non-raft membranes and whether this method provides solid evidence to conclude that HIV-1 assembles at these specific Triton X-100-resistant lipid raft domains. Our results strongly indicate that detergent extraction yields highly variable results depending on the starting material used for the extraction protocol. We also show that VLPs emerging from cells do not possess raft-like membranes around them suggesting that classical cholesterol and sphingolipid rafts are not necessarily assembly platforms for extracellular HIV particles. We imply that Pr55^{gag} associates with Brij98-resistant membrane structures instead of classical, Triton X-100-resistant raft membranes.

5.1 CONSIDERATIONS OVER EXPERIMENTAL APPROACHES TO ANALYZE DETERGENT RESISTANT MEMBRANES

Lipid raft hypothesis has received over the past years much attention and is no doubt appealing as such, but the correlation between detergent extraction and cholesterol depletion data and membrane domain formation in living cells needs apparently some further consideration. Although cell membranes clearly are not only homogenous mixture of lipids and proteins providing cells just a boundary against the outer world, almost all aspects defining rafts, such as their size, composition, variation in composition, life-time and biological relevance have remained controversial (Pike 2006). One apparent reason for this is that lipid rafts are too small to be seen with light microscopy and all other conventional methods to study rafts, like detergent extraction at cold, cholesterol depletion or domain patching with antibodies are more or less perturbing for the cells (Hao et al. 2001, Harder et al. 1998, Kwik et al. 2003) and do not necessarily provide any information of the existence of rafts in natural settings (Lai 2003, Munro 2003).

Cholesterol- and sphingolipid-enriched domains are expected to be resistant to Triton X-100 at +4°C and they can be separated from detergent soluble bulk membranes by means of density gradients (Brown and Rose 1992). This traditional method to define and analyze detergent resistant microdomains has however evoked a great deal of criticism since temperature and detergents interfere with normal cellular dynamics and organization and therefore these extracts do not reflect the real state and functions of living cells (Hao et al. 2001, Kwik et al. 2003, Lichtenberg et al. 2005, Pike et al. 2005). In addition, non-standardized extraction procedures, gradient conditions and experimentation with synthetic membranes have further generated confusion over the existence and relevance of detergent resistant membranes (Hancock 2006). On the other hand, it has been suggested that lipid-independent protein-protein interactions alone can create distinct domains at the plasma membrane and these interactions in turn determine which molecules are retained in or excluded from certain microdomain assemblies (Douglass and Vale 2005).

Our data is consistent with the considerations over the relevance of the detergent-extracted membrane fractions. We clearly show that the classical detergent treatment with Triton X-100 at cold followed by a density gradient centrifugation gives highly variable results depending on which cellular fraction is used as a starting material for extraction. We have identified cellular chromatin as a possible source of artifacts. Cellular chromatin is sticky and thus may give a flotation phenotype for unspecific, non-raft-associated cellular components, such as nucleic acid binding proteins easily leading to misinterpreted conclusion about raft association of a given protein. Works by others have pointed out another plausible source of artifacts such as actin cytoskeleton which has differential solubility depending on detergent concentration used (Babiyhuk and Draeger 2006).

What is creating further confusion in the field of detergent resistant microdomains or lipid rafts, is the usage of different kinds of detergents for the membrane extraction. Detergents are a vast group of chemical compounds with different properties including their differential capacity to solubilise physiological membranes (Pike et al. 2005, Schuck et al. 2003). Alongside Triton X-100, the Brij-series of detergents and for instance Lubrol WX, can be used for membrane extraction (Drevot et al. 2002, Roper et al. 2000, Yanez-Mo et al. 2009). Each of these, according to their solubilisation capacity produces different flotation patterns of membrane lipids and proteins in density gradients (Pike et al. 2005, Schuck et al. 2003). It have however remained to be determined, whether these different types of detergent resistant membranes are distinct or at least relevant functional entities in the living cells.

Cholesterol is essential for the structural integrity of lipid rafts and cholesterol depletion is a widely used method in studying these domains as they are dispersed upon extraction. Cholesterol is typically depleted from the cells by inhibitors of synthesis (statins) in combination with cholesterol-free culture medium or by extracting it from the membranes with methyl- β -cyclodextrin (Hao et al. 2001). The cholesterol extraction method is however also prone to misinterpretations. Cholesterol depletion has been shown to alter drastically the composition of the plasma membrane by inducing domain segregation, decreasing the diffusion rate of both raft and non-raft proteins, alter the bilayer permeability and affecting the endocytic pathways (Goodwin et al. 2005, Hao et al. 2001, Hao et al. 2004, Kenworthy et al. 2004, Subtil et al. 1999). Furthermore, cholesterol depletion has been shown to affect the phosphoinositide content of the membrane and reorganize cellular actin (Kwik et al. 2003). Cholesterol depletion has vast adverse global effect on the cell biology. In other words, cholesterol depletion makes cells sick and therefore it is questionable whether this method is suitable for drawing definitive conclusions of the existence or functions of cholesterol and sphingolipid-enriched membrane domains.

5.2 HIV-1 ASSEMBLY AND LIPID RAFTS

The assembly and budding of HIV-1 have been linked to the raft domains biochemically by non-ionic detergent extractions and fractionations of these extracts

on different kinds of density gradients (Lindwasser and Resh 2001, Nguyen and Hildreth 2000, Ono and Freed 2001) or by simple pelletation of cell extracts (Rousso et al. 2000). Further evidence for raft association of HIV-1 has been gained from confocal microscopy studies where the co-localization of viral proteins with raft markers has been examined (Nguyen and Hildreth 2000).

To evaluate the possible role of detergent resistant microdomains in HIV-1 assembly, our first approach was a classical detergent extraction assay with Triton X-100 followed by a density gradient. Our results first suggested that Pr55^{gag} associates with a heterogenous group of light and intermediate density membranes implicating that Pr55^{gag} may be a raft-associated protein as reported by others (Lindwasser and Resh 2001, Nguyen and Hildreth 2000, Ono and Freed 2001, Zheng et al. 2001). However, our control proteins that are not associated with lipid rafts surprisingly also floated to the intermediate density fractions. This prompted us to believe that buoyant Pr55^{gag} complexes were not true detergent resistant membrane-associated complexes but structures most likely representing post-lysis artifacts. Moreover, Triton X-100 extraction at physiological temperatures did not resolve these buoyant Pr55^{gag} complexes further suggesting that these structures were not genuine lipid rafts. After modifications made in membrane isolation protocol that quantitatively removed the artificial floating we could conclude, that newly synthesized Pr55^{gag} associates with membranes with intermediate density in a time-dependent manner, but these do not represent genuine Triton X-100-resistant lipid rafts because of their increased density and inability to float through 30% iodixanol.

Consistent with our results these intermediate density complexes have been also observed by other groups (Ding et al. 2003, Lindwasser and Resh 2001). The former concluded that multimerization of Gag promotes its localization to barges, which are described to be more dense raft domains than classical light density Triton X-resistant lipid rafts. Remarkably though, even when Pr55^{gag} was found to float to these intermediate density fractions instead of light ones, this floatation phenotype was interpreted to as a raft association, nevertheless of a special kind, but without any further analysis of these floating barges. Given that intermediate density flotation phenotype is obtained also for membranes largely devoid of cholesterol and sphingomyelin, i.e. essential raft components, we disagree with the interpretation of barges possessing raft-like properties. The latter study was in agreement with us as they clearly interpreted these intermediate density complexes to be distinct of lipid rafts based on flotation analyses, cholesterol extraction studies and electron microscopy data.

Two other major studies implicating that rafts are important for HIV-1 assembly (Nguyen and Hildreth 2000, Ono and Freed 2001) cannot be directly compared with our results, as in flotation assays different types of density gradients were employed. In the former study it was reported that ³H-myristic acid-labelled intracellular Pr55^{gag} is associated with light density Triton X-100-resistant structures in cold cell lysates. However, the density gradient used in this study did not have dense enough separating layer (38% sucrose corresponds to ~30% iodixanol) that could be capable of separating intermediate buoyant complexes from light density Triton X-100-resistant membranes. In addition, Gag complexes floating through 38% sucrose were

not further characterized and the buoyant phenotype was directly interpreted as Pr55^{gag} being a raft-associated protein.

In the report of Ono and Freed (Ono and Freed 2001) it was shown that about half of the membrane-associated Pr55^{gag} is found at detergent resistant fractions and association of newly synthesized Pr55^{gag} with this subset of membranes occurs after the initial targeting of Pr55^{gag} to the membranes. On the basis of the flotation phenotype of Pr55^{gag} they concluded that rafts play a critical role in HIV assembly and release. Unfortunately though, the density gradient used in this study was not apparently capable of separating complexes possessing light and intermediate densities since the gradient consisted of only a relatively dense separating layer made of 65% sucrose solution. The use of such a dense separation layer leads to an apparent flotation of both intermediate density as well as light density complexes to the top fractions of the gradient and therefore these results cannot be used to validate the association of Pr55^{gag} with lipid rafts.

However, this study (Ono and Freed 2001) is consistent with our results in regard to the kinetic studies where they show that maturation of membrane-bound complexes takes place during the chase. Our results also indicated that initially Triton X-100 soluble Pr55^{gag} becomes associated with intermediate density membranes over time (paper I). Furthermore, their results are also in agreement with ours in respect that Pr55^{gag} domains present affect the degree of Pr55^{gag} complexes that remain attached to detergent resistant membranes. Our preliminary data analysing truncated Gag variants showed that Pr55^{gag} lacking NC-domain and sequences downstream does not associate with membranes possessing intermediate density, but remains soluble even after longer periods of chase (unpublished results), most likely due to their incapability to form higher order oligomers (Burniston et al. 1999).

To gain further insight whether Pr55^{gag} associates with lipid rafts, we turned into confocal microscopy. Inherently rafts are too small to be visualized by light microscopy, but raft domains can be cross-linked by antibodies against raft components in order to coalesce them into larger visible patches. Our data showed that significant amount of Pr55^{gag} indeed colocalized with GM1-cholera toxin B subunit cross-linked raft domains. According to our data from detergent extraction experiments, these domains are apparently sensitive to Triton X-100. On the other hand, one can speculate that artificially patched raft domains do not exist in the native membranes, so to my opinion it is questionable whether this data bring about anything conclusive upon putative association of Pr55^{gag} with rafts. Furthermore, apparent colocalization in general does not have to mean direct association of the components under study, so based on our confocal images it is still highly speculative whether Pr55^{gag} is raft-resident or not.

The fluorescent microscopy studies by others (Nguyen and Hildreth 2000) have shown that HIV-1 viral proteins colocalize with raft markers such as GM1 and Thy-1 but no colocalization is observed with a raft-excluded protein CD45. Furthermore, when Jurkat cells were loaded with raft-specific and non-raft partitioning fluorescent lipid analogs it was shown that HIV-1 proteins colocalize preferentially with raft-specific lipid analog, but not with fluid phase associating analog. Interestingly, the

staining pattern of HIV-1 in chronically infected Jurkat cells seemed to concentrate to discrete regions at one side of the cells, to the uropods, rising a possibility that these subcellular structures may in itself contain factors that lead to this particular virus protein distribution and the primary cause for such distribution lies far away from lipid raft-induced phenomena.

One method to assess whether lipid rafts play a role in HIV-1 assembly and budding is apparently the analysis of VLP lipid composition as the viral envelope is derived from the actual budding site. Furthermore, VLPs can be easily purified from the most contaminants of cellular origin hence providing a relatively uniform material for analyses.

Our data suggested that VLPs do not possess raft-like membranes around them as they exclusively ended up on intermediate density fractions on iodixanol density gradients and the extraction at physiological temperatures did not change this flotation pattern. In accordance with this, the cholesterol and phospholipid analysis of Triton X-100-extracted VLPs showed that these buoyant complexes are largely devoid of cholesterol and sphingolipids further confirming that lipid rafts are not an inherent component of extracellular virions. Consistent with our findings it was also confirmed by others that Triton X-100-extracted VLPs floated to the intermediate densities (Ding et al. 2003, Lindwasser and Resh 2001), resembling the flotation phenotype of intracellular Pr55^{gag}. Lindwasser and Resh interpreted these buoyant complexes as Triton X-resistant rafts containing oligomeric Pr55^{gag}, but no lipid analysis whatsoever was performed to confirm the raft-like nature of these floating objects.

The direct analyses of virion lipids have also spoken on behalf of HIV-1 budding from raft domains. The virion envelope has been shown to be enriched in cholesterol and sphingomyelin, the main constituents of lipid rafts suggesting that HIV egress occurs through special membrane domains (Aloia et al. 1993, Brugger et al. 2006). A recent study however suggested that overall lipid content of virions matched closely to that of the plasma membrane except with enrichment in cholesterol, ceramide and phosphoinositides. These authors did not find any evidence of HIV budding from the rafts with unusual lipid composition (Chan et al. 2008). This is in line with our results as our data did not either support the conclusion of Pr55^{gag} being associated with lipid rafts.

5.3 BRIJ98-RESISTANT MICRODOMAINS

It is well established that not all rafts are resistant to Triton X-100 (Drevot et al. 2002, Roper et al. 2000) and different detergents vary in their capacity to solubilise biological membranes (Pike et al. 2005, Schuck et al. 2003). To assess whether Pr55^{gag} associates membrane rafts other than classical Triton X-100 rafts we tested the sensitivity of membrane-bound Pr55^{gag} to various other detergents. Our analysis suggested that Pr55^{gag} is partially resistant to many other detergents, such as members of Brij-series. We decided to focus on polyoxyethylene ether Brij98, as this detergent detects rafts at physiological temperatures (Drevot et al. 2002) and thus may provide more relevant information of lipid microdomain behaviour on the

native membranes than extraction at cold with Triton X-100. We also slightly modified the density gradient in order to achieve more accurate separation of the detergent resistant membranes from soluble complexes.

Our results indicated that at the steady state Pr55^{gag} is largely resistant to Brij98 extraction at physiological temperatures. We implicate that these buoyant complexes are most likely precursors for the assembling particles as newly synthesized Pr55^{gag} accumulate to these membrane domains in a time-dependent manner and recovered extracellular particles also exhibit comparable buoyant density when analyzed on density gradients. Our analysis of Brij98-extracted VLPs suggested that cholesterol and phospholipids are quantitatively retained on the particles upon detergent treatment. These data all together suggest that instead of usurping Triton X-100-resistant lipid rafts for assembly, Pr55^{gag} may egress through Brij98-resistant membrane microdomains. Our data is consistent with a work showing that infectious wild-type HIV-1 particles contains Brij98 rafts but only minimal amounts of Triton X-100 rafts (Campbell et al. 2004).

The membrane microdomains owing resistance to other detergents than Triton X-100 are less characterized entities in the raft field. We are here first to implicate, based on careful analysis of detergent extracted buoyant complexes that HIV-1 may associate with lipid domains other than classical Triton X-100-resistant lipid rafts. We strongly emphasize that anything that owns a floating phenotype on a density gradient cannot directly taken as a proof of HIV-1 Gag being associated with lipid rafts as suggested previously (Lindwasser and Resh 2001, Nguyen and Hildreth 2000, Ono and Freed 2001).

It is worth discussion what is, indeed, the biological relevance of lipid microdomains. At least classical Triton X-100-resistant lipid rafts have been assigned to many cellular functions such as signal transduction, formation of immunological synapse upon B- and T-cell activation and clathrin-independent endocytosis (Kirkham et al. 2005, Simons and Toomre 2000, Sohn et al. 2008, Zech et al. 2009), but also serious concerns have been raised on this issue (Lai 2003, Munro 2003). One should consider critically what is the biological "gain" of such a compartmentalization in living biological systems; are these domains true composites of membranes directing certain cellular functions or are they just by-products induced by protein-protein or physically preferred protein-lipid interactions (Lingwood and Simons 2010).

Whatever is the case, the assignment of Brij98-resistant lipid domains in the assembly of Pr55^{gag} provided a fresh insight in the raft field. These membrane domains seem to exist at physiological temperatures rendering them at least conceptually more relevant in the biological sense than lipid domains that can be scored only when extracted at low temperatures. On the other hand, extractability at cold does not necessarily render Triton X-100-resistant non-existent in the native membranes as rapid cooling of a biological system is known to slow down free diffusion on the membranes and preserve cellular structures. Clearly more studies are needed in order to elucidate whether the Brij98-rafts possess any relevance on native cellular functions or in the context of HIV-1 assembly.

New advanced microscopy-based techniques have been applied to study lipid rafts in the context of living cells (Simons and Gerl 2010). Some of these methods, such as total internal reflection fluorescence microscopy have also been used in order to

visualize the assembly of HIV-1 at the cell membrane (Ivanchenko et al. 2009, Jouvenet et al. 2008). In these studies virus particles have been seen to assemble at discrete punctate areas at the plasma membrane. Whether these discrete clusters reflect the assembly of HIV-1 in specialized microdomains, such as Brij98-resistant rafts remains to be elucidated.

According to another view, interaction of Gag with phosphatidylinositol-4,5-bisphosphate (PI(4,5)P₂) at the plasma membrane could promote the membrane reorganization and formation of lipid rafts. Upon Gag binding and insertion of Gag N-terminal saturated myristic acid into the cytoplasmic leaflet of the plasma membrane the unsaturated acyl chain of PI(4,5)P₂ flips out from the membrane. This exchange has been speculated to cluster saturated lipids at the site of virus assembly and promote raft formation (Simons and Gerl 2010, Waheed and Freed 2009). In this case though, HIV-1 assembly merely drives the formation of certain microenvironment rather than targeting itself into the pre-existing lipid rafts for the assembly. Moreover, the composition and functional relevance of these virus-driven nanoclusters remain to be assessed.

5.4 TARGETING OF PR55^{GAG}

In this work (paper II) we have analyzed the targeting of newly synthesized Pr55^{GAG} to the site of assembly in HeLa H1-cells by pulse-chase studies and subcellular fractionations. Our results suggested that Pr55^{GAG} first arrives at the plasma membrane of an infected cell and is subsequently directed either to the extracellular space as virions or alternatively endocytosed. We were first to show that Vpu, a viral accessory protein is an essential regulator of the intracellular trafficking of Pr55^{GAG}. Furthermore, we found that integrity of p6-domain of Pr55^{GAG} and endogenous Tsg101 are required for the endocytic uptake of Pr55^{GAG}.

The intracellular targeting of Pr55^{GAG} has been under vigorous study during past years. Electron microscopy and fluorescent microscopy studies initially suggested that in T-cells and laboratory adapted model cell lines such as HeLa, Cos-1 and 293T Pr55^{GAG} predominantly assembles at the plasma membrane of the infected cells and that the plasma membrane was also the site of production of progeny virions (Hermida-Matsumoto and Resh 2000, Ono et al. 2004). In compelling studies however, Pr55^{GAG} and virus particles have been found both at the plasma membrane and in internal, endosome-like compartments (Grigorov et al. 2006, Nydegger et al. 2003, Sherer et al. 2003).

There are basically three options how Pr55^{GAG} can achieve its steady state localization both at the plasma membrane and at the internal membranes: (i) Pr55^{GAG} is initially targeted to the plasma membrane and endosomal localization could be achieved via endocytosis of the cell surface-associated protein, (ii) Pr55^{GAG} is first inserted into endosomal membranes and is then either retained in these compartments or transported further to the plasma membrane, or (iii), Pr55^{GAG} promiscuously associates simultaneously with both type of membranes. To distinguish between these three possibilities, we employed pulse-chase studies combined to subcellular fractionations to follow the targeting of newly synthesized Pr55^{GAG} in HeLa H1-cells.

Subcellular fractionation was carried out by using silica coating of the plasma membrane by cationic colloidal silica beads. In this method, the outface of the plasma membrane is covered by silica beads and the plasma membrane fraction can be separated from the internal membranes in a discontinuous density gradient based on increased density of the bead-coated plasma membrane (Spector et al. 1998). Our results suggested that an efficient separation of the plasma membrane from internal membranes in HeLa H1-cells can be achieved by using this method. However, our observations suggested that this method can be employed only with cell types with a rigid cytoskeleton (data not shown) as cells with less firm cytoskeleton, which is typical for suspension cells, (e.g. Jurkat T-cells) tended to disrupt during the coating procedure leading to binding of silica beads to internal membranes as well. Similarly, we found that we could not test the involvement of cytoskeleton on Pr55^{gag} endocytosis (paper III) as pharmacological agents disrupting cytoskeletal fibers also rendered cells unsuitable for silica coating. In contrast to our observations, others have successfully used silica coating for suspension cells as well (Chan et al. 2008). This discrepancy may be explained by Jurkat T-cells being extraordinary sensitive to plasma membrane modifying silica beads or experimental differences employed in these studies.

5.5 VPU REGULATES THE ENDOCYTOSIS OF PR55^{GAG}

Our pulse-chase analyses of newly synthesized Pr55^{gag} revealed that Pr55^{gag} is first targeted to the plasma membrane in HeLa H1-cells and remains at the cell surface when coexpressed with the viral accessory protein Vpu. In contrast, in the absence of Vpu the cell surface-associated Pr55^{gag} was endocytosed from the plasma membrane and considerably less extracellular virions were released from these cells (data not shown). This study was the first to show that Vpu regulates the intracellular trafficking of Pr55^{gag} by preventing the endocytosis of the protein from the cell surface. Similar results were suggested soon by others (Neil et al. 2006). Several studies are also in line with our results showing that in established model cell lines Pr55^{gag} primarily localizes at the plasma membrane (Finzi et al. 2007, Gomez and Hope 2006, Jouvenet et al. 2006, Jouvenet et al. 2008).

Targeting of newly synthesized Pr55^{gag} to the plasma membrane in HeLa-cells was observed also in study using tetracycline tagged Gag-molecules (Rudner et al. 2005) while this study failed to show the dependence of Pr55^{gag} trafficking on Vpu. These contrary results may be due to the fusion tags inserted into Pr55^{gag} as our data also indicated that carboxyterminal tag may interfere with Pr55^{gag} endocytosis in the absence of Vpu. This observation raises a general concern when using tagged proteins. Fusion tags are extremely common method in studying the localization and functions of proteins because of facilitated detection with commercially available antibodies, but as shown by us, unexpected consequences may occur when using these tagged variants leading easily to false conclusions.

5.6 TSG101 IS INVOLVED IN THE ENDOCYTOSIS OF PR55^{GAG}

Our results further show that a host cell protein Tsg101 is required for the endocytosis of Pr55^{gag} in the absence of Vpu. Tsg101 binds to the late PTAP-domain of Pr55^{gag} and recruits ESCRT-machinery to the site of budding (Martin-Serrano et al. 2001, Morita and Sundquist 2004). Cellular levels of Tsg101 were downmodulated by siRNAs designed to be specific for Tsg101. As a control,

siRNAs with scrambled sequence was used. Despite of control siRNA, RNA interference may sometimes cause unexpected off-target effects or induce activation of antiviral state which grossly modulates the gene expression of the cell (Scacheri et al. 2004, Sledz et al. 2003). To ensure that Tsg101-PTAP interaction was indeed essential for Pr55^{gag} endocytosis and was not due to the adverse effects caused by RNAi, we deleted the whole p6-domain from our proviral construct. As expected, the newly synthesized Pr55^{gag}/Δp6 was targeted to the plasma membrane and even in the absence of Vpu, remained there. These data confirmed that Tsg101 interaction with Pr55^{gag} is required for the endocytic uptake of the plasma membrane-associated HIV-1 Gag. Given that interaction of Pr55^{gag} p6-domain with Tsg101 and ESCRT-machinery is expected to facilitate the release of virus particles from the cell surface it was in slightly unexpected that Tsg101 also regulated the uptake of Pr55^{gag} in the absence of Vpu. We could speculate that Tsg101 may have yet-undefined functional roles early in HIV-1 assembly other than so far recognized. As suggested by others (Neil et al. 2006) the role of Tsg101 in Pr55^{gag} endocytosis most likely included the requirement of completed budding process with a disclosure of the membrane stalk prior to endocytosis.

5.7 PR55^{GAG} IS ENDOCYTOSED AS VIRIONS

In our original discussion (paper II) we speculated that in the absence of Vpu, the over time accumulating internal Pr55^{gag} signal was due to endocytosis of the plasma membrane-associated Pr55^{gag} rather than originating from extracellular particles taken up by endocytosis. We thought that was a case since very little Pr55^{gag} signal was detected at the internal membranes when Pr55^{gag} was expressed in the presence of Vpu and in the context where approximately 18-fold more VLPs were released (data not shown). Furthermore, when exploring the data from electron micrographs, in the absence of Vpu we could detect occasional budding structures at MVB-like compartments and the steady state analysis of Gag/Pol -expressing cells by Western blotting revealed the presence of Pr55^{gag} processing intermediates at the internal membrane fraction.

In the work of others (Neil et al. 2006) and confirmed also later by us (paper III) it was conclusively shown that in the absence of Vpu, fully formed VLPs can be stripped from the surface of infected cells by a protease treatment suggesting that nascent virions remained captured at the cell surface by a protein-mediated manner. In the light of this newer data we would re-discuss that accumulating internal Pr55^{gag} signal in the absence of Vpu expression is due to these tethered VLPs endocytosed from the cell surface. The processing intermediates observed in the internal membrane fraction and few budding structures seen in MVB-like compartment most likely arose from endocytosis of plasma membrane regions containing incomplete buds. Furthermore, we cannot exclude the possibility that internally scored MVB-resembling structures were not true endosomes since deep membrane invaginations containing tethered particles can sometimes be difficult to distinguish from the genuine internal compartments (Deneka et al. 2007, Welsch et al. 2007). Retrospectively speaking, the fact that Pr55^{gag} is internalized as fully formed virions would also explain why HA-tagged variant of Pr55gag was retained at the plasma membrane in the absence of Vpu. This engineered protein is defective in forming virus particles and as completed budding is required for the subsequent endocytosis in the absence of Vpu, it is now obvious that Pr55^{gag} with C-terminal HA-tag was not internalized in our experiments.

To date, the ultimate fate of endocytosed particles have remained unclear. As our subcellular fractionation method did not separate different intracellular compartments from each other we could not conclude to which compartment endocytosed particles were directed. Our fluorescence microscopy analysis (data not shown) indicated that internalized Pr55^{Gag}-signal gave a vesicular staining pattern but did not extensively co-localize with early or late endosomal markers or recycling endosomal compartment.

This paper was first to assign Vpu to the intracellular trafficking of Pr55^{Gag} and is therefore a precursor for expanded understanding behind the old observations of Vpu being an enhancer of particle release (Klimkait et al. 1990). Data published during the past couple of years have gone far beyond from our initial observations and revealed more of the molecular mechanism behind the action of Vpu. Vpu have been shown to counteract a host restriction factor CD317, also known as tetherin. In the absence of Vpu, nascent viral particles remain attached at the host cell surface via an anchor formed by tetherin and are subsequently endocytosed instead of released as extracellular virions (Neil et al. 2006, Neil et al. 2007). It has been speculated that Vpu may counteract tetherin by blocking the insertion of the tetherin N-terminal transmembrane domain into virion envelopes (McNatt et al. 2009, Perez-Caballero et al. 2009) and relocating tetherin from the plasma membrane to the intracellular compartments in order to sequester it away from the site of productive particle assembly (Dube et al. 2009, Dube et al. 2010, Habermann et al. 2010, Van Damme et al. 2008). However, the analyses of virus particles (Fitzpatrick et al. 2010, Habermann et al. 2010) have shown that tetherin is incorporated into virions even in the presence of Vpu implicating that mechanisms other than suggested above may contribute to the activity of Vpu. Interestingly, nascent virus particles could also be released from Vpu expressing cells by protease treatment (Karetnikov and Suomalainen 2010) further implicating that Vpu action is not yet conclusively studied.

5.8 PR55^{GAG} ENDOCYTOSIS IS CLATHRIN-INDEPENDENT

As the viral accessory protein Vpu was found to be an important regulator of Pr55^{Gag} endocytosis, in this work (paper III) we further assessed the endocytosis phenotype of Pr55^{Gag} in the absence of Vpu. Mammalian cells harbor several endocytic routes of which the three major uptake modes are clathrin-mediated endocytosis, macropinocytosis and cholesterol-dependent (lipid raft-mediated) uptake. We used inhibitors of these three uptake modes to probe the mechanism of Pr55^{Gag} endocytosis.

We used RNAi-mediated knockdown of clathrin heavy chain (CHC) to determine whether the endocytosis of Pr55^{Gag} occurred by a clathrin-dependent mechanism. SiRNA against CHC efficiently inhibited the endocytosis of transferrin, but did not block the uptake of Pr55^{Gag} in the absence of Vpu. This suggested that endocytosis of Pr55^{Gag} is not directed by clathrin-mediated endocytosis. However, in case of Gag/Pol coexpression, we observed a minor inhibition in uptake of processed Pr55^{Gag}. This prompted us to further investigate whether a subpopulation of Gag is indeed internalized via clathrin-mediated endocytosis or whether the RNAi against CHC had an effect on assembly. To distinguish between these possibilities, we analyzed VLP production from Pr55^{Gag} and Gag/Pol-expressing cells. Our results

indicated that CHC-knockdown did not interfere with particle production from Pr55^{gag} expressing cells, but did significantly reduce the amount of VLPs rescued by protease-treatment from Gag/Pol-expressing cells. This suggested that overall assembly in the Gag/Pol-expressing cells is somehow perturbed when CHC-level is downmodulated.

Our conclusion that Pr55^{gag} endocytosis is clathrin-independent is in concordance with studies showing that inhibition of clathrin-mediated endocytosis by chlorpromazine did not block Gag endocytosis (Finzi et al. 2007). In contrast, when using dominant negative forms of regulators of clathrin-mediated endocytosis, such as dynamin, EPS15 or Rab5a, a clear inhibition in Pr55^{gag} endocytosis was observed (Jouvenet et al. 2006, Neil et al. 2006). This discrepancy may be due to different experimental approaches or different cells used. In these studies C-terminally tagged Pr55^{gag} variants were used instead of our proviral expression of wild type Pr55^{gag} and furthermore, it is recognized also in other studies that expression of dominant negative proteins may yield different results than depletion of endogenous proteins by RNAi (Martin-Serrano et al. 2001, Ward et al. 2005). Also, it is possible although not likely that in our study the remaining CHC-levels in cells could be still enough to carry out clathrin-mediated endocytosis required for Pr55^{gag} uptake as RNAi at best only reduces the amount of targeted protein but do not eliminate it completely. The applicability of RNAi in studies of coated-pits has been generally questioned because of short half life of these pits and redundancy of coated-pit proteins (Sorkin 2004).

It would be intriguing to test whether results from above studies would be more uniform if for instance dynamin was targeted by a small-molecule inhibitor dynasore, that blocks the GTPase activity of the protein (Macia et al. 2006) and have been shown to diminish HIV-1 particle endocytosis from the cell surface (Miyauchi et al. 2009). Despite of certain drawbacks of dynasore studies, such as batch-dependent variation, that approach may be more specific to block clathrin-mediated endocytosis than either RNAi or usage of dominant negative mutants as both are prone to off-target effects and can induce unexpected global changes in cells. Likewise, new small-molecule inhibitors called pitstops that regulate coated pit dynamics (von Kleist et al. 2011) would be worth trying in addressing the role of clathrin-mediated endocytosis in Pr55^{gag} uptake.

In the scope of this thesis it remained unresolved why CHC-knockdown reduced virus assembly in the context of Gag/Pol co-expression. Most likely the assembly defect occurred at very late step in the assembly process as Pr55^{gag} processing had already started. A careful inspection of the Western blot comparing the internalization of processed Pr55^{gag} in mock-treated and CHC-knockdown cells might suggest that there is actually a processing defect in question as in CHC-knockdown cells more higher molecular weight complex (p25) seems to remain at the plasma membrane fraction than in mock cells. This analysis is however very speculative. It seems like only fully processed Pr55^{gag} (p24) could have been endocytosed, being in concordance of VLPs taken up as fully formed virions (Neil et al. 2006).

5.9 PR55^{GAG} ENDOCYTOSIS OCCURS INDEPENDENTLY OF MACROPINOCYTOSIS

We next tested whether the endocytosis of Pr55^{GAG} could occur through macropinocytosis like activity. As HeLa-cells are a type of epithelial cells, macropinocytosis is not a constitutive process in this cell type and if Pr55^{GAG} uptake occurred by this process the viral infection would need to stimulate this uptake mechanism. When the uptake of fluorescein-conjugated dextran into HeLa H1-cells was measured by flow cytometry the low levels of macropinocytosis-like activity was observed in both infected and uninfected cells, indicating that infection itself did not induce any gross changes in macropinocytosis. The effect of amiloride, a potent inhibitor of an induced macropinocytosis was tested (Meier et al. 2002, West et al. 1989). Our analysis indicated that amiloride did not block the shift of newly synthesized Pr55^{GAG} from the plasma membrane to the internal membranes suggesting that Pr55^{GAG} endocytosis did not occur by a macropinocytosis-like activity. Unfortunately we could not confirm this result by for instance disrupting the actin fibers which are essential for the macropinocytic ruffle formation by pharmacological agents such as latrunculin B because actin disrupted cells were too fragile for silica coating.

5.10 THE EFFECT OF MEMBRANE CHOLESTEROL ON PR55^{GAG} ENDOCYTOSIS IS INDIRECT

The possible dependence of Pr55^{GAG} endocytosis on cholesterol was probed with filipin, an antibiotic used to disrupt lipid raft domains on the cell surface. Our results indicated that cholesterol sequestration by filipin reduced the amount of Pr55^{GAG} at internal membranes when added in the beginning of the chase, but the effect was less pronounced if the drug was applied at the time point where the membrane insertion of the newly synthesized Pr55^{GAG} was maximal (Tritel and Resh 2001). As cholesterol is required for the efficient membrane binding and higher-order oligomerization of HIV-1 Gag (Ono et al. 2007) and as Pr55^{GAG} is most likely endocytosed as a fully assembled virion (Neil et al. 2006) we reasoned that the observed effect of filipin could be due to the inability of plasma membrane-bound Pr55^{GAG} to multimerize in the presence of filipin. As processing of Pr55^{GAG} is a convenient marker for the multimerization of Pr55^{GAG}, we tested the effect of filipin on Gag endocytosis in the context of Gag/Pol co-expression. Our results indicated that Pr55^{GAG} processing is largely inhibited in the presence of filipin suggesting a defect in Pr55^{GAG} multimerization. However, there was not significant difference in the intracellular distribution of the capsid protein in filipin treated or in mock treated cells indicating that Gag endocytosis occurred in a cholesterol-independent fashion. This is also consistent with the assumption that Gag is endocytosed as assembled virions (Neil et al. 2006) since only processed Gag was found in the internal membrane fraction. Our data implicates that membrane cholesterol is required for efficient assembly, but once assembled, the endocytosis of the virions does not require intact cholesterol or in other words, the endocytosis of virions is not occurring in a lipid raft-mediated manner.

To date it has been remained to be elucidated by which mechanism Gag internalization is taking place in the absence of Vpu. As we did not find any direct evidence of uptake proceeding by the three most common modes of endocytosis,

this most likely suggests that endocytosis of Gag occurs by a yet-undefined or less studied internalization pathway (Gong et al. 2008). It also remains to be determined what is the fundamental function of removing the membrane-tethered virions from the cell surface. One could speculate that tethered virions at the plasma membrane may promote some kind of a scavenging activity to maintain the membrane homeostasis and organized membrane trafficking or alternatively, virions can be just endocytosed by a default pathway.

Our results also demonstrate that in the absence of Vpu the cell confluency can have an impact on the targeting phenotype of Pr55^{gag} as shown also before (Deora and Ratner 2001). Our results indicated that in subconfluent cells the newly synthesized Pr55^{gag} simultaneously appeared both at the plasma membrane and at the internal membranes while in confluent cell cultures Pr55^{gag} is first detected exclusively at the plasma membrane. What is the basis for this more promiscuous membrane association in subconfluent cells is currently unresolved. One could argue that membrane trafficking is highly accelerated in rapidly growing cells instead of being compromised somehow when cells are subconfluent. This interpretation is consistent with our data showing that a tagged Pr55^{gag} variant that is not internalized was targeted directly to the plasma membrane also in subconfluent cells showing no apparent association with internal membrane fraction.

It should be noted that our results have been obtained by using a laboratory-adapted model cell line, and these results may not be directly applicable to the natural target cells of HIV-1. This work has however brought up the fact that anything that interferes with proper assembly would most likely also interfere with Pr55^{gag} intracellular trafficking and endocytosis in the absence of Vpu. We also imply that membrane trafficking events are highly dynamic in nature and when studying HIV-1 localization by using cell culture applications, it should be kept in mind that even slight modifications in assay conditions, expression systems and cell types may easily yield variable data that could explain different outcomes produced by some other laboratories.

6 CONCLUDING REMARKS

This thesis, which consists of three original publications covers two different entities. First (paper I) we have analyzed the possible lipid raft association of HIV-1 structural precursor protein Pr55^{gag}. It has been widely accepted in the literature that HIV-1 assembles at lipid domains that are resistant for Triton X-100 extraction at cold. Our data however strongly suggests that during virus assembly, Pr55^{gag} associates with lipid domains other than classical Triton X-100-resistant lipid rafts. We show here that Triton X-100 extraction actually produces highly variable floating complexes depending on the starting material used for detergent extraction and that floating phenotype alone cannot be taken as a proof of a protein being associated with lipid rafts. Our data further implicates that Pr55^{gag} associates with lipid domains that are resistant for Brij98 extraction at physiological temperatures and these lipid microdomains may serve as assembly platforms for Pr55^{gag}.

In the second part of this thesis (paper II and III) we discuss about the assembly site of HIV-1 Pr55^{gag}. There has been an intense debate over the issue whether the assembly takes place initially at the plasma membrane of an infected cell or at the endosomal compartment. Our results from the kinetic analyses and subcellular fractionations show clearly that in HeLa H1-cells the newly synthesized Pr55^{gag} first arrives exclusively at the plasma membrane and is subsequently either released as extracellular virus like particles or endocytosed. We were first to show that HIV-1 accessory protein Vpu and the cellular protein Tsg101 both regulate the endocytosis of the plasma membrane-associated Pr55^{gag}. We then probed for the mechanism by which the plasma membrane-associated Pr55^{gag} is internalized in the context of Vpu-negative virus. Our results implicated that neither clathrin-mediated endocytosis nor macropinocytosis is involved in Pr55^{gag} uptake. Membrane cholesterol sequestration had an effect on Pr55^{gag} endocytosis, but only if employed prior to the extensive multimerization of newly synthesized Pr55^{gag} at the plasma membrane suggesting that cholesterol-dependent uptake is most likely only indirectly involved in Pr55^{gag} internalization. We, therefore, conclude that factors that interfere with Pr55^{gag} assembly also have an effect on subsequent Pr55^{gag} endocytosis.

Taken together, the original publications in this thesis have provided valuable new insight concerning the assembly attributes of HIV-1 and the host cell components related to the assembly process.

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9 ORIGINAL PAPERS I-III

I

Human Immunodeficiency Virus Type 1 Assembly and Lipid Rafts: Pr55^{gag} Associates with Membrane Domains That Are Largely Resistant to Brij98 but Sensitive to Triton X-100

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The assembly and budding of human immunodeficiency virus type 1 (HIV-1) at the plasma membrane are directed by the viral core protein Pr55^{gag}. We have analyzed whether Pr55^{gag} has intrinsic affinity for sphingolipid- and cholesterol-enriched raft microdomains at the plasma membrane. Pr55^{gag} has previously been reported to associate with Triton X-100-resistant rafts, since both intracellular membranes and virus-like Pr55^{gag} particles (VLPs) yield buoyant Pr55^{gag} complexes upon Triton X-100 extraction at cold temperatures, a phenotype that is usually considered to indicate association of a protein with rafts. However, we show here that the buoyant density of Triton X-100-treated Pr55^{gag} complexes cannot be taken as a proof for raft association of Pr55^{gag}, since lipid analyses of Triton X-100-treated VLPs demonstrated that the detergent readily solubilizes the bulk of membrane lipids from Pr55^{gag}. However, Pr55^{gag} might nevertheless be a raft-associated protein, since confocal fluorescence microscopy indicated that coalescence of GM1-positive rafts at the cell surface led to copatching of membrane-bound Pr55^{gag}. Furthermore, extraction of intracellular membranes or VLPs with Brij98 yielded buoyant Pr55^{gag} complexes of low density. Lipid analyses of Brij98-treated VLPs suggested that a large fraction of the envelope cholesterol and phospholipids was resistant to Brij98. Collectively, these results suggest that Pr55^{gag} localizes to membrane microdomains that are largely resistant to Brij98 but sensitive to Triton X-100, and these membrane domains provide the platform for assembly and budding of Pr55^{gag} VLPs.

The plasma membrane is partially composed of ordered domains, called “rafts,” which are enriched in sphingolipids and cholesterol and contain a specific set of proteins (5, 43). Rafts are resistant to extraction with nonionic detergents at low temperatures, and thus rafts and raft-associated proteins can be separated from detergent-solubilized material by fractionation of cell lysates on density gradients (6). The plasma membrane apparently contains different types of rafts, which exhibit differential sensitivities to different detergents (11, 41). The best-characterized rafts are Triton X-100-resistant rafts, which have been implicated in playing a critical role in numerous cellular processes (28, 43, 45). Triton X-100-resistant rafts have also been proposed to provide a platform for assembly and budding of several different enveloped viruses (38, 53, 55). One of these viruses is human immunodeficiency virus type 1 (HIV-1) (32, 35, 36, 42). Assembly and budding of HIV-1 occur at the plasma membrane and are directed by the viral core protein precursor Gag (Pr55^{gag}), since Pr55^{gag} expressed in the absence of other viral components induces formation of enveloped virus-like particles (VLPs) (18, 30). Pr55^{gag} associates with the cytoplasmic leaflet of the plasma membrane via an amino-terminal dual motif that consists of a covalent my-

ristic acid modification and a cluster of basic amino acid residues (7, 13, 18, 19, 26, 47, 48, 58). Through a mechanism that is poorly understood, the membrane-bound Pr55^{gag} proteins oligomerize into core structures and concomitantly deform the membrane into a bud (14, 17). Five recent reports have concluded that Triton X-100-resistant rafts play an important role in this Pr55^{gag}-mediated assembly and budding (32, 35, 36, 42, 57). Four of these reports assigned Pr55^{gag} to the Triton X-100-resistant rafts, since density gradient analyses indicated that a significant fraction of intracellular Pr55^{gag} displayed buoyant density in cold Triton X-100 cell lysates (32, 35, 36, 57). Results from Lindwasser and Resh (32), however, implied that Pr55^{gag} does not localize to “classical” Triton X-100-resistant rafts, but instead localizes to distinct, dense Triton X-100-resistant rafts, which were given the name “barges.” It was speculated that higher density of barges was caused by the presence of extensive arrays of oligomeric Pr55^{gag} assembly intermediates in a raft-like membrane. It was suggested that assembly of HIV-1 occurs at the raft-like barge-membranes, since Triton X-100-solubilized Pr55^{gag} complexes from extracellular VLPs had a density similar to that of intracellular barges, and mutant Pr55^{gag} proteins that exhibited increased affinity for barges were found to produce VLPs more efficiently than wild-type Pr55^{gag} (32). In addition to localization of Pr55^{gag} to rafts or barges, three other lines of evidence have been put forward in support of rafts playing a critical role in assembly and budding of HIV-1. (i) Cholesterol-depleting agents, which among other things cause alterations in raft structures, have been demonstrated to decrease the release of virus particles from HIV-1-infected cells (36), as well as to reduce the infectivity of released particles (36, 57). (ii) There is localization (partial) of

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HIV-1 Env proteins to Triton X-100-resistant rafts (42). (iii) The presence of raft-associated host proteins and lipids in HIV-1 particles has been interpreted to signify selective budding of HIV-1 through (Triton X-100-resistant) rafts (35).

In this study, we have analyzed raft association of HIV-1 Pr55^{gag} by using Triton X-100 and Brij98 extractions as well as confocal fluorescence microscopy. Our results demonstrate that Triton X-100 extraction when cold readily solubilizes bulk of VLP envelope lipids. Thus, it is questionable whether the buoyancy of Triton X-100-treated Pr55^{gag} complexes can be taken as evidence for localization of the protein to rafts. However, confocal fluorescence microscopy analyses indicated extensive colocalization of Pr55^{gag} at the cell surface with patched GM1-positive rafts. Furthermore, Brij98 extractions indicated that a population of intracellular Pr55^{gag}, as well as the VLP envelope, was largely resistant to this detergent. This raises the possibility that cell surface raft structures that are resistant to Brij98 but sensitive to Triton X-100 could provide a platform for Pr55^{gag}-mediated assembly and budding of HIV-1.

MATERIALS AND METHODS

Cell culture. Jurkat cells were maintained in RPMI 1640 medium containing 10% fetal calf serum, 10 mM HEPES (pH 7.4), 2 mM glutamine, 100 U of penicillin per ml, and 0.1 mg of streptomycin per ml (all tissue culture reagents were from Invitrogen Life Technologies). 293T cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 2 mM glutamine, 100 U of penicillin per ml, 0.1 mg of streptomycin per ml, nonessential amino acids, and 1 mM sodium pyruvate.

Preparation of infectious SFV-C/HIVgag. Plasmid pSFV-C/HIVgag was used for the production of recombinant Semliki Forest virus (SFV) particles directing the synthesis of HIV-1 Pr55^{gag}. In this plasmid, the Pr55^{gag} coding sequence is fused to the C gene of SFV. The first step in the construction of pSFV-C/HIVgag was cloning of the Pr55^{gag} open reading frame from pSP64-HIV11b-BH10 (20) as a 1,720-bp *Bam*HI-*Stu*I fragment into the *Bam*HI-*Sma*I-linearized pSFV1 (31) to give pSFV1-HIVgag. The fusion between SFV-C and HIV-1 Pr55^{gag} genes was engineered by PCR such that the second codon of Pr55^{gag} was fused to the last codon of SFV-C by using Vent DNA polymerase (New England Biolabs, Beverly, Mass.) in accordance with the manufacturer's instructions (27, 54). The terminal primers used for the amplification of the fused DNA fragment were 5'-CAACGGAAACGACGACGAC-3' (the C 5' primer) and 5'-CCCCCTGGCC TTAACCGAATTTTCCC-3' (the Pr55^{gag} 3' primer). The fusion primers were 5'-GGGGTCCGAAGAGTGGGGTGGCGAGAGCGTCA-3' (C fragment primer) and 5'-TGACGCTCTCGACCCCACTCTTCGGACCCC-3' (Pr55^{gag} fragment primer). After amplification, the fusion fragment was cut with *Asu*II and *Cl*aI and cloned into *Asu*II-*Cl*aI-digested pSFV1-HIVgag to give pSFV-C/HIVgag(1). Subsequently the pSFV-C/HIVgag (1) was modified by site-directed mutagenesis (9) with the primers 5'-CGTGCTCAAGAGTTCCAAGTTGGCC-3' (*Sac*I site eliminator) and 5'-GTACTGAGAGACAGGCTAACTTCCTGGGGAAGATCTGGCCTTCC-3' to mutate the frameshift signal so that only Pr55^{gag} sequences from the Gag gene insert could be translated. The mutations were silent mutations with respect to Pr55^{gag} coding sequence. Another modification of pSFV-C/HIVgag(1) was to delete one of the two amino-terminal repeats (LQSRPEPTAPP) of the BH10 p6 domain by using the primer 5'-GGAAGGCCAGGGAATTTTCTTCAGAGCAGACAGGCCAACAGCC CCACCAGAAGAGAGCTTCAGGTCTGGG-3'. This gave the final version pSFV-C/HIVgag that was used in the study. Infectious SFV particles directing the synthesis of HIV-1 Pr55^{gag} (SFV-C/HIVgag) were prepared by coelectroporating equal amounts of in vitro-transcribed SFV-C/HIVgag and helper 1 (31) RNAs into 10⁷ BHK-21 cells as previously described (49, 50).

Analysis of raft association of Pr55^{gag}. (i) **Total cell lysates.** Jurkat cells (3.5 × 10⁶) were infected with SFV-C/HIVgag in RPMI-BSA medium (RPMI 1640 containing 0.2% bovine serum albumin [BSA], 10 mM HEPES, pH 7.4, 2 mM glutamine, 100 U of penicillin per ml, and 0.1 mg of streptomycin per ml), and 4 h postinfection, cells were switched to methionine-free minimum essential medium (MEM; supplemented with 0.2% BSA, 10 mM HEPES, pH 7.4, 2 mM glutamine, 100 U of penicillin per ml, and 0.1 mg of streptomycin per ml) for 30 min. Cells were subsequently metabolically labeled in methionine-free modified

Eagle's medium containing 100 μCi of [³⁵S]methionine per ml for 15 min and chased for 0 to 60 min in RPMI-BSA medium containing 10-fold excess of cold methionine. After chase, cells were placed on ice and washed once with ice-cold phosphate-buffered saline (PBS; with MgCl₂ and CaCl₂), and then the cells were lysed in 200 μl of lysis buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 20 μg of phenylmethylsulfonyl fluoride per ml, 1 μg of CLAP [chymostatin, leupeptin, aprotinin, and pepstatin A] per ml). After 30 min of incubation at 0°C, lysate was mixed with 3 ml of 56% iodixanol (Optiprep; Nycomed Pharma A/S) and overlaid with 3 ml of 40% iodixanol, 5 ml of 30% iodixanol, and 0.5 ml of 5% iodixanol (all iodixanol solutions were in 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100). Gradients were centrifuged in an SW41 rotor at 77,100 × g for 14 h at 4°C. One-milliliter fractions were collected from the top, aliquots of each fraction were mixed with sodium dodecyl sulfate (SDS) sample buffer, and equal amounts of fractions were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE). Radioactivity in Pr55^{gag} bands was determined by using a Bas-III Image Plate and the Bio-Image analyzer system Bas 2000 (Fuji Photo film Co.), and the amount of Pr55^{gag} in each of the different fractions was expressed as a percentage of the total (the sum of all fractions). Influenza virus NP expressed from SFV-C/NP genome (56), transferrin receptor (TR) expressed from SFV-C/TRΔ2 (49), and CD55 expressed from SFV1-CD55 were used as controls. Raft association of influenza virus NP was analyzed after a 15-min pulse and 1-h chase, and that of TR was analyzed after a 60-min pulse and 120-min chase. Cell lysates from SFV1-CD55-infected cells were prepared 5 h postinfection, and proteins in gradient fractions were precipitated with 10% trichloroacetic acid and analyzed by SDS-PAGE followed by Western blotting with anti-CD55 antibody (H-319) from Santa Cruz Biotechnology, Inc. Lck was analyzed from gradient fractions by Western blotting with anti-Lck antibody (3A5) from Santa Cruz Biotechnology, Inc.

(ii) **Membrane-associated Pr55^{gag}-crude cell homogenates.** Jurkat cells (10⁷) were infected with SFV-C/HIVgag and metabolically labeled with [³⁵S]methionine as described above. After chase, cells were placed on ice, washed once with ice-cold PBS (with MgCl₂ and CaCl₂), and homogenized in buffer containing 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 20 μg of phenylmethylsulfonyl fluoride per ml, and 1 μg of CLAP per ml with a tight-fitting Dounce homogenizer. Immediately after homogenization, NaCl was added to 150 mM, and the homogenate was diluted with 100% Nycodenz (Nycomed Pharma A/S) to give a final 60% Nycodenz concentration. The sample (2 ml) was overlaid with 1.5 ml of 50% Nycodenz and 1 ml of homogenization buffer (with 150 mM NaCl), and the gradient was centrifuged in an SW50.1 rotor at 63,000 × g for 45 min at 4°C. The total membrane fraction at the buffer–50% Nycodenz interphase was collected. About 200 to 400 μl of this membrane fraction was mixed with Triton X-100 (final concentration, 1%) and after 30 min of incubation at 0 or 37°C, the solubilized membranes were analyzed on iodixanol step gradients as described above. TR was analyzed after a 60-min pulse and 30-min chase, influenza virus NP was analyzed after a 60-min pulse, and CD55 and Lck were analyzed by Western blotting as described above.

Subconfluent 293T cells grown on 10-cm-diameter plates were transfected with 28 μg of pCMV-HIVgag plasmid by a calcium phosphate precipitation technique. The pCMV-HIVgag plasmid was derived from pCMVΔR8.91 (a generous gift from Didier Trono) (59) by deleting a *Bcl*I-*Eco*RI fragment from the Pol region. The transfected cells were collected ~22 to 24 h posttransfection and processed as described above for the SFV-C/HIVgag-infected Jurkat cells. Total Pr55^{gag} in gradient fractions was visualized by Western blotting using anti-p24 antibody from Biogenesis.

(iii) **Membrane-associated Pr55^{gag} postnuclear supernatants.** Jurkat cells (10⁷) were infected with SFV-C/HIVgag and metabolically labeled with [³⁵S]methionine as described above. Transfected 293T cells were analyzed ~22 to 24 h posttransfection. After chase, cells were placed on ice, washed once with ice-cold PBS (with MgCl₂ and CaCl₂), and homogenized in buffer containing 25 mM HEPES-KOH (pH 7.4), 0.25 M sucrose, 4 mM MgCl₂, 20 μg of phenylmethylsulfonyl fluoride per ml, and 1 μg of CLAP per ml by using a tight-fitting Dounce homogenizer. The crude cell homogenates were centrifuged at 1,400 × g for 4 min at 4°C to pellet nuclei and cell debris. About 70 to 80% of total labeled Pr55^{gag} was found in the postnuclear supernatant (PNS), which was subsequently fractionated on the Nycodenz step gradient described above to separate membranes from cytosolic material (gradient solutions were prepared in 25 mM HEPES-KOH, pH 7.4, 150 mM NaCl and 1 mM EDTA). The total membrane fraction from the buffer–50% Nycodenz interphase was mixed with Triton X-100 (final concentration, 1%) and protease inhibitors, and after 30 min of incubation at 0°C, or at 37°C, the solubilized membranes were analyzed on iodixanol step gradients as described above. For Brij98 extraction, the total membranes were mixed with protease inhibitors and 0.5% Brij98, and after a 5-min incubation at

37°C, the extracts were mixed with 3.5 ml of 56% iodixanol and overlaid with 2 ml of 30% iodixanol, 2 ml of 25% iodixanol, 2 ml of 20% iodixanol, and 0.5 ml of 5% iodixanol (all iodixanol solutions were in 25 mM HEPES-KOH, pH 7.4, 150 mM NaCl, 1 mM EDTA). Gradients were centrifuged in an SW41 rotor at $77,100 \times g$ for 14 h at 4°C.

(iv) **VLPs.** Jurkat cells (10^7) were infected with SFV-C/HIVgag and metabolically labeled with [35 S]methionine for 3 h as described above. Culture media were collected and clarified by centrifugation at $200 \times g$ for 4 min. VLPs from clarified culture media were purified by a two-step gradient centrifugation. The clarified culture supernatant was first applied to a linear 5 to 20% iodixanol gradient (21). After centrifugation in an SW41 rotor at $160,000 \times g$ for 1 h at 4°C, fractions were collected from the top, and Pr55^{gag}-containing fractions were pooled, 1 ml of pooled fractions was mixed with 3 ml of 56% iodixanol, and this mixture was overlaid with 4 ml of 27% iodixanol, 3 ml of 20% iodixanol, and 0.5 ml of 5% iodixanol. The iodixanol solutions were made in a mixture of 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 1 mM EDTA (Triton X-100 extraction) or a mixture of 25 mM HEPES-KOH (pH 7.4), 150 mM NaCl, and 1 mM EDTA (Brij98 extraction). The gradients were centrifuged in a SW41 rotor at $77,100 \times g$ for 14 h at 4°C, and VLPs from the 20 to 27% iodixanol interphase were collected and extracted with Triton X-100 or Brij98 as described above and analyzed on iodixanol step gradients as described above.

Production and analysis of [32 P]orthophosphate- and [35 S]methionine-labeled VLPs. Jurkat cells were prelabeled with [32 P]orthophosphate as described in reference 21. Prelabeled Jurkat cells (10^7) were infected with SFV-C/HIVgag and metabolically labeled with [35 S]methionine for 3 h as described above. VLPs were harvested from clarified culture supernatants as described above. Material from SFV-C/NP-infected Jurkat cells prepared in a similar way was used as a control to estimate contamination of VLPs by microvesicles. Triton X-100- or Brij98-extracted VLPs or NP controls were fractionated on iodixanol step gradients as described above, except that gradients were centrifuged for 4.5 h. Buoyant Pr55^{gag} complexes from the 30 to 40% iodixanol interphase (Triton X-100 extraction) or the 25% iodixanol zone (Brij98 extraction) were collected, diluted fourfold with a mixture of 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA (Triton X-100) or 25 mM HEPES-KOH (pH 7.4), 150 mM NaCl, 1 mM EDTA (Brij98), and pelleted by centrifugation in a SW41 rotor ($160,000 \times g$ for 1 h at 4°C). The pelleted Pr55^{gag} complexes and the control intact, nonextracted VLPs were solubilized with 3% SDS at 70°C, mixed with SDS-PAGE buffer, and analyzed by SDS-PAGE with 20% polyacrylamide gels (21). Radioactivity in mixed SDS-lipid micelles was determined by using a Bas-III Image Plate and the Bio-Image analyzer system Bas 2000. [35 S]methionine label in Pr55^{gag} bands (analyzed by SDS-PAGE with 10% polyacrylamide gels) was used to ensure that similar amounts of Pr55^{gag} from the intact particles and the buoyant detergent-treated Pr55^{gag} complexes were loaded on 20% polyacrylamide gels.

Production and analysis of [3 H]cholesterol-labeled VLPs. Jurkat cells (10^7) were infected with SFV-C/HIVgag or with SFV-C/NP for 30 min. The virus inoculum was removed, and the cells were metabolically labeled with 10 μ Ci of [$1\alpha,2\alpha(n)^3$ H]cholesterol per ml for 4 h in RPMI-BSA medium. The radioactive cholesterol label was removed and cells were incubated in RPMI-BSA medium for 3 h. VLPs were harvested from clarified culture supernatants by the two-step centrifugation procedure described above. After the last flotation gradient, the peak VLP fractions were pooled and then diluted threefold with buffer, and VLPs were pelleted by a 2-h centrifugation in an SW50.1 rotor at $173,000 \times g$ (4°C). For Triton X-100 extraction, the pellets were placed into 160 μ l of mixture containing 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 20 μ g of phenylmethylsulfonyl fluoride per ml, 1 μ g of CLAP per ml, and 1% Triton X-100 and incubated at 0°C for 30 min, and the extracts were mixed with 1 ml of 56% iodixanol and overlaid with solutions of 400 μ l of 40% iodixanol, 400 μ l of 30% iodixanol, and 200 μ l of 5% iodixanol. The gradients were centrifuged in TLS-55 rotors at $166,000 \times g$ for 4 h at 4°C, 400- μ l fractions were collected from the top, and [3 H]cholesterol in fractions was quantified by scintillation counting. The counts from the NP control were subtracted from the VLP values, and the amount of [3 H]cholesterol radioactivity in different fractions was expressed as a percentage of the total (the sum of all fractions). For Brij98 extraction, the pelleted VLPs were placed into a mixture containing 160 μ l of 25 mM HEPES-KOH (pH 7.4), 150 mM NaCl, 1 mM EDTA, 20 μ g of phenylmethylsulfonyl fluoride per ml, 1 μ g of CLAP per ml, and 0.5% Brij98, incubated at 37°C for 5 min, and the extracts were mixed with 550 μ l of 56% iodixanol and overlaid with 400 μ l of 30% iodixanol, 800 μ l of 20% iodixanol, and 200 μ l of 5% iodixanol solutions. The gradients were centrifuged and collected as described for the Triton X-100 extraction.

Confocal fluorescence microscopy. Subconfluent 293T cells seeded onto coverslips (3-cm-diameter plates) were transfected with 3 μ g of pCMV-HIVgag.

After 20 to 22 h, cells were placed on ice and washed twice with cold PBS (with MgCl₂ and CaCl₂) and then incubated for 20 min on ice with fluorescein isothiocyanate (FITC)-conjugated cholera toxin B subunit (10 μ g/ml in PBS with MgCl₂ and CaCl₂; Sigma). After two washes in PBS, cells were fixed in 4% formaldehyde for 30 min at room temperature (unpatched cells) or incubated on ice in PBS (with MgCl₂ and CaCl₂) containing anti-cholera toxin antibodies (Calbiochem) for 30 min and subsequently transferred to 37°C for 30 min for patching. After two washes in PBS, cells were fixed in 4% formaldehyde as described above, permeabilized with 0.3% Triton X-100, and stained with mouse anti-Pr55^{gag} antibodies (EF-7 or 38-9; generous gifts from Jorma Hinkula, Department of Virology, SMI/KI, Sweden) and tetramethyl rhodamine isocyanate (TRITC)-conjugated anti-mouse immunoglobulin G (IgG) antibodies (Southern Biotechnology Associates, Inc.). Preparations were mounted in glycerol-PBS (pH 8.0) containing *p*-phenyldiamine and analyzed with the Carl Zeiss LSM 510 confocal system. The system is based on the compact scanning module, which has been fitted to an inverted (Axiovert 200 M) microscope. The LSM system is equipped with Zeiss software (LSM modes) and is controlled via a standard high-end Pentium PC. Two lasers were selected—one with a wavelength of 488 nm and the other with a wavelength of 514 to 543 nm. In most cases, 40 to 50 optical horizontal sections with intervals about 0.25 μ m were taken through the whole preparation (Z-stack). Selected single optical sections were then analyzed, and fluorescence images were saved and processed in Photoshop 6.0. In some cases, the Z-stack was analyzed in the pseudo-three-dimensional LSM mode with orthogonal function allowing us to obtain a membrane-bound intracellular section through the whole cell.

RESULTS

Analyses of raft association of Pr55^{gag} by using Triton X-100. We chose to use SFV-driven expression of Pr55^{gag} in Jurkat cells to analyze raft association of the protein. In the recombinant SFV genome SFV-C/HIVgag, the coding sequence of Pr55^{gag} is fused to the C gene of SFV, and the resulting C-Pr55^{gag} fusion protein is cotranslationally processed into C and Pr55^{gag} proteins by the autoproteolytic activity of SFV C protein (1, 34). [3 H]myristic acid labeling of cells indicated that Pr55^{gag} expressed in SFV-C/HIVgag-infected cells was efficiently myristoylated (data not shown). We also tested kinetics and efficiency of membrane association of newly synthesized Pr55^{gag}. Cell homogenates of SFV-C/HIVgag-infected Jurkat cells were prepared after 15 min of metabolic labeling with [35 S]methionine and a 0- or 30-min chase, and membranes were separated from cytosolic material by fractionation on a flotation gradient. The results obtained indicated that, already by the 0-min chase point, ~34% of labeled Pr55^{gag} was membrane associated, and the membrane-bound pool increased to ~50% after a 60-min chase (data not shown). Thus, the newly synthesized Pr55^{gag} in SFV-C/HIVgag-infected cells becomes membrane associated with similar kinetics to Pr55^{gag} expressed from proviral constructs (52).

Pr55^{gag} has been previously assigned to Triton X-100-resistant rafts based on results from a biochemical flotation assay that isolates lipid rafts on the basis of their insolubility in this detergent at low temperatures (32, 35, 36). However, as shown in Fig. 1, the assay is problematic, since the flotation phenotype of Pr55^{gag} varies, depending on which cellular fraction is used as the starting material for Triton X-100 extraction. Figure 1A shows the results from crude cell lysates. Recombinant SFV-infected Jurkat cells expressing Pr55^{gag} or control marker proteins were extracted with 1% Triton X-100 at 0°C for 30 min, and the extracts were analyzed on an iodixanol step gradient, which consisted of a 56% loading zone overlaid with 40, 30, and 5% iodixanol solutions. After ultracentrifugation, the Triton X-100-insoluble rafts float to the 5 to 30% iodixanol inter-

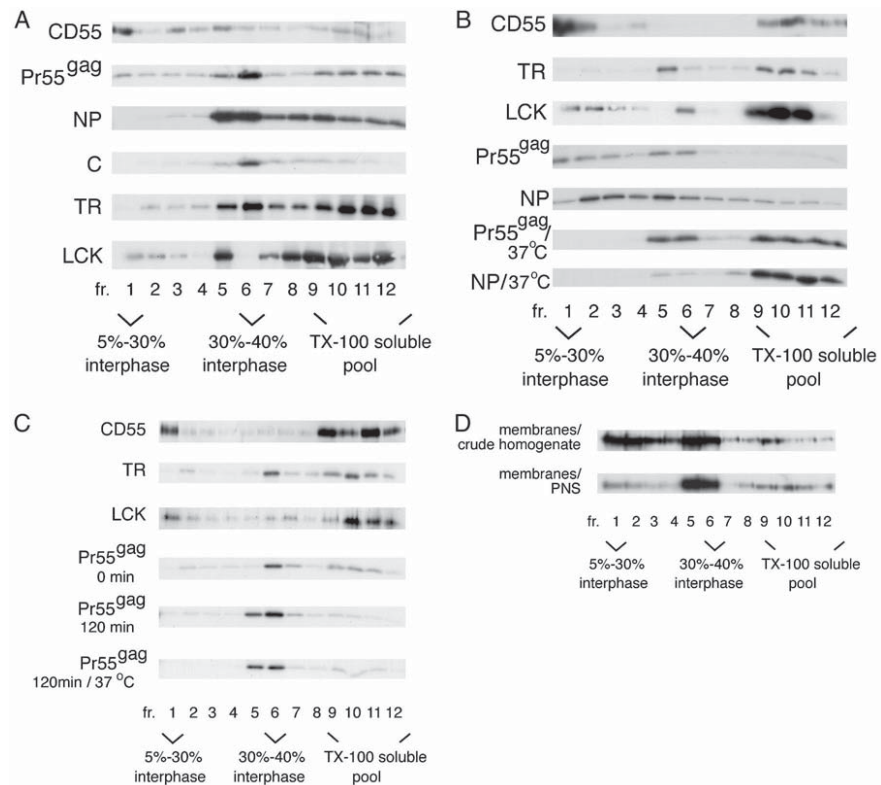


FIG. 1. Cold Triton X-100 extraction of cells yields variable complexes for Pr55^{gag}, depending on which cellular fraction is used as a starting material for the detergent treatment. (A) Analyses of crude cell lysates. Pr55^{gag}, CD55, influenza virus NP, the nucleocapsid protein C of SFV, and the TR were expressed in Jurkat cells from recombinant SFV genomes. Cells were extracted with 1% Triton X-100 (TX-100) at 0°C, and lysates were subjected to flotation on iodixanol step gradients. Aliquots of gradient fractions were analyzed by SDS-PAGE. The Pr55^{gag}, NP, and C proteins were analyzed after a 15-min pulse with [³⁵S]methionine and a 60-min chase, and TR was analyzed after a 60-min pulse and 120-min chase, whereas CD55 and the endogenous Lck were detected by Western blotting. (B) Analyses of the total membrane fraction from Jurkat crude cell homogenates. Pr55^{gag} was analyzed after a 15-min pulse with [³⁵S]methionine and a 60-min chase, NP was analyzed after a 60-min pulse, and TR was analyzed after a 60-min pulse and 30-min chase. Total CD55 and Lck were visualized by Western blotting. (C) Analyses of the total membrane fraction from the PNS of Jurkat cells. Pr55^{gag} was analyzed after a 15-min pulse with [³⁵S]methionine and a 0- or 120-min chase, and TR was analyzed after a 90-min pulse. Total CD55 and Lck were visualized by Western blotting. (D) Analyses of membrane-bound Pr55^{gag} from transfected 293T cells. Total Pr55^{gag} in gradient fractions was detected by Western blotting.

phase (fraction 1), whereas detergent-soluble proteins stay in the loading zone (fractions 9 to 12) (4). Our positive control for a raft-associated protein, the glycosylphosphatidylinositol (GPI)-anchored protein CD55 (16, 46), efficiently floated to fraction 1. In contrast, e.g., after a 15-min pulse with [³⁵S]methionine and a 60-min chase, only minor amounts of labeled Pr55^{gag} were found in fraction 1, but a clear concentration of the protein was seen at the 30 to 40% iodixanol interphase in fraction 6. About 37% of labeled Pr55^{gag} was in the Triton X-100-soluble pool (fractions 9 to 12), which could represent cytosolic Pr55^{gag}, or be of both cytosolic and membranous origins. The Pr55^{gag} complexes at the 30 to 40% interphase correspond to the “barges” described by Lindwasser and Resh

(32). Barges have been interpreted to represent raft-like membranes that have a higher density than classical Triton X-100-resistant rafts due to the presence of multimeric Pr55^{gag} complexes in barges (32). However, our control experiments indicated that both the influenza virus NP, a predominantly nuclear protein (56), and the cytosolic nucleocapsid protein C of SFV (50, 51) efficiently floated to the 30 to 40% iodixanol interphase after cold Triton X-100 extraction (Fig. 1A). Significant amounts of the non-raft membrane marker TR ended up in the 30 to 40% iodixanol interphase as well (Fig. 1A). We also analyzed the distribution of the endogenous Src family kinase Lck, which like Pr55^{gag} associates with the cytoplasmic leaflet of the plasma membrane. As previously reported (24),

the majority of Lck was found in the Triton X-100-soluble pool (fractions 9 to 12). Some Lck was in fraction 1, but significant amounts of the protein concentrated in fraction 5. Taken together, these controls demonstrated that different types of proteins in crude Triton X-100 cell lysates float to the 30 to 40% iodixanol interphase, and thus this flotation phenotype does not necessarily signify association of a protein with raft-like membranes. The extreme viscosity of the SDS-solubilized fraction 5 and 6 samples suggested that this gradient region contained large amounts of chromatin. This raised the possibility that flotation of some of the marker proteins, and possibly also that of Pr55^{gag}, to the 30 to 40% iodixanol interphase region could actually be a postlysis artifact caused by unspecific trapping of proteins to nuclear remnants or chromatin.

Since the results from crude cell lysates were difficult to interpret, we also tested alternative cellular fractions in the assay. Figure 1B shows results from experiments in which the total membrane fraction of cells was used as the starting material for Triton X-100 extraction. Crude cell homogenates were first fractionated on a flotation gradient to separate membrane-associated proteins from cytosolic components. The total membrane fraction was extracted with 1% Triton X-100 at cold temperatures, and the extracts were analyzed on the iodixanol step gradient described above. Our positive control CD55 still efficiently floated to fraction 1 in this modified raft fractionation scheme. The flotation phenotype of TR was otherwise unchanged as well, except that the buoyant TR had now shifted to fraction 5. In the case of Lck, there was a clear reduction in the relative amount of the protein in the middle of the gradient compared to the crude cell lysates, and the buoyant Lck complexes in this gradient region were now concentrated in fraction 6. Our total membrane fraction apparently still contained significant amounts of unspecified nuclear remnants, since ~64% of intracellular influenza virus NP cofractionated with cellular membranes in the crude cell homogenates (data not shown). When this "membrane-associated" NP was extracted with Triton X-100 at cold temperatures, this led to scattering of the protein along the gradient, although with some concentration in fractions 2 to 5. Also the small amounts of the cytosolic nucleocapsid protein of SFV that copurified with membranes were predominantly found in fractions 2 to 5 (data not shown). Analyses of membrane-bound Pr55^{gag} after a 15-min pulse with [³⁵S]methionine and a 60-min chase indicated that labeled Pr55^{gag} was predominantly found in buoyant complexes, but in contrast to the crude cell lysates, these buoyant complexes were now scattered in fractions 1 to 6. The Pr55^{gag} panel in Fig. 1B suggests also that relatively more Pr55^{gag} ended up in fraction 1 in this fractionation scheme than in crude cell lysates. However, the relative amounts of labeled Pr55^{gag} in fraction 1 versus fraction 6 varied significantly between different experiments (data not shown), thus suggesting that fraction 1 material in fact could be a postlysis artifact of fraction 6 complexes, or vice versa. Furthermore, when comparing the gradient profiles of Pr55^{gag} and NP in Fig. 1B, it is obvious that the buoyant complexes of Pr55^{gag} are strikingly similar to those of NP. Since NP and Pr55^{gag} are both nucleic acid binding proteins, these heterogeneous buoyant structures could represent yet another type of postlysis artifact induced by nuclear remnants. In an attempt to determine whether the Pr55^{gag} buoyant complexes were artifacts or rafts, we tested the

sensitivity of the complexes to Triton X-100 at 37°C. Rafts are solubilized with Triton X-100 if the extraction is performed at 37°C (6). As expected, extraction at 37°C shifted essentially all CD55 to the detergent-soluble pool (fractions 9 to 12) (data not shown). In the case of Pr55^{gag}, floating of the protein to fractions 1 to 4 was suppressed, and increased amounts of labeled Pr55^{gag} were found in the Triton X-100-soluble pool (Fig. 1B). However, the material in fractions 5 and 6 was largely resistant to extraction at 37°C. But since the buoyant NP complexes were efficiently solubilized at 37°C as well (Fig. 1B), the Triton X-100 solubility of the Pr55^{gag} complexes in fractions 1 to 4 at 37°C cannot be taken as proof for these complexes representing rafts. In addition to solubilization of rafts, 37°C could also activate an enzymatic function or functions that destabilize the buoyant Pr55^{gag} and NP complexes.

As a third approach to assess association of Pr55^{gag} with Triton X-100-resistant rafts, we used membranes from PNS as the starting material for the detergent extraction. In experiments described above, we had used EDTA in the cell homogenization buffer, but the NP control indicated that nuclear remnants in EDTA-homogenized cell extracts were not quantitatively pelleted by the low-speed spin used to produce PNS (data not shown). We therefore replaced EDTA in the homogenization buffer with Mg²⁺, which is known to promote nuclear integrity during homogenization and thus to improve the removal of nuclear material by low-speed spin. PNS was first fractionated on a flotation gradient to separate membranes from cytosol, and the total membrane fraction was extracted with 1% Triton X-100 at cold temperatures, and the extracts were analyzed on the iodixanol step gradient. Under these conditions, no NP cofractionated with cellular membranes (data not shown). As shown in Fig. 1C, the relative amount of CD55 in fraction 1 was somewhat reduced compared to that of the membranes from crude cell homogenate, whereas the gradient profile of TR was essentially unchanged. Relatively more Lck was now found in fraction 1 than that shown in Fig. 1B, but most significantly, only trace amounts of Lck were present in fractions 5 and 6. In contrast, the majority of Pr55^{gag} was concentrated in the middle of the gradient. After a 15-min pulse with [³⁵S]methionine and a 0-min chase, about equal amounts of labeled Pr55^{gag} were found in fraction 6 and the Triton X-100-soluble pool (fractions 9 to 12), whereas after a 60-min chase (data not shown) or 120-min chase, the protein was predominantly in fractions 5 and 6 (Fig. 1C). Similar results were obtained if the detergent concentration was reduced to 0.25% (data not shown). As shown in the lowest panel in Fig. 1C, the Pr55^{gag} complexes at the 30 to 40% iodixanol interphase were resistant to Triton X-100 at 37°C.

Taken together, the results in Fig. 1A to C demonstrate that cold Triton X-100 extraction yields variable buoyant complexes for Pr55^{gag} depending on which cellular fraction is used in the experiment. To ascertain that the variability is not an artifact of the SFV expression system, we also analyzed the Triton X-100 resistance of Pr55^{gag} expressed in transfected 293T cells. Western blotting of the gradient fractions was used to visualize total Pr55^{gag}. Also in these transfected cells, the different cellular fractions yielded variable buoyant complexes for Pr55^{gag}: Triton X-100 extraction of membranes of crude homogenates yielded buoyant Pr55^{gag} complexes that were scattered in fractions 1 to 6, whereas the membranes of PNS gave Pr55^{gag}

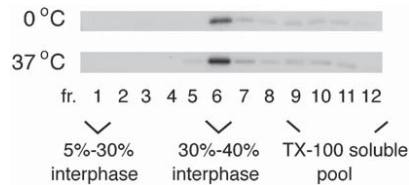


FIG. 2. Triton X-100 extraction of VLPs at 0°C or 37°C yields buoyant Pr55^{gag} complexes that float to the 30 to 40% iodixanol interphase. VLPs were collected from SFV-C/HIVgag-infected Jurkat cells which had been metabolically labeled with [³⁵S]methionine for 180 min. VLPs were extracted with 1% Triton X-100 (TX-100) and fractionated on iodixanol step gradients.

complexes that were concentrated into fractions 5 and 6 (Fig. 1D). Thus, the variability is a feature of Pr55^{gag}, and is not due to the expression system used.

Triton X-100-treated buoyant Pr55^{gag} complexes from VLPs are not physically associated with a raft-like membrane. The technical problems associated with the flotation assay made it difficult to determine with certainty what was a genuine Triton X-100-resistant complex and what was an artifact in the case of the intracellular Pr55^{gag}. However, one factor common to all of the fractionation schemes used in Fig. 1 was that some Pr55^{gag} consistently floated to the 30 to 40% iodixanol interphase (fraction 6). In agreement with previously published results (32), we found that extraction of VLPs with Triton X-100 at either 0 or 37°C also yielded Pr55^{gag} complexes that floated to fraction 6 (Fig. 2). Thus, the Pr55^{gag} complexes at the 30 to 40% iodixanol interphase most likely are not artifacts but represent genuine Triton X-100-resistant complexes. Figure 2 shows analyses of VLPs produced from SFV-C/HIVgag-infected Jurkat cells, but VLPs from transfected 293T cells gave similar results (data not shown).

Since VLPs can be obtained in a relatively pure form, this prompted us to determine whether floating of VLP-derived Pr55^{gag} to the 30 to 40% iodixanol interphase indeed is a result of these complexes being physically associated with Triton X-100-resistant membranes as has been previously suggested (32). To determine whether envelope cholesterol was retained in the Triton X-100-extracted Pr55^{gag} complexes, we labeled SFV-C/HIVgag-infected Jurkat cells with [³H]cholesterol and purified VLPs from the culture media by a two-step gradient centrifugation as described in Materials and Methods. Since cultured cells are known to shed microvesicles, which have densities similar to that of retrovirus particles, we first controlled how much of the total ³H radioactivity in our VLP sample was of microvesicular origin. To this end, we collected medium from [³H]cholesterol-labeled Jurkat cells infected with a recombinant SFV encoding influenza virus NP and subjected that medium to the same purification procedure used for VLPs. The ³H counts in VLPs and the NP control samples produced from parallel experiments were determined by liquid scintillation counting, and comparison of the two values indicated that the radioactivity in the NP control amounted to only ~10% of that of the VLPs (data not shown). Thus, the vast majority of the radioactive cholesterol in our VLP sample was likely to reside on the VLP envelope. To

determine whether this envelope-associated radioactive cholesterol was retained on Pr55^{gag} complexes after extraction with Triton X-100, the [³H]cholesterol-labeled VLPs and control NP samples were treated with Triton X-100 at 0°C, and the extracts were analyzed on an iodixanol step gradient otherwise similar to that in Fig. 1, except the gradient used was shorter. [³⁵S]methionine-labeled VLPs were used as a control to track the position of Pr55^{gag} complexes on the gradient. Five fractions were collected from the top of the gradient, and aliquots of fractions were analyzed by SDS-PAGE ([³⁵S]methionine-labeled VLPs) or by scintillation counting ([³H]cholesterol-labeled VLPs and the NP control; the counts from the NP sample were subtracted from the VLP values). As already demonstrated in Fig. 2, the [³⁵S]methionine-labeled Pr55^{gag} complexes from the Triton X-100-extracted VLPs floated to the 30 to 40% iodixanol interphase (fraction 2, Fig. 3). In contrast, the majority of the radioactive cholesterol counts (88% of total) were found in the Triton X-100-soluble pool (fractions 3 to 5). These results indicated that Triton X-100 extraction efficiently stripped envelope cholesterol from the VLP-Pr55^{gag} complexes.

We used VLPs containing radioactively labeled phospholipids to determine whether the Triton X-100-treated Pr55^{gag} complexes retain envelope phospholipids. The VLPs were collected from SFV-C/HIVgag-infected Jurkat cells that had been metabolically labeled with [³²P]orthophosphate. The [³²P]orthophosphate label is incorporated into both glycerophospholipids and sphingomyelin of VLPs. The VLPs were purified from the culture media by the same two-step centrifugation procedure used in the [³H]cholesterol experiments described above. Material collected from [³²P]orthophosphate-labeled Jurkat cells infected with the recombinant SFV encoding influenza virus NP was used as a control for microvesicular contamination. The radioactively labeled phospholipids were quantitated by solubilization of samples with excess hot SDS and by analyzing the solubilized material by SDS-PAGE. On 20% polyacrylamide gels, the mixed SDS-lipid micelles are separated from other ³²P-labeled components (proteins and RNA) (21), and this enables easy quantification of the gly-

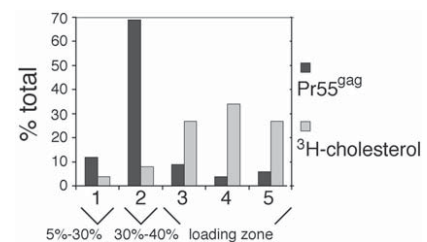


FIG. 3. Triton X-100 extraction efficiently strips envelope cholesterol from VLP-Pr55^{gag}. VLPs were collected from SFV-C/HIVgag-infected Jurkat cells that had been metabolically labeled with [³⁵S]methionine or [³H]cholesterol. VLPs were extracted with 1% Triton X-100 at 0°C, and the extracts were fractionated on iodixanol step gradients. Aliquots of gradient fractions were analyzed by SDS-PAGE ([³⁵S]methionine-labeled VLPs) or by scintillation counting ([³H]cholesterol-labeled VLPs). Labeled Pr55^{gag} and [³H]cholesterol in gradient fractions are expressed as percentages of the total (sum of all fractions).

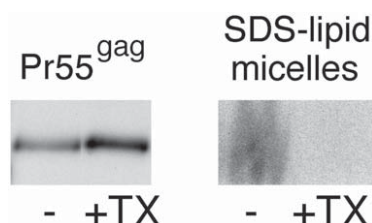


FIG. 4. Triton X-100 extracts the bulk of envelope phospholipids from the VLP-Pr55^{gag}. VLPs were collected from SFV-C/HIVgag-infected Jurkat cells that had been metabolically labeled with [³⁵S]methionine and [³²P]orthophosphate. VLPs were extracted with 1% Triton X-100 at 0°C, and the lysate was fractionated on an iodixanol step gradient as shown in Fig. 1. Buoyant Pr55^{gag} complexes from the 30 to 40% iodixanol interphase were concentrated by ultracentrifugation. The resulting pellet (+TX), as well as an intact, non-Triton X-100-extracted VLP sample (–), were solubilized with hot SDS and analyzed by SDS-PAGE. Equivalent amounts of samples were loaded on 10% (Pr55^{gag}) and 20% (SDS-lipid micelles) polyacrylamide gels. On 20% polyacrylamide gels, mixed SDS-lipid micelles separate from other ³²P-labeled material. Comparison of the 10 and 20% polyacrylamide gels demonstrates that although the intact VLP and the Triton X-100-treated samples contained similar amounts of Pr55^{gag} complexes, the two samples significantly differed in their phospholipid contents. The radioactive signal in the mixed SDS-lipid micelles originates from both glycerophospholipids and sphingomyelin.

erophospholipid and sphingomyelin contents of samples. We first estimated what percentage of the radioactivity in the VLP-derived SDS-lipid micelles truly originated from VLPs by comparing the radioactive signal of VLP sample to that of the control NP sample from a parallel experiment. Surprisingly, the SDS-lipid micelles from both the VLP and the control NP samples contained considerable amounts of radioactivity (data not shown), thus suggesting that Jurkat cells shed large amounts of microvesicles that were rich in phospholipids, but apparently poor in cholesterol, since the [³H]cholesterol-experiments described above had indicated only ~10% microvesicular contamination for our VLP sample. The microvesicles were not an artifact of the SFV expression system used, since similar amounts of vesicles were also shed from noninfected Jurkat cells labeled with [³²P]orthophosphate (data not shown). The exact degree of contamination of the VLP samples by microvesicles was difficult to establish, because the relative ratios of the VLP- and control NP-associated radioactivities differed between different experiments. From three different experiments though, we estimated that the microvesicle contamination accounted for 30 to 51% of the total phospholipid signal in VLP samples. To test what percentage of the VLP-envelope phospholipids were retained on Pr55^{gag} complexes after extraction with Triton X-100, we collected VLPs from cells that had been metabolically labeled with both [³²P]orthophosphate and [³⁵S]methionine. The VLPs were extracted with Triton X-100 at 0°C, and extracts were fractionated on the iodixanol step gradient described in the legend to Fig. 1. Material from the 30 to 40% iodixanol interphase was recovered and concentrated by pelleting. The pellet was solubilized in hot SDS, and equal volumes of the solubilized sample were analyzed on 10 and 20% gels along with intact VLPs. A representative experiment is shown in Fig. 4. [³⁵S]me-

thionine label in Pr55^{gag} bands (10% gels) was used to quantitate the amount of Pr55^{gag} in the two samples. In the experiment shown in Fig. 4, the Triton X-100-treated sample contained ~30% more Pr55^{gag} than the intact VLP sample. However, as shown by the right-hand panel in Fig. 4, the two samples significantly differed in their phospholipid contents: the radioactive lipid signal was readily detected in the intact VLP sample, whereas the lipid signal was very weak in the Triton X-100-extracted sample. Assuming that 49 to 70% of the total phospholipid signal from the intact VLP sample was from VLP envelopes, quantitation of the Pr55^{gag} and lipid signals of the two samples suggested that only 12 to 17% of the envelope phospholipids were retained on the Triton X-100-extracted buoyant Pr55^{gag} complexes. Taken together, these results and the [³H]cholesterol results described above strongly suggest that cold Triton X-100 extraction solubilizes the bulk of VLP envelope lipids. Thus floating of Triton X-100-treated VLP-Pr55^{gag} to the 30 to 40% iodixanol interphase is not due to these complexes being associated with a Triton X-100-resistant raft-like membrane.

Analyses of raft association of Pr55^{gag} by confocal fluorescence microscopy. The results shown in Fig. 1 to 4 suggested that Pr55^{gag} does not associate with Triton X-100-resistant rafts. However, the results do not exclude the possibility that Pr55^{gag} is a raft-associated protein, since not all rafts are resistant to Triton X-100 (11, 41). We therefore probed raft association of Pr55^{gag} by confocal fluorescence microscopy as well. Individual raft domains at the plasma membrane are small (~50 nm in diameter) (40) and thus are below the detection level of standard light microscopes. However, cell surface rafts can be induced to form larger patches by antibody-mediated cross-linking of raft components (23). Since the raft-lipid marker GM1 has been reported to be incorporated into HIV-1 virions (35), we decided to cross-link rafts at the cell surface via GM1 and to test whether Pr55^{gag} localizes to these cross-linked raft structures. Transfected 293T cells were used in the experiment. Pr55^{gag} has been previously reported to localize at the cell surface in a punctate pattern (25). However, as shown in Fig. 5A, the staining pattern for plasma membrane-associated Pr55^{gag} is dependent on the antibody used: the monoclonal EF-7 anti-Pr55^{gag} antibody gave a smooth staining pattern (Fig. 5A.1), whereas a punctate pattern was obtained with another anti-Pr55^{gag} monoclonal antibody (MAb 38:9; Fig. 5A.2). Since a possible raft localization of Pr55^{gag} would be easier to score with a staining pattern that was initially smooth, we decided to use the EF-7 antibody in our raft cross-linking experiments. Cholera toxin B subunit specifically binds to GM1 (23), and the GM1-containing rafts were patched with this toxin and antibodies directed against it. The cells were subsequently fixed, permeabilized, and stained for Pr55^{gag}. Figure 5B shows confocal analyses of nonpatched cells. Pr55^{gag} (red, Fig. 5B.1) and GM1 (green, Fig. 5B.2) were both uniformly distributed on the plasma membrane of these nonpatched cells. Figure 5C.1 to C.3 show three representative examples of cross-linked cells with the Pr55^{gag} and GM1 signals superimposed. Both Pr55^{gag} and GM1 were distributed in a patchy, dot-like pattern on these cells. Three different types of patches could be distinguished: patches that were positive only for GM1 (green), patches that were strongly positive for both GM1 and Pr55^{gag} (yellow), and patches that appeared to

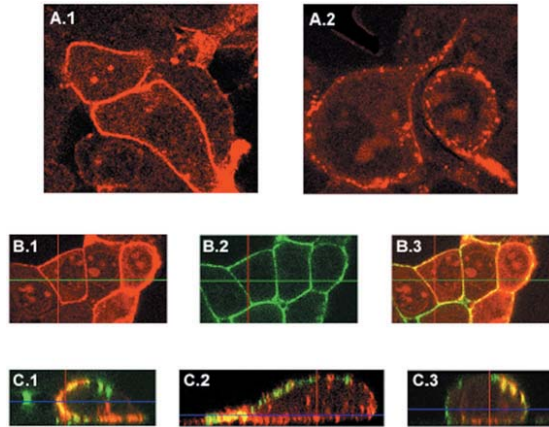


FIG. 5. Confocal fluorescence microscopy of transfected 293T cells. (A) Different anti-Pr55^{gag} antibodies give different staining patterns for Pr55^{gag} at the cell surface. (A.1) MAb EF-7. (A.2) MAb 38:9. (B) Both Pr55^{gag} and GM1 exhibit smooth staining patterns on transfected 293T cells if GM1-containing rafts have not coalesced. Live cells were first incubated with FITC-conjugated cholera toxin B subunit at a cold temperature to detect GM1, and the cells were subsequently fixed, permeabilized, and incubated with MAb EF-7 and TRITC-conjugated antimouse IgG antibodies to detect Pr55^{gag}. (B.1) Pr55^{gag}. (B.2) GM1. (B.3) Pr55^{gag} and GM1 images superimposed. (C) Coalescence of GM1-positive rafts at the cell surface transforms the smooth staining pattern of Pr55^{gag} into a patchy one. Live cells were first incubated with FITC-conjugated cholera toxin B subunit at a cold temperature, and the GM1-positive rafts were subsequently patched by brief incubation at 37°C in the presence of anti-cholera toxin antibodies. Cells were then fixed, permeabilized, and immunostained with MAb EF-7 and TRITC-conjugated antimouse IgG antibodies to detect Pr55^{gag}. Three representative examples of patched cells (C.1 to C.3) are shown with Pr55^{gag} (red) and GM1 (green) images superimposed. Yellow indicates extensive colocalization of Pr55^{gag} and GM1. A single optical section is shown in panels A and B, whereas all optical sections (the Z-stack) were superimposed in panel C and tilted with the orthogonal function to show the view through the whole cell.

be positive only for Pr55^{gag} (red). However, from the nonoverlapped images, it was evident that the latter Pr55^{gag}-containing patches were actually positive for GM1 as well, but the GM1 signal was weak in these patches (data not shown). Collectively, these results suggest that a significant fraction of Pr55^{gag} at the cell surface might be localized to GM1-positive rafts, but according to the results from Fig. 1 to 4, these rafts apparently are Triton X-100 sensitive.

Intracellular membrane-bound Pr55^{gag}, as well as VLP-associated Pr55^{gag}, is largely resistant to extraction with Brij98. To obtain further evidence for localization of Pr55^{gag} to distinct Triton X-100-sensitive rafts, we turned back to the biochemical assay and tested the sensitivities of membrane-bound Pr55^{gag} to different detergents by using transfected 293T cells. Since we judged that chromatin and nuclear remnants were the main sources of artifacts in the biochemical assay, we chose the total membrane fraction of PNS as the starting material for detergent extractions. Our pilot assays indicated that the membrane-bound Pr55^{gag} was partially resistant to Brij35, Brij56, Brij58, Brij98, Lubrol, and CHAPS (data not shown). We decided to focus on Brij98, since this is a detergent that detects rafts at physiological temperatures (11). Figure 6A shows analysis of Pr55^{gag}-containing membranes either nonextracted or extracted with 0.5% Brij98 at 37°C for 5 min. The samples were analyzed on an iodixanol step gradient consisting of a 56% loading zone overlaid with 30, 25, 20, and 5% iodixanol solutions. Western blotting of the gradient fractions was used to visualize total Pr55^{gag}. In the nonextracted sample, Pr55^{gag} was predominantly found in fractions 1 to 4. After extraction with

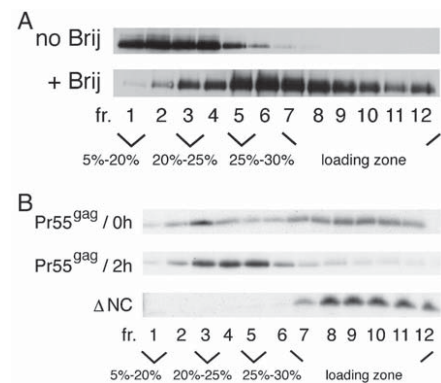


FIG. 6. Intracellular membrane-bound Pr55^{gag} is largely resistant to Brij98. (A) Analyses of transfected 293T cells. The total membrane fraction from PNS was extracted with Brij98 for 5 min at 37°C or left untreated, and the samples were subjected to flotation on iodixanol step gradients. Total Pr55^{gag} in gradient fractions was visualized by Western blotting. (B) Brij98 resistance of newly synthesized Pr55^{gag}. The wild type and Δ NC (MA-CA-p2) mutant of Pr55^{gag} were expressed in Jurkat cells from recombinant SFV genomes. Cells were metabolically labeled with [³⁵S]methionine for 15 min and chased for 0 min and 2 h (Pr55^{gag}) or for 2 h (Δ NC). The total membrane fraction from PNS was extracted with Brij98 as described above, and the extracts were fractionated on iodixanol step gradients. Aliquots of gradient fractions were analyzed by SDS-PAGE.

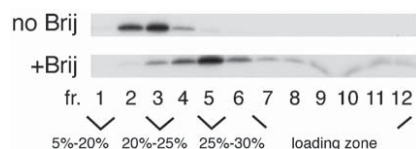


FIG. 7. Brij98-treated VLPs retain low buoyant density. VLPs were collected from SFV-C/HIVgag-infected Jurkat cells that had been metabolically labeled with [35 S]methionine for 180 min. The VLPs were extracted with Brij98 and analyzed as described in the legend to Fig. 6. No Brij indicates intact, nonextracted VLPs.

Brij98, the protein was scattered in fractions 2 to 12 (i.e., from the 20% iodixanol zone to the loading zone). Most importantly, significant amounts of the protein were found in the light-density fractions 2 to 5, thus suggesting that at steady state, a population of the membrane-bound Pr55^{gag} was largely resistant to Brij98. To test the sensitivity of newly synthesized Pr55^{gag} to Brij98, we turned to Jurkat cells and the SFV expression system. SFV-C/HIVgag-infected Jurkat cells were metabolically labeled with [35 S]methionine for 15 min, and membrane-bound Pr55^{gag} was analyzed after a 0- or 120-min chase. As shown in Fig. 6B, after a 0-min chase, labeled Pr55^{gag} was broadly distributed along the gradient, with a minor concentration of the protein in fraction 3. After a 120-min chase, the amount of the protein in the loading zone was decreased, and a clear accumulation of the protein (~61% of total) was observed in fractions 3 to 5. This suggested that Pr55^{gag} accumulated in Brij98-resistant membrane domains of light density in a time-dependent fashion. In contrast to the wild-type Pr55^{gag}, a carboxy-terminally-deleted mutant form of the protein consisting of MA-CA-p2-domains (Δ NC) remained sensitive to Brij98 even after 2 h of chase.

In order to determine whether the Brij98-resistant intracellular Pr55^{gag} complexes were precursors for VLPs, we tested the sensitivity of VLPs to Brij98 extraction. Extracellular VLPs were extracted with 0.5% Brij98 at 37°C for 5 min, and the extracts were fractionated on an iodixanol step gradient similar to that shown in Fig. 6. As shown in Fig. 7, nonextracted VLPs produced from SFV-C/HIVgag-infected Jurkat cells floated through 25% iodixanol to fractions 2 and 3. After extraction with 0.5% Brij98 (5 min at 37°C), a slight shift in the density was evident, and Pr55^{gag} peaked in fraction 5, thus overlapping the intracellular Brij98-resistant complexes. Similar results were obtained with VLPs produced from transfected 293T cells (data not shown). These results suggested that VLP envelope was largely resistant to Brij98, and the intracellular Brij98-resistant Pr55^{gag} complexes banding at 25% iodixanol most likely represented precursors for VLPs.

To confirm that the VLP envelope was indeed largely resistant to Brij98, we monitored the extractability of envelope lipids by the detergent. To determine whether envelope cholesterol was retained on Brij98-extracted Pr55^{gag} complexes, we performed an assay similar to that described above for Triton X-100 extractions. [3 H]cholesterol- or [35 S]methionine-labeled VLPs were extracted with 0.5% Brij98 for 5 min at 37°C, and the extracts were analyzed on an iodixanol step gradient consisting of a 56% loading zone overlaid with 30, 20, and 5% iodixanol solutions. As shown in Fig. 8A, the majority

of both the [35 S]methionine-labeled Pr55^{gag} complexes and the envelope [3 H]cholesterol label (80 and 58% of the total, respectively) was found in fraction 3 at the 20 to 30% iodixanol interphase. This indicated that a large fraction of the envelope cholesterol was resistant to Brij98.

To test the extractability of the envelope phospholipids by Brij98, we carried out an assay similar to that described above for Triton X-100 extractions. [35 S]methionine- and [32 P]orthophosphate-labeled VLPs and the [32 P]orthophosphate-labeled NP control material were extracted with 0.5% Brij98 for 5 min at 37°C, and the extracts were fractionated on an iodixanol step gradient as described in the legend to Fig. 6. The buoyant Pr55^{gag} complexes or the control NP material from fractions 3 to 5 was pooled and concentrated by pelletation, and the pellets were solubilized with excess hot SDS and analyzed on 10 and 20% polyacrylamide gels along with intact VLPs. As shown in Fig. 8B, the Brij98-treated Pr55^{gag} complexes retained significant amounts of the envelope phospholipids. Comparison with the Brij-treated NP control indicated that

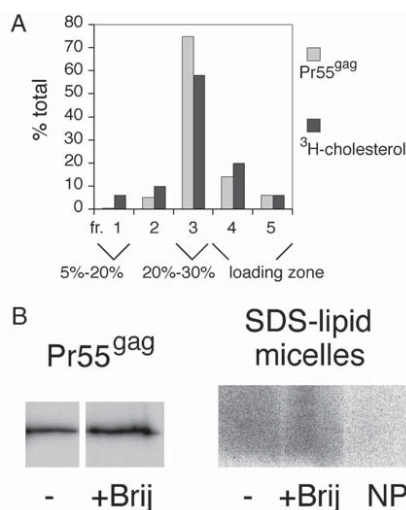


FIG. 8. Large fraction of envelope phospholipids and cholesterol remains attached to Brij98-treated VLP-Pr55^{gag}. (A) Brij98 extraction of [35 S]methionine- or [3 H]cholesterol-labeled VLPs produced from SFV-C/HIVgag-infected Jurkat cells. The VLPs were extracted with Brij98 for 5 min at 37°C, and the samples were processed as described in the legend to Fig. 3, with the exception that the iodixanol step gradient used was slightly different. (B) Brij98 extraction of [35 S]methionine- and [32 P]orthophosphate-labeled VLPs produced from SFV-C/HIVgag-infected Jurkat cells. The VLPs were extracted with Brij98 for 5 min at 37°C, and the extracts were fractionated on iodixanol step gradients as described in the legend to Fig. 6. The buoyant Pr55^{gag} complexes from fractions 3 to 5 were pooled and concentrated by pelletation. The pellets, as well as intact, nonextracted VLP samples (–) were analyzed as described in the legend to Fig. 4. NP is a Brij98-treated control sample that was collected from [32 P]orthophosphate-labeled Jurkat cells infected with recombinant SFV encoding influenza virus NP. The NP control demonstrates that the majority of phospholipid signal in the Brij98-treated VLP sample originates from VLPs, not from contaminating microvesicles.

majority of the Brij-resistant Pr55^{gag} signal originated from VLPs. Assuming that microvesicles accounted for ~30 to 51% of the total phospholipid signal in intact VLPs, quantitations of the Pr55^{gag} and the SDS-lipid micelle signals from two separate experiments suggested that ~37 to 53% of envelope phospholipids were retained on Brij98-treated Pr55^{gag} complexes. Taken together, these results indicated that the VLP envelope was largely resistant to extraction with Brij98.

DISCUSSION

HIV-1 is one of the enveloped viruses whose assembly and budding has been proposed to be critically dependent on lipid rafts (32, 35, 36, 42). The strongest evidence for this raft connection has been the localization of Pr55^{gag}, the protein that drives the assembly of virus, to Triton X-100-resistant rafts (32, 35, 36, 57). Localization of Pr55^{gag} to the Triton X-100-resistant rafts was based on results from the commonly used biochemical raft fractionation assay, which involves extraction of cells with Triton X-100 at cold temperatures. Triton X-100-resistant rafts retain buoyant density after cold detergent treatment and float to a low density when the detergent extracts are fractionated on density gradients. However, our results indicate that the buoyancy of Triton X-100-treated Pr55^{gag} complexes does not provide solid evidence for raft localization of Pr55^{gag}.

Nguyen and Hildreth (35) have reported that the majority of [³H]myristic acid-labeled intracellular Pr55^{gag} is associated with buoyant Triton X-100-resistant structures in cold cell lysates, and in a report by Zheng et al. (57), Nef was found to increase cofractionation of Pr55^{gag} with Triton X-100-resistant buoyant structures. However, in these two reports, the buoyant structures were not further characterized. Lindwasser and Resh (32) reported that cold Triton X-100-extracted crude cell lysates, as well as Triton X-100-treated VLPs, yield buoyant Pr55^{gag} complexes that band at the 30 to 40% iodixanol interphase. These buoyant Pr55^{gag} complexes were of higher density than "classical" Triton X-100-resistant rafts, which float through 30% iodixanol. Ono and Freed (36) analyzed raft association of Pr55^{gag} by using postnuclear supernatants of HIV-1-infected cell homogenates. They estimated that up to approximately half of membrane-bound Pr55^{gag} associated with Triton X-100-resistant buoyant structures in cold lysates, and these structures were soluble in the detergent if the extraction was carried out at 37°C. In contrast to the study by Lindwasser and Resh (32), the buoyant Pr55^{gag} complexes in the study by Ono and Freed (36) appeared to have a density similar to that of classical raft markers, but this discrepancy was most likely due to the different gradients used. Ono and Freed separated Triton X-100-sensitive and -resistant pools of proteins from each other by using sucrose gradients with a single, rather dense (65% [wt/vol] sucrose) separating layer. Nguyen and Hildreth (35), Lindwasser and Resh (32), Zheng et al. (57), and Ono and Freed (36) all interpreted that their buoyant Pr55^{gag} complexes reflected association of the protein with rafts. However, our data presented in Fig. 1 indicate that, in the case of Pr55^{gag}, the results from the biochemical raft fractionation assay have to be interpreted with caution, since the assay is very prone to artifacts. Figure 1 demonstrates that variable buoyant complexes are obtained for Pr55^{gag}, depend-

ing on which cellular fraction is used as the starting material for the Triton X-100 treatment. Whole-cell lysates, as well as membranes from PNS, predominantly yielded buoyant Pr55^{gag} complexes, which banded at the 30 to 40% iodixanol interphase, whereas membranes from crude cell homogenates gave buoyant complexes of more heterogeneous density. Furthermore, the influenza virus NP control (Fig. 1B) demonstrated that the sensitivity of a buoyant protein structure to Triton X-100 at 37°C does not necessarily mean that the structure represents rafts. Although it is difficult to determine with certainty which of the buoyant Pr55^{gag} complexes reflect true *in vivo* Pr55^{gag} structures and which are artifacts, our control experiments suggested that chromatin and other nuclear remnants could comprise a major source for artifacts. We therefore favor the interpretation that membranes from PNS are the best starting material for the detergent treatment, and, consequently, the Pr55^{gag} complexes at the 30 to 40% iodixanol interphase represent the true intracellular Triton X-100-resistant structures of Pr55^{gag}. This interpretation is further supported by the fact that similar complexes are obtained from Triton X-100-treated VLPs as well. Due to their higher relative purity, the VLP preparations are less likely to yield artifacts than the molecularly complex cell extracts.

Lindwasser and Resh coined the name "barges" for the Triton X-100-treated buoyant Pr55^{gag} structures at the 30 to 40% iodixanol interphase (32). Since their results indicated that localization of Pr55^{gag} to barges correlated with oligomerization of the protein, their interpretation was that barges represent Triton X-100-resistant rafts, which have an increased density due to the presence of high-valence Pr55^{gag} multimers (32). However, they did not validate their interpretation by performing lipid analyses of the barge-associated Pr55^{gag}. Our lipid analyses of the [³²P]orthophosphate- and [³H]cholesterol-labeled VLPs do not support the notion that floating of the Triton X-100-treated Pr55^{gag} to the 30 to 40% iodixanol interphase reflects association of the protein with Triton X-100-resistant rafts. [³²P]orthophosphate labeling of cells produces VLPs with radioactively labeled glycerophospholipids and sphingomyelin, and comparison of SDS-lipid micelles from intact VLPs and the Triton X-100-treated Pr55^{gag} complexes indicated that the buoyant Pr55^{gag} complexes were largely devoid of envelope glycerophospholipids and sphingomyelin. Furthermore, fractionation of [³H]cholesterol-labeled Triton X-100-extracted VLPs on an iodixanol step gradient indicated that the detergent extraction efficiently stripped envelope cholesterol from the VLP-Pr55^{gag}. Since glycerophospholipids, sphingomyelin, and cholesterol together constitute the vast majority of HIV-1 envelope lipids (2, 3), these results strongly suggest that envelope lipids do not remain attached to the buoyant Triton X-100-treated Pr55^{gag} complexes in large amounts. This conclusion is further supported by the fact that the Triton X-100 extraction causes a considerable shift in the density of membrane-bound Pr55^{gag} (compare, e.g., Fig. 1D, PNS panel, with Fig. 6A, no-detergent-panel). The most plausible explanation for this shift is that the detergent extracts the bulk of membrane lipids from the Pr55^{gag} complexes. Recently, Ding et al. (10) published a study demonstrating that only Pr55^{gag}, but not classical raft markers, undergoes this clear shift in density upon cold Triton X-100 extraction. Thus, it is questionable whether the buoyant Triton X-100-resistant

Pr55^{gag} complexes can be interpreted to signify localization of the protein to rafts. Our finding that the buoyant Pr55^{gag} complexes at the 30 to 40% iodixanol interphase, unlike classical raft markers, are insensitive to Triton X-100 at 37°C or that the buoyant Pr55^{gag} complexes are insensitive to cholesterol depletion (10) provides a further argument against the buoyant Pr55^{gag} structures representing Triton X-100-resistant raft membranes. At present, however, we have no explanation to offer for the floating phenotype of the Triton X-100-treated Pr55^{gag}. Since we estimated that 12 to 17% of total membrane phospholipids could remain attached to the Triton X-100-treated Pr55^{gag}, it is possible that these residual phospholipids, perhaps together with membrane lipids that were not scored in our assay (e.g., gangliosides), cause the buoyancy of the Pr55^{gag} complexes. Our attempts to reliably measure the retention of GM1 on the Triton X-100-treated Pr55^{gag} complexes were unsuccessful due to the low specific GM1 signal from VLPs (data not shown). Interestingly, only the unprocessed HIV-1 Pr55^{gag} yielded buoyant Triton X-100-resistant complexes, whereas MA and CA of processed HIV-1 cores, as well as the unprocessed Gag precursor of Moloney murine leukemia virus VLPs, fractionated with the detergent-soluble pool (data not shown).

Triton X-100 has been the most commonly used detergent in raft studies, but not all rafts are resistant to Triton X-100 (11, 41). We therefore probed the possible raft association of Pr55^{gag} by confocal fluorescence microscopy and with detergents other than Triton X-100. The smooth cell surface staining pattern of Pr55^{gag} in transfected 293T cells could be transformed into a patchy one by antibody-mediated coalescence of GM1-positive rafts. Furthermore, extraction of the total membrane fraction of PNS from transfected 293T cells suggested that at steady state, a population of membrane-bound Pr55^{gag} was largely resistant to Brij98. In contrast to the Triton X-100-treated buoyant Pr55^{gag} complexes, the Brij98-resistant complexes were of lighter density and banded at 25% iodixanol. Pulse-chase studies in Jurkat cells indicated that newly synthesized Pr55^{gag} accumulated in these low-density Brij98-resistant structures in a time-dependent manner. In contrast, a mutant form of Pr55^{gag} consisting of MA-CA-p2 remained sensitive to Brij98 even after extended chase periods. Since the MA-CA-p2-mutant is impaired in oligomerization (8; K. Holm and M. Suomalainen, unpublished data), the time-dependent accumulation of wild-type Pr55^{gag} in the low-density Brij98-resistant structures could reflect oligomerization of the protein. The intracellular Brij98-resistant complexes most likely represent assembly intermediates, since Brij98 extraction of VLPs produced Pr55^{gag} complexes of similar density. Analyses of [³²P]orthophosphate- and [³H]cholesterol-labeled VLPs confirmed that the buoyant density of the floating Brij98 complexes was due to these complexes being associated with a membrane that was largely resistant to the detergent. We estimated that ~37 to 53% of envelope phospholipids and ~53% of cholesterol remained attached to the buoyant Brij98-treated VLP-Pr55^{gag}. One possible explanation for this partial resistance is that the VLP membrane is a mosaic, consisting of both Brij98-resistant and Brij98-sensitive membrane domains. Taken together, these confocal and Brij98 results could indicate that Pr55^{gag} associates with raft microdomains at the cell surface that are resistant to Brij98, and these microdomains

form a part of the platform for Pr55^{gag}-mediated assembly and budding of HIV-1. However, at present, we cannot exclude the possibility that oligomerization of Pr55^{gag} induces a structural alteration in the membrane that results in increased Brij98 resistance (i.e., that the Brij98 resistance of membrane-bound Pr55^{gag} has nothing to do with cellular rafts). Since Brij98-resistant rafts are newcomers in the raft field and are still poorly characterized, it is unknown at present whether any cellular rafts have a similar density to the Brij98-resistant Pr55^{gag} structures described here. Of our three cellular marker proteins, only TR partially overlapped with Pr55^{gag} complexes after Brij98 treatment, whereas CD55 floated through 20% iodixanol and Lck stayed in the loading zone (data not shown).

The growing list of viral raft-associated structural proteins has created a lot of excitement in the field of virus assembly. Even a partial localization of a viral structural protein to rafts has commonly been taken as evidence for rafts playing a critical role in the assembly of the virus in question. However, concrete evidence for rafts being mechanistically important for assembly and budding of enveloped viruses is actually scarce. In the case of HIV-1, it amounts to one observation—namely, that cholesterol-depleting agents inhibit particle release from HIV-1-infected cells (36). Cholesterol is essential for structural integrity of rafts (5, 45), and disruption of rafts by cholesterol depletion is expected to reduce virus production if assembly and budding of HIV-1 are dependent on rafts. However, since cholesterol depletion affects many different cellular processes (28, 29, 44, 45), it could bring about reduction in HIV-1 particle production entirely through indirect effects as well. The observations that viral spike proteins appear to localize (partially) to rafts and that cellular raft lipids and proteins are found in HIV-1 particles have been put forward as another piece of evidence for the importance of rafts in the assembly and budding of HIV-1 (35, 38, 42, 57). However, whether these observations indicate an obligatory linkage between rafts and the assembly and budding mechanisms of HIV-1 is unknown. With respect to the presence of host raft lipids and proteins in the HIV-1 envelope, it is worthwhile to remember that there are indications that rafts actually might be relatively abundant structures at the cell surface (12, 15, 22, 33, 39) and that retroviruses seem to be quite indiscriminate in choosing their envelope components (21, 37). Furthermore, in the case of viral spike proteins, the rafts in question appear to belong to the class of Triton X-100-resistant rafts. It is unclear how these rafts correlate with viral assembly intermediates, since according to our data, Pr55^{gag} does not appear to have intrinsic affinity for Triton X-100-resistant rafts, and there are no data on Triton X-100 sensitivity or resistance of spike proteins in virus particles. In principle, it is possible that the Triton X-100-resistant rafts containing viral spike proteins and the Brij98-resistant Pr55^{gag} membrane domains described here are similar types of rafts and that Pr55^{gag} and viral envelope proteins only exhibit different sensitivities to Triton X-100 within these raft structures. However, we did not find any indications for Triton X-100-resistant raft-like lipid domains in the envelope of Pr55^{gag} VLPs with cold Triton X-100 extractions: both the radioactive cholesterol (Fig. 3) and the bulk of radioactively labeled phospholipids (data not shown) in the VLP envelope were readily solubilized with cold Triton X-100. In summary, before it can be firmly concluded that rafts play a critical role

in the assembly of HIV-1, direct evidence is needed that rafts or raft-associated host factors are important for some discrete step or steps in the viral assembly process.

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II

Vpu and Tsg101 Regulate Intracellular Targeting of the Human Immunodeficiency Virus Type 1 Core Protein Precursor Pr55^{gag}

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Assembly of human immunodeficiency virus type 1 (HIV-1) is directed by the viral core protein Pr55^{gag}. Depending on the cell type, Pr55^{gag} accumulates either at the plasma membrane or on late endosomes/multivesicular bodies. Intracellular localization of Pr55^{gag} determines the site of virus assembly, but molecular mechanisms that define cell surface or endosomal targeting of Pr55^{gag} are poorly characterized. We have analyzed targeting of newly synthesized Pr55^{gag} in HeLa H1 cells by pulse-chase studies and subcellular fractionations. Our results indicated that Pr55^{gag} was inserted into the plasma membrane and, when coexpressed with the viral accessory protein Vpu, Pr55^{gag} remained at the plasma membrane and virions assembled at this site. In contrast, Pr55^{gag} expressed in the absence of Vpu was initially inserted into the plasma membrane, but subsequently endocytosed, and virus assembly was partially shifted to internal membranes. This endocytosis of Pr55^{gag} required the host protein Tsg101. These results identified a previously unknown role for Vpu and Tsg101 as regulators for the endocytic uptake of Pr55^{gag} and suggested that the site of HIV-1 assembly is determined by factors that regulate the endocytosis of Pr55^{gag}.

Assembly and budding of human immunodeficiency virus type 1 (HIV-1) is driven by the viral core protein precursor Gag (Pr55^{gag}), which assembles into enveloped virus-like particles (VLPs) when expressed in the absence of other viral proteins (9, 21). Pr55^{gag} is a myristoylated, peripheral membrane protein (3, 9, 14, 40, 47). Concomitantly with assembly and budding, Pr55^{gag} is processed into matrix, capsid (CA), nucleocapsid, and p6 proteins and two smaller peptides SP1 and SP2 by a viral protease present in the minor core component, the Gag-Pol precursor. An intriguing feature of the Pr55^{gag}-mediated assembly of HIV-1 is that different cellular membranes serve as a platform for virus assembly in different cell types. Electron microscopy (EM) and fluorescence microscopy studies have indicated that Pr55^{gag} predominantly accumulates at the plasma membrane, and progeny virions are formed at this site in T cells and model cell lines such as HeLa, 293T, and Cos-1 (see, for example, references 8, 16, and 27), although some Pr55^{gag} has also been identified on late endosomes in these cells (6, 10, 26, 39, 46). In contrast, in macrophages Pr55^{gag} almost exclusively concentrates on late endosomes/multivesicular bodies (MVBs) and progeny virions bud into the lumen of MVBs (25, 28, 31, 33). Egress of virus particles from the cell surface, and probably also from the endosomal assembly sites, is critically dependent on interaction of the Pr55^{gag} p6 domain with the host protein Tsg101 (tumor susceptibility gene 101) (7, 23, 45, 46), which normally functions in formation of MVB vesicles (24). Since ablation of Pr55^{gag}-Tsg101 interactions arrests virus budding at a stage

after membrane distortion (7, 46), the primary function of Tsg101 in HIV-1 assembly and budding is thought to be the recruitment of downstream factors essential for the completion of budding and release of virions from the donor membrane (24, 46).

Molecular mechanisms that control cell surface versus endosomal accumulation of Pr55^{gag}, and consequently the choice of virus budding site, are poorly understood. One aspect that remains to be clarified is the route by which Pr55^{gag} reaches its cell surface or endosomal steady-state localization. Like other myristoylated proteins, Pr55^{gag} is expected to be synthesized on cytosolic ribosomes and inserted into membranes posttranslationally (20). It is assumed that the classical endoplasmic reticulum-Golgi secretory route to the plasma membrane and endosomes is not involved in intracellular targeting of Pr55^{gag}, since inhibitors of this transport pathway, such as brefeldin A or monensin, have no effect on the assembly of Pr55^{gag} into extracellular particles (29, 30). Since Pr55^{gag} has been observed on endosomal membranes in a number of different cell types (6, 10, 26, 39, 46), it is possible that the protein is first inserted into endosomal membranes and either retained in these compartments (macrophages) or transported further to the plasma membrane (e.g., HeLa, 293T, or T cells). Alternatively, newly synthesized Pr55^{gag} could be first targeted to the plasma membrane, and endosomal localization could be achieved via endocytosis of the cell surface-associated protein. A third possibility is that a newly synthesized Pr55^{gag} is selectively inserted either into the plasma membrane or endosomal membranes depending on the host cell type.

In the present study, we analyzed targeting of newly synthesized Pr55^{gag} in HeLa H1 cells by pulse-chase studies and a subcellular fractionation assay that efficiently separated plasma

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membrane from internal membranes. Our results demonstrated that a newly synthesized Pr55^{gag} was initially targeted to the plasma membrane. Pr55^{gag} accumulated at the plasma membrane when coexpressed with the viral accessory protein Vpu but, in the absence of Vpu, a significant fraction of the cell surface-associated Pr55^{gag} proteins was retargeted to internal membranes by endocytosis. This retargeting partially shifted virus assembly from the cell surface to internal membranes. RNA interference (RNAi)-mediated knockdown of Tsg101, or deletion of the p6 domain from Pr55^{gag} suppressed the endocytic uptake of Pr55^{gag} when the protein was expressed in the absence of Vpu. Taken together, these results suggest that the site of HIV-1 assembly is determined by factors that regulate endocytosis of Pr55^{gag}, and the results identified a previously unknown role for Vpu and Tsg101 as regulators of the endocytic uptake of Pr55^{gag}.

MATERIALS AND METHODS

Cell culture and transfection. HeLa H1 cells (American Type Culture Collection) were grown in Dulbecco's modified Eagle medium supplemented with 7% fetal bovine serum (EuroClone) and nonessential amino acids (i.e., HeLa growth medium). 293T cells were cultured as previously described (18). Vesicular stomatitis virus (VSV) G-protein-pseudotyped infectious lentivirus vectors carrying modified HIV-1 genomes were produced by cotransfecting pCMVΔR8.91 and pMD2.VSV.G (generous gifts from Didier Trono [48]), together with a plasmid directing the synthesis of a recombinant HIV-1 genome into 293T cells by a calcium phosphate precipitation technique.

Plasmid constructs. pHXB2D and pNL4-3 were originally obtained from the NIBSC Centralized Facility for AIDS Reagents (the plasmids were donated by Robert Gallo and Mikulas Popovic and by Malcolm Martin, respectively). pHXB2D is a HIV-1 molecular clone with a defective *vpu* gene and truncated *vpr* and *nef* genes (38). Plasmid pHXB2D-Gag is a derivative of pHXB2D that expresses an unprocessed Pr55^{gag} due to an inactivation of the Pol coding region. The plasmid was constructed by replacing the 423-bp ApaI-Bell fragment with a PCR-derived 297-bp ApaI-Bell fragment in which the stop codon of Pr55^{gag} was immediately followed by SmaI and Bell sites. pHXB2D-GagHA and pHXB2D-Gagflag are derivatives of pHXB2D-Gag which express unprocessed Pr55^{gag} proteins that contain hemagglutinin (HA) or FLAG epitopes at their carboxy termini. pNL4-3(Gag), a derivative of pNL4-3 (1), has an inactive Pol-region and expresses an unprocessed Pr55^{gag}. The plasmid was constructed by replacing the 3,779-bp ApaI-SalI fragment with the equivalent ApaI-SalI fragment of pHXB2D-Gag. pNL4-3(Gag/ΔVpu), a derivative of pNL4-3(Gag), has a defective *vpu* gene due to a point mutation in the AUG start codon of *vpu*. The construct was created by replacing the 558-bp SalI-KpnI fragment with the equivalent fragment of pHXB2D. The plasmid pNL4-3(GagΔp6/ΔVpu) encoding a carboxy terminally truncated unprocessed Pr55^{gag} lacking the p6 domain was made from pNL4-3(Gag/ΔVpu) by replacing the 791-bp SpeI-SmaI fragment with a PCR-created 635-bp SpeI-SmaI fragment in which a stop codon and a SmaI site were placed immediately after the last codon of SP2 peptide. pNL4-3(ΔEnv/ΔNef) is a derivative of pNL4-3 with defective *env* and *nef* genes. The first step in construction of pNL4-3(ΔEnv/ΔNef) involved inactivation of the *env* gene on pNL4-3(Gag) by deleting the 580-bp BglII-BglII fragment from the *env* coding region. This created the plasmid pNL4-3(Gag/ΔEnv). pNL4-3(ΔEnv/ΔNef) was made by replacing the 2,680-bp SalI-BamHI fragment of pNL4-3-R71.Nef (35): the plasmid contains an inactivated *nef* gene with the equivalent fragment from pNL4-3(Gag/ΔEnv). pHXB2D-(ΔVpu/ΔEnv/ΔNef) contains inactive *vpu*, *env*, and *nef* genes. The first step in the construction of pHXB2D-(ΔVpu/ΔEnv/ΔNef) was inactivation of the *env* gene on pHXB2D by deleting a 1,278-bp region between KpnI and BglII sites on *env* which in addition to an internal deletion also introduced a frameshift mutation on the gene. This created the plasmid pHXB2D-(ΔVpu/ΔEnv). pHXB2D-(ΔVpu/ΔEnv/ΔNef) was made from pHXB2D-(ΔVpu/ΔEnv) by inactivating the truncated *nef* gene via converting cohesive ends of XhoI-linearized pHXB2D-(ΔVpu/ΔEnv) into blunt ends by Klenow and subsequently religating the plasmid.

Subcellular fractionations. HeLa H1 cells were seeded on 10-cm plates at a density which gave ~70% confluence after o/n growth. Cells were infected the following day with VSV-G-pseudotyped lentivirus vectors carrying modified HIV-1 genomes. At ~24 h postinfection the non-confluent cell monolayers were

metabolically labeled with 50 μ Ci of [³⁵S]methionine for 30 min (18) and chased for various times in HeLa growth medium containing a 10-fold excess of cold methionine. After the chase, cell monolayers were washed twice with phosphate-buffered saline containing 0.02% EDTA (PBS-EDTA), and the cells were brought into suspension by a 2-min incubation in PBS-EDTA at 37°C. Subsequently, the suspension cells were coated at 4°C with cationic silica beads essentially as previously described (4, 41), except that the coating buffer used was 20 mM 2-[N-morpholine]ethanesulfonic acid (pH 6.66 to 6.68) and 150 mM NaCl. After coating, cells were homogenized with a tight-fitting Dounce homogenizer in buffer containing 25 mM HEPES (pH 7.4), 150 mM NaCl, 2 mM MgCl₂, 20 μ g of phenylmethylsulfonyl fluoride, 1 μ g of CLAP (chymostatin, leupeptin, aprotinin, and pepstatin A), and 2.5 mg of heparin per ml (the heparin was included to suppress binding of Pr55^{gag} to chromatin released from broken nuclei). The crude cell homogenates were mixed with 100% Nycodenz solution (AXIS-SHIELD PoC AS) to give a final 60% Nycodenz concentration. The sample (2 ml) was placed on a 1-ml cushion of 70% Nycodenz and overlaid with 1.5 ml of 50% Nycodenz and 0.5 ml of 25 mM HEPES (pH 7.4), 150 mM NaCl, and 1 mM EDTA. The gradient was centrifuged in an SW55 Ti rotor at 63,000 \times g for 45 min at 4°C. The pellet (i.e., the plasma membrane fraction) was resuspended in hot 0.5% sodium dodecyl sulfate (SDS)-0.5 M NaCl buffer and then boiled for 5 min, and silica beads were removed from the resuspended samples by centrifugation in an Eppendorf centrifuge at full speed for 1 min. The internal membrane fraction at the 50% Nycodenz-buffer interphase was collected and diluted fivefold with 25 mM HEPES (pH 7.4), 150 mM NaCl, and 1 mM EDTA, and membranes were pelleted by centrifugation at 100,000 \times g for 1 h at 4°C. The internal membrane pellet was solubilized as described above for the plasma membrane, and the samples were diluted with a 10-fold excess of lysis buffer (1% Triton X-100, 25 mM HEPES [pH 7.4], 150 mM NaCl, 1 mM EDTA, 20 μ g of phenylmethylsulfonyl fluoride, and 1 μ g of CLAP per ml). Pr55^{gag} proteins were immunoprecipitated from the lysates with mouse anti-HIV-1 CA monoclonal antibody 38:9 (17), the Pr55^{gag} processing intermediates and CA with a mixture of monoclonal antibody 38:9 and mouse monoclonal anti-p24 antibody 32/5.17.76 (Abcam, Ltd.), and the HA-tagged Pr55^{gag} with a rabbit polyclonal anti-HA antibody (Sigma).

For biotinylation of the cell surface proteins, HeLa H1 cells were brought into suspension by treatment with PBS-EDTA and incubated in PBS (with MgCl₂ and CaCl₂) containing 0.5 mg per ml of EZ-Link Sulfo-NHS-SS-biotin (Pierce) for 30 min at 0°C. The sample was quenched with 50 mM NH₄Cl before silica coating. Biotin-tagged proteins in the plasma membrane and the internal membrane fractions were visualized by dot blots using streptavidin-peroxidase polymer (Sigma). Biotin-tagged transferrin proteins (15 μ g per ml; Molecular Probes) that had been bound to HeLa H1 cells at 0°C or internalized for 20 min at 37°C in serum-free medium were used as markers for the plasma membrane and recycling endosomes, respectively. In the case of the internalized transferrin, cells were incubated in a pH 4.5 buffer containing 25 mM citric acid, 24 mM trisodiumcitrate, 280 mM sucrose, and 10 μ M deferoxamine mesylate for 2 min to remove cell surface-bound transferrin proteins prior to silica coating. Transferrin proteins in the plasma membrane and internal membrane fractions were visualized by Western blotting with streptavidin-peroxidase polymer. The amounts of the endoplasmic reticulum (ER) marker protein calnexin and the late endosome/lysosome marker protein Lamp-1 in the plasma membrane and internal membrane fractions were determined by Western blotting with rabbit polyclonal anti-calnexin (StressGen Biotechnologies Corp.) and goat polyclonal anti-Lamp-1 (C-20; Santa Cruz Biotechnology, Inc.), respectively.

Electron microscopy. Cells were fixed at 24 h postinfection with 2% glutaraldehyde in 100 mM HEPES (pH 7.4). The fixed samples were first stained with 1% osmium tetroxide, followed by staining with 0.5% alcoholic uranyl acetate, dehydration in ethanol, and embedding in Agar 100 resin. Sections (60 nm) were cut on an LKB ultramicrotome and imaged in an FEI Tecnai Spirit transmission electron microscope.

RNAi. HeLa H1 cells were seeded on 10-cm plates at a density which gave ~50% confluence after overnight growth. siRNAs directed against the Tsg101 (7) or a control siRNA (GGUCUCUUCGACGUGCdTdT; BLAST searches against the human genome sequence did not identify any target for this siRNA) were transfected into HeLa H1 cells by using Oligofectamine (Invitrogen) in accordance with the manufacturer's instructions. Transfections were carried out in HeLa growth medium containing 7% fetal bovine serum, and 40 μ l oligofectamine and 20 pmol siRNA per 10-cm plate were used. Mock-transfected cells were treated with Oligofectamine alone. At about 22 h posttransfection the cells were infected with recombinant lentivirus vectors and then analyzed about 24 h postinfection. The intracellular levels of Tsg101 in mock- and siRNA-treated cells were determined from postnuclear supernatants of cell extracts by Western blotting with a monoclonal anti-Tsg101 antibody (C-2; Santa Cruz

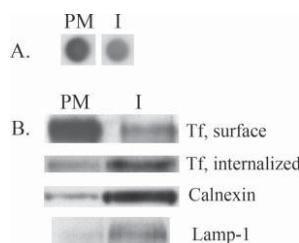


FIG. 1. Coating of HeLa H1 cells with cationic silica beads enables separation of the plasma membrane from internal membranes. HeLa H1 cells were coated with cationic silica beads at 4°C prior to homogenization, and crude cell extracts were fractionated on a Nycodenz step gradient to yield a plasma membrane fraction (PM) and an internal membrane fraction (I). Distribution of marker proteins between PM and I fractions was determined by dot blotting (A) or by Western blotting (B). (A) Plasma membrane-associated proteins were tagged by biotin prior to coating and fractionation, and biotinylated proteins were visualized by streptavidin-peroxidase polymer. (B) Biotin-tagged transferrin proteins bound to cells at 0°C degrees were used as a marker for the plasma membrane (Tf, surface; biotinylated transferrin was visualized by streptavidin-peroxidase polymer). Biotin-tagged transferrin internalized into cells for 20 min at 37°C was used as a marker for recycling endosomes (Tf, internalized). Calnexin and Lamp-1 were used as markers for the ER and late endosomes/lysosomes, respectively.

Biotechnology, Inc.). The monoclonal anti-actin antibody (Chemicon) was used as a control.

RESULTS

Subcellular fractionation method to separate plasma membrane from internal membranes. We have expressed Pr55^{gag} in HeLa H1 cells and analyzed the targeting of the newly synthesized Pr55^{gag} by pulse-chase studies and subcellular fractionations of cell extracts. HeLa H1 cells were chosen since our pilot experiments indicated that Pr55^{gag} can accumulate in these cells either at the plasma membrane or at the internal membranes. Thus, these cells provide a good model system for studying how plasma membrane versus internal localization of the protein is achieved. To determine whether Pr55^{gag} is initially targeted to the plasma membrane or internal membranes requires efficient separation of these membranes. During homogenization, plasma membrane breaks into vesicles and membrane sheets of various density (see, for example, reference 42) that are difficult to separate from, for example, endosomal membranes on conventional sucrose or iodixanol density gradients. Therefore, we chose a fractionation method in which intact cells were coated with cationic silica beads at 4°C prior to homogenization (4, 41), and crude cell extracts were fractionated on a Nycodenz step gradient. The step gradient consisted of a 70% cushion and a 60% sample loading zone overlaid with 50% Nycodenz and buffer solutions. Since the bound silica beads greatly increase density of the plasma membrane, the plasma membrane-derived vesicles and membrane sheets pellet through the 70% Nycodenz cushion during ultracentrifugation (the PM fraction), whereas internal membranes float to the 50% Nycodenz-buffer interphase (I, the internal membrane fraction). Figure 1 shows controls for this fraction-

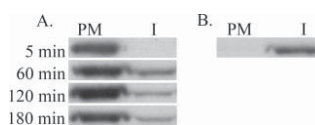


FIG. 2. Newly synthesized Pr55^{gag} is targeted to the plasma membrane and remains at the cell surface when coexpressed with Vpu. (A) NL4-3(Gag)-infected HeLa H1 cells were metabolically labeled with [³⁵S]methionine for 30 min and chased for the indicated times before coating and fractionation of the cell extracts on a Nycodenz step gradient. Pr55^{gag} proteins were immunoprecipitated from the plasma membrane (PM) and internal membrane (I) fractions, and immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis. (B) Distribution of labeled Pr55^{gag} between the PM and I fractions when silica coating was omitted. NL4-3(Gag)-infected cells were metabolically labeled for 210 min and chased for 45 min.

ation method. In Fig. 1A, externally exposed cell surface-associated proteins were tagged with biotin prior to coating, and biotinylated proteins in the PM and I fractions were visualized by dot blots with horseradish peroxidase-conjugated streptavidin. The majority of biotin-tagged proteins were found in the PM fraction. Similarly, biotin-conjugated transferrin that was bound to cells at 0°C prior to coating was predominantly in the PM fraction (Fig. 1B, Tf surface). In contrast, biotin-conjugated transferrin that had been concentrated into recycling endosomes by internalization at 37°C for 20 min was in the I fraction (Fig. 1B, Tf internalized). The ER protein calnexin, as well as the endosomal/lysosomal marker lysosome-associated membrane protein 1 (Lamp-1), were predominantly in the I fraction (Fig. 1B). Taken together, these results demonstrate that the fractionation method can separate the plasma membrane from internal membranes in HeLa H1 cell extracts.

Newly synthesized Pr55^{gag} is inserted into the plasma membrane. To analyze targeting of newly synthesized Pr55^{gag}, HeLa H1 cells were infected with VSV-G-pseudotyped lentivirus vectors carrying modified HIV-1 genomes. At ~24 h postinfection, cells were metabolically labeled with [³⁵S]methionine for 30 min and chased for various times prior to coating and fractionation. Confluent cell monolayers were used in the studies. Amounts of labeled Pr55^{gag} proteins in the PM and I fractions were determined by immunoprecipitation and by SDS-polyacrylamide gel electrophoresis analysis of the immunoprecipitates. Figure 2A shows analysis of newly synthesized, unprocessed Pr55^{gag} expressed from a NL4-3 proviral genome from which the Pol region was deleted [the NL4-3(Gag) genome]. After a 5-min chase, a significant amount of labeled Pr55^{gag} was detected in the PM fraction, but essentially no labeled Pr55^{gag} was present in the I fraction. Similar results were obtained with a 15-min chase (data not shown). Beginning from the 60-min chase point, small amounts of labeled Pr55^{gag} appeared in the I fraction, but the majority of the protein (>80%) was found in the PM fraction at all chase points tested. The NL4-3(Gag) proviral construct efficiently produced VLPs, since large amounts of labeled Pr55^{gag} was found in the culture medium already after 2 h of chase (data not shown). If silica coating was omitted, all labeled Pr55^{gag} was in the I fraction (Fig. 2B), thus confirming that Pr55^{gag} found in the PM fraction in coated cells represented cell surface-associated proteins, and no unspecific protein aggregates. A nonmyristoy-

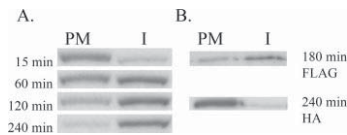


FIG. 3. Newly synthesized Pr55^{gag} is first inserted into the plasma membrane but subsequently endocytosed when expressed in the absence of Vpu. (A) Distribution of newly synthesized Pr55^{gag} proteins between the plasma membrane (PM) and internal membranes (I) at different chase points in NL4-3(Gag/ΔVpu)-infected HeLa H1 cell extracts. Cells were metabolically labeled and analyzed as described in the legend for Fig. 2. (B) Carboxy-terminal HA tag but not FLAG tag inhibits the endocytosis of cell surface-associated Pr55^{gag} expressed in the absence of Vpu. HXB2D-GagHA- and HXB2D-GagFLAG-infected HeLa H1 cells were metabolically labeled for 30 min and chased for the indicated times prior to coating and fractionation.

lated mutant of Pr55^{gag} in which the amino-terminal glycine residue was mutated to alanine failed to associate with either the PM or I fractions (data not shown). Taken together, these results indicated that newly synthesized Pr55^{gag} was inserted into the plasma membrane, and the majority of the proteins remained at the cell surface.

Deletion of the *vpu* gene induces endocytosis of the plasma membrane-associated Pr55^{gag}. We next tested whether localization of Pr55^{gag} to the plasma membrane was an intrinsic property of the protein, or whether other viral proteins had an effect on intracellular targeting of Pr55^{gag}. Elimination of *env* or *nef* genes did not significantly alter the targeting of newly synthesized Pr55^{gag}, i.e., the protein was inserted into the plasma membrane and the majority of labeled Pr55^{gag} remained at the plasma membrane in the absence of Env or Nef expressions (data not shown). In contrast, deletion of the *vpu* gene produced a dramatic change in the intracellular localization of Pr55^{gag}. As shown in Fig. 3A, after a 15-min chase, or 30-min chase (data not shown), the vast majority of labeled Pr55^{gag} was present in the PM fraction in NL4-3(Gag/ΔVpu)-infected cells. Beginning from the 60-min chase point, significant amounts of labeled Pr55^{gag} appeared in the I fraction. By 2 h of chase, ~70% of labeled Pr55^{gag} was shifted to internal membranes. As previously reported for HeLa cells (22), Vpu was also required for efficient virus production from HeLa H1 cells, since NL4-3(Gag)-infected cells produced ~18-fold more VLPs than NL4-3(Gag/ΔVpu)-infected cells (data not shown). Since newly synthesized cytosolic Pr55^{gag} is rapidly targeted to membranes, within 30 min of synthesis (43), the results presented in Fig. 3A suggest that in the absence of Vpu expression Pr55^{gag} is initially targeted to the plasma membrane but is subsequently redistributed to the internal membranes by endocytosis. As shown in Fig. 3B, a carboxy-terminal HA tag, but not a FLAG tag, suppressed endocytosis of cell surface-associated Pr55^{gag}. After 15-min chase, Pr55^{gag}/HA and Pr55^{gag}/FLAG expressed in the absence of Vpu were primarily at the plasma membrane (data not shown) but after 3 h of chase, ~60% of labeled Pr55^{gag}/FLAG had redistributed to internal membranes, whereas >80% of Pr55^{gag}/HA remained at the cell surface even after 4 h of chase.

The effect of Vpu was also tested in the context of Gag and Gag-Pol coexpression. Figure 4A shows an analysis of pro-

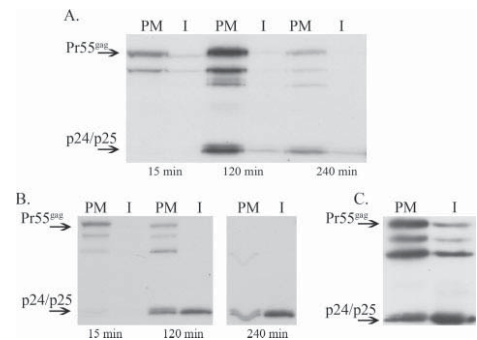


FIG. 4. Vpu inhibits endocytosis of cell surface-associated Pr55^{gag} also when the protein is expressed together with Gag-Pol. (A) Analysis of NL4-3(ΔEnv/ΔNef)-infected HeLa H1 cells. Cells were metabolically labeled and analyzed as described in the legend to Fig. 2. Pr55^{gag} and the p25/p24 CA forms are indicated. Labeled bands between Pr55^{gag} and p25/p24 represent processing intermediates. (B) Analysis of metabolically labeled HXB2D-(ΔVpu/ΔEnv/ΔNef)-infected HeLa H1 cells. (C) Western blot analysis of steady-state distribution of Pr55^{gag} and its processing products between PM and I fractions in HXB2D-(ΔVpu/ΔEnv/ΔNef)-infected cell extracts. The sample was collected at 26 h postinfection.

cessed Pr55^{gag} that was expressed from a modified NL4-3 proviral genome lacking *env* and *nef* genes but expressing Vpu [the NL4-3(ΔEnv/ΔNef)]. At all of the time points tested, most of the labeled Pr55^{gag}, its processing intermediates, or the p25/p24 CA forms were found in the PM fraction. Only trace amounts of the proteins could be detected in the I fraction. In contrast, if in addition to the *env* and *nef* genes the *vpu* gene was also deleted [the HXB2D-(ΔVpu/ΔEnv/ΔNef) construct], the intracellular localization of CA forms was changed (Fig. 4B). After 15 min of chase, Pr55^{gag} and two of its early processing intermediates were detected in the PM fraction but not in the I fraction, thus indicating that Pr55^{gag} was first targeted to the plasma membrane. After 2 h of chase, Pr55^{gag} and its early processing intermediates were still found in the PM fraction but, significantly, ~60% of the processed CA forms were in the internal membrane fraction. At the 4-h chase point, only the p25 and p24 CA forms were evident, and the majority of these proteins was found in internal membranes. Although CA was the only Gag form detected in the I fraction in the radioactively labeled samples, Western blot analysis of the PM and I fractions indicated that small amounts of Pr55^{gag} and its processing intermediates were present in internal membranes as well (Fig. 4C). Taken together, the results in Fig. 4 demonstrate that deletion of *vpu* gene induced uptake of plasma membrane-associated core proteins also when Gag and Gag-Pol were coexpressed. These results were confirmed by EM. EM analysis of NL4-3(ΔEnv/ΔNef)-infected cells identified numerous budding profiles at the cell surface (Fig. 5A). Budding profiles were detected at the plasma membrane in HXB2D-(ΔVpu/ΔEnv/ΔNef)-infected cells as well (Fig. 5C), but these cells also contained endosome-like intracellular compartments full of VLP-sized vesicles (Fig. 5B). Occasionally, budding profiles resembling nascent VLPs could be observed on the lim-

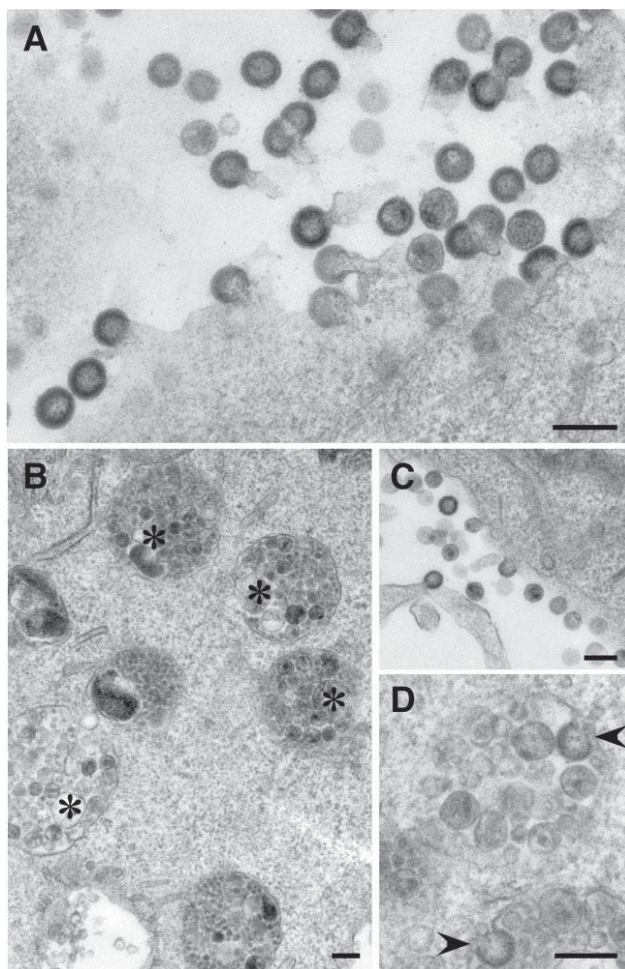


FIG. 5. EM analysis of NL4-3(Δ Env/ Δ Nef)- and HXB2D-(Δ Vpu/ Δ Env/ Δ Nef)-infected HeLa H1 cells. (A) NL4-3(Δ Env/ Δ Nef)-infected cells. Numerous budding structures are seen at the cell surface. (B to D) HXB2D-(Δ Vpu/ Δ Env/ Δ Nef)-infected cells. In addition to the budding structures at the plasma membrane (C), the cells also contain endosome-like structures (*) full of VLP-sized vesicles (B). (D) Virus-like budding profiles (arrow heads) on the internal VLP-containing compartments. Bar, 200 nm.

iting membrane of these compartments (Fig. 5D). When 10 randomly selected NL4-3(Δ Env/ Δ Nef)- and HXB2D-(Δ Vpu/ Δ Env/ Δ Nef)-infected cells, i.e., cells that had budding structures at the cell surface, were scored for VLP-containing endosomal structures, these structures were found in all of the inspected HXB2D-(Δ Vpu/ Δ Env/ Δ Nef)-infected cells. In contrast, only one of the NL4-3(Δ Env/ Δ Nef)-infected cells had clearly identifiable endosomal VLPs.

Knockdown of Tsg101 inhibits targeting of Pr55^{gag} to internal membranes. The PTAP motif on the p6 domain of Pr55^{gag}

binds Tsg101, and this interaction is necessary for the efficient release of progeny particles (7, 23, 45). We tested the effect of a previously described Tsg101-specific small interfering RNAs (siRNAs) (7) in NL4-3(Gag/ Δ Vpu)-infected cells to probe whether Tsg101 is involved in endocytosis of Pr55^{gag}. Western blot analysis of cell lysates demonstrated that Tsg101 siRNAs effectively reduced intracellular levels of Tsg101 (Fig. 6A). After a 30-min pulse and 3 h of chase, 58% of labeled Pr55^{gag} remained at the cell surface in Tsg101 siRNA-transfected cells, whereas 73% of labeled Pr55^{gag} was in internal membranes in

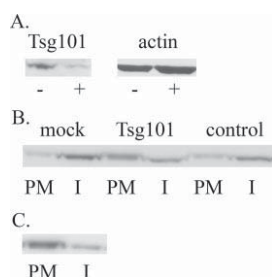


FIG. 6. The p6 domain of Pr55^{gag}, as well as the host protein Tsg101, are required for the endocytic uptake of Pr55^{gag}. (A) Tsg101-siRNAs effectively reduce the intracellular levels of Tsg101. HeLa H1 cells were transfected with Tsg101-specific siRNAs (+) or mock-transfected (-) prior to infection with NL4-3(Gag/ΔVpu) recombination viruses. Tsg101 levels in postnuclear supernatants were probed by Western blotting, and actin was used as a control to confirm that equal amounts of the two samples were analyzed. (B) HeLa H1 cells were transfected with Tsg101-siRNAs, with control siRNAs of scrambled sequence (control) or mock-transfected (mock) prior to infection with the NL4-3(Gag/ΔVpu) recombination viruses. Cells were metabolically labeled for 30 min and chased for 3 h prior to silica coating and fractionation. (C) HeLa H1 cells were infected with NL4-3(GagΔp6/ΔVpu) recombination virus that directs the synthesis of a carboxy-terminally truncated Pr55^{gag} lacking the p6 domain. Cells were metabolically labeled for 30 min and chased for 3 h.

mock-transfected cells (Fig. 6B). Cells transfected with control siRNAs of scrambled sequence had 63% of labeled Pr55^{gag} in internal membranes (Fig. 6B). The less-efficient Pr55^{gag} uptake in Tsg101 siRNA-transfected cells could be due to ablation of Pr55^{gag} interaction with Tsg101, or, alternatively, due to unspecific and/or pleiotropic effects of the Tsg101 siRNAs. To distinguish between these possibilities, we tested uptake of a carboxy-terminally truncated Pr55^{gag} lacking the p6 domain. The truncated protein was expressed from the recombinant proviral genome NL4-3(GagΔp6/ΔVpu). After a 30-min pulse and a 15-min chase, the labeled GagΔp6 protein was in the PM fraction (data not shown). Deletion of the p6 domain inhibited endocytosis of the cell surface-associated protein, since 75% of the truncated proteins were still at the plasma membrane after 3 h of chase (Fig. 6C). Taken together, these results suggest that endocytosis of Pr55^{gag} is critically dependent on interaction of Tsg101 with the p6 domain of Pr55^{gag}.

DISCUSSION

In the present study, we have analyzed targeting of newly synthesized Pr55^{gag} in HeLa H1 cells by pulse-chase studies and by using a subcellular fractionation method that efficiently separates plasma membrane from internal membranes. Our results indicate that Pr55^{gag} is initially targeted to the plasma membrane, and the protein is delivered to internal membranes by endocytosis. Our study also identified Vpu as a key regulator of intracellular targeting of Pr55^{gag}. In the presence of Vpu, the bulk of Pr55^{gag} remained at the plasma membrane after the initial membrane insertion, whereas in the absence of coexpressed Vpu a significant fraction of the cell surface-associated Pr55^{gag} proteins was retargeted to internal membranes. Similar

retargeting was observed regardless of whether Pr55^{gag} was expressed as a nonprocessed precursor protein or whether Pr55^{gag} was expressed together with Gag-Pol. These results are in agreement with previous reports indicating that deletion of the *vpu* gene increases virus budding into intracellular compartments in HeLa and T cells (13, 22). We observed a similar increase of VLPs in internal membrane compartments in the absence of Vpu when NL4-3(ΔEnv/ΔNef)- and HXB2D-(ΔVpu/ΔEnv/ΔNef)-infected cells were compared by EM. Furthermore, it is noteworthy that in all of the studies that have identified Pr55^{gag} in internal membranes in transfected or infected, but otherwise nonperturbed HeLa, Cos-1, or 293T cells, the protein was expressed in the absence of Vpu (6, 10, 26, 39). Consistent with our results, Rudner et al. (34) recently identified plasma membrane as the primary target membrane for Pr55^{gag} in HeLa cells. The method used was the fluorescent imaging of cells expressing a tetracycline-tagged Pr55^{gag} and sequential staining of the cells with two biarsenical compounds that produced different fluorescent signals upon binding to the tetracycline tag. The dual-labeling procedure enabled the detection of newly synthesized Pr55^{gag}. However, in contrast to our results, the presence or absence of Vpu was reported to have no effect on the association of Pr55^{gag} with the plasma membrane. This discrepancy could perhaps be explained by the carboxy-terminal tetracycline tag; as exemplified by our HA-tagged Pr55^{gag}, a carboxy-terminal tag can inhibit the endocytosis of cell surface-associated Pr55^{gag}. Since our subcellular fractionation method did not separate internal membrane compartments from each other, the identity of the internal membranes harboring Pr55^{gag} was not established. Fluorescence microscopy analysis of HXB2D-(ΔVpu/ΔEnv/ΔNef)-infected cells indicated a vesicular staining pattern for intracellular Gag (data not shown), but the intracellular Gag-signal in this particular viral genetic background did not extensively colocalize with any of the tested early or late endosomal markers (CD63, Lamp-1, EEA1, or internalized fluorescein isothiocyanate-conjugated transferrin; data not shown).

It is formally possible that the Gag signal in the internal membrane fraction in our silica coating experiments originated from internalized extracellular VLPs rather than from endocytosed plasma membrane-associated Pr55^{gag}. However, we think this is unlikely for the following reasons. First, very little Gag was detected in the internal membrane fraction in NL4-3(Gag)- and NL4-3(ΔEnv/ΔNef)-infected cells despite the fact that these cells released ~18-fold more VLPs than cells expressing the respective ΔVpu-genomes (data not shown). Second, although rare, virus-like budding structures could be identified on internal membrane compartments, and early processing intermediates of Pr55^{gag}, although not as abundant as CA, were observed in internal membrane fraction by Western blotting. Thus, unless VLPs produced in the absence of Vpu have some feature that promotes their rapid uptake after budding from the cell surface, these observations strongly suggest that Gag localization to internal membranes is the consequence of endocytosis of plasma membrane-associated proteins rather than internalization of extracellular VLPs. The mechanism by which Vpu suppresses endocytosis of Pr55^{gag} remains to be elucidated. Since there are no indications that Vpu directly interacts with Pr55^{gag}, Vpu most likely exerts its effect indirectly, e.g., by inducing modifications on the plasma

membrane or by exerting broader modifying effects on the membrane trafficking pathways of the host cell. In order to elucidate how Vpu suppresses endocytosis of Pr55^{gag}, it is essential to understand the molecular basis of endocytosis of Pr55^{gag} when the protein is expressed in the absence of Vpu. Our initial analyses suggest that this uptake requires interactions between the cell surface-associated Pr55^{gag} and Tsg101. siRNAs directed against Tsg101, or deletion of the p6 domain that mediates Pr55^{gag} and Tsg101 interactions (7, 23, 45), inhibited the endocytosis of Pr55^{gag}. siRNAs can cause various off-target effects that are difficult to control for in conventional experimental settings (32, 36), and therefore RNAi-mediated knockdown results have to be interpreted with caution. However, since the endocytosis of Pr55^{gag} was suppressed by deletion of p6 domain, this suggests that the effect of Tsg101 siRNAs was due to ablation of Tsg101 and Pr55^{gag} interactions. Tsg101 was initially identified as being essential for efficient egress of progeny particles from the cell surface assembly sites (7, 23, 45). Tsg101 is a component of the ESCRT-I (endocytic sorting complex required for trafficking) and recruits the so-called class E proteins, which normally function in MVB biogenesis, to the site of HIV-1 assembly and budding (24). The class E proteins also participate in the budding of other retroviruses, filoviruses, and arenaviruses (24). Since HIV-1 assembly is arrested at a late stage (after membrane distortion) when p6-Tsg101 interactions are disrupted (24), it has been assumed that Tsg101 and other class E proteins facilitate the final steps in budding. However, since our results indicate that Tsg101 also has an impact on intracellular localization of Pr55^{gag}, this suggests that the function of Tsg101 in HIV-1 assembly and budding might be more complex than was previously thought.

Several biological activities have been attributed to Vpu (2, 19, 44). One of these is the enhancement of virus release from HIV-1-infected cells. Interestingly, Vpu can also increase virus release from cells infected by other retroviruses (13) and even alphaviruses (11). Since Vpu enhances virus production from most human cells, as well as from human-simian heterokaryons, but not from simian cells, Varthakavi et al. (44) postulated that Vpu counteracts a human cell-specific restriction factor that inhibits HIV-1 particle production. Although our present study by no means excludes the existence of such an assembly/release inhibitor, the data suggest a possible alternative explanation for the Vpu-mediated enhancement of HIV-1 particle release in human cells. Namely, Vpu could affect particle production indirectly via its effects on intracellular targeting of Gag. Since deletion of the *vpu* gene subjected plasma membrane-associated Gag to endocytosis and partially shifted virus assembly to internal membranes, and if these endosomal viruses were only inefficiently released into the extracellular medium, then Vpu-deficient viruses would be expected to produce less extracellular particles than their wild-type counterparts. Vpu has also been reported to enhance overall membrane association of Gag when Gag was expressed from a T7-driven vaccinia virus expression system (5). Although we did not rigorously test the distribution of Gag between cytosolic and membrane fractions in the present study, we, or others (15), have not observed any dramatic reduction in membrane-associated Gag with Vpu-deficient proviral expression systems.

The biological significance of the plasma membrane versus the endosomal assembly of HIV-1 is not fully understood at present, but emerging information suggests that the site of

virus assembly could impact HIV-1 pathogenesis. For example, intracellular assembly may promote viral persistence since intracellular virions in macrophages retain infectivity for an extended period of time and can efficiently transfer the infection to lymphocytes upon contact (37). Furthermore, it has been speculated that the cellular site of assembly might have a direct impact on immune recognition and infectivity of the formed virions (12). Thus, understanding the molecular mechanisms that regulate the choice of viral assembly site is important. Our results suggest that cell surface versus internal assembly of HIV-1 in HeLa H1 cells is determined by factors that regulate endocytic uptake of plasma membrane-associated Pr55^{gag}. Our results provide compelling evidence that Vpu promotes cell surface assembly of HIV-1 by inhibiting endocytosis of plasma membrane-associated Pr55^{gag} and that newly synthesized Pr55^{gag} proteins reach internal membrane compartments in HeLa H1 cells via endocytosis from the plasma membrane. The presence of VLPs in internal compartments in HXB2D-(ΔVpu/ΔEnv/ΔNef)-infected cells implies that internalized Pr55^{gag} proteins can engage in productive particle assembly and that the internalization process does not merely represent garbage disposal, i.e., the removal of defective Gag forms from the cell surface. Since HeLa cells recapitulate the main features of HIV-1 assembly in T cells, our results most likely apply to T cells as well. Unfortunately, we could not directly test the targeting of Pr55^{gag} in T cells, since, e.g., Jurkat T cells tended to disrupt during silica coating, and this led to binding of silica beads to internal membranes as well (data not shown). Whether endocytosis from the plasma membrane is also the mechanism by which Pr55^{gag} is targeted to the MVBs in macrophages is unclear at present, since intracellular targeting of Pr55^{gag} in HeLa H1 cells and macrophages differs at least in two aspects. Namely, Gag is apparently targeted to MVBs in macrophages even when the protein is coexpressed with Vpu (25, 31, 33) and targeting to MVBs in macrophages appears to be independent of Pr55^{gag}-Tsg101 interactions (27). Consequently, either the uptake mechanism is different in macrophages or, alternatively, the initial membrane insertion does not occur at the plasma membrane in these cells. We are currently testing whether the silica coating method can be used to determine the targeting phenotype of newly synthesized Pr55^{gag} in macrophages.

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III



The Vpu-regulated endocytosis of HIV-1 Gag is clathrin-independent

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Abstract

Recent results by us and others have shown that the accessory protein Vpu determines plasma membrane versus endosomal accumulation of the HIV-1 core protein Gag and progeny virions in the HeLa model of HIV-1 infection, since Vpu suppresses endocytosis of cell surface-associated Gag. In this report, we used pulse-chase studies and subcellular fractionations to investigate endocytosis of newly synthesized Gag in HeLa H1 cells. The uptake of Gag in Δ Vpu-virus background was not blocked by inhibitors of clathrin-mediated endocytosis and macropinocytosis. The cholesterol-sequestering drug filipin inhibited the uptake, but only if the drug was applied before extensive multimerization of Gag had taken place. Thus, the uptake mechanism most likely is only indirectly dependent on cholesterol. Our results also indicated that targeting phenotype of Gag was different in confluent versus subconfluent cell cultures, which could perhaps explain some of the controversies in intracellular targeting of Gag.

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Keywords: HIV-1; Gag trafficking; Endocytosis; Vpu; Virus assembly

Introduction

Assembly of human immunodeficiency virus type 1 (HIV-1) is directed by the viral core protein precursor Gag (Pr55^{Gag}) (Göttlinger, 2001; Resh, 2005). Pr55^{Gag} is a myristoylated, peripheral membrane protein, and it binds to cellular membranes and assembles into enveloped virus-like particles (VLPs) independently of other viral proteins (Bryant and Ratner, 1990; Gheysen et al., 1989; Göttlinger et al., 1989; Spearman et al., 1994; Zhou et al., 1994), but release of progeny virions from infected cells is enhanced by the viral accessory protein Vpu (Bour and Strebel, 2003), and by the viral protease present in the other core protein precursor, the Gag-Pol (Kaplan et al., 1994). During or shortly after budding, Pr55^{Gag} is cleaved into matrix,

capsid (CA), nucleocapsid, and p6 proteins and two peptides SP1 and SP2 by the viral protease.

One intriguing aspect of the Gag-mediated assembly of HIV-1 is that the assembly phenotype is cell type-dependent. Electron microscopy (EM) and fluorescence microscopy studies have indicated that Gag and progeny virions concentrate within large internal vacuoles in macrophages (Greene Nguyen et al., 2003; Orenstein et al., 1988; Pelchen-Matthews et al., 2003; Raposo et al., 2002). These vacuoles have been assumed to represent late endosomes/multivesicular bodies, but recent data indicate that they in fact are internally sequestered plasma membrane domains (Deneka et al., 2007; Welsch et al., 2007). In contrast, Gag and virus particles have been found both at the plasma membrane and in internal, endosome-like compartments in T cells and model cell lines such as 293T, HeLa, and Cos (see, for example, Grigorov et al., 2006; Nydegger et al., 2003; Sherer et al., 2003). The intracellular route by which Gag reaches its cell surface or internal localization, as well as the intracellular site where virus assembly is initiated, have been subjects of intense debate.

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Since the virus-filled vacuoles of macrophages have been found to be continuous with the plasma membrane, the cell surface is the primary site for HIV-1 assembly in these cells (Deneka et al., 2007; Welsch et al., 2007). This conclusion is supported by cell imaging and biochemical studies as well (Jouvenet et al., 2006). The plasma membrane appears to be the primary site for HIV-1 assembly in other cell types as well, since several recent studies have provided strong evidence that the internal Gag and virus particles in HeLa and 293T cells originate from uptake of newly assembled virions from the cell surface (Finzi et al., 2007; Harila et al., 2006; Jouvenet et al., 2006; Neil et al., 2006). We and others recently identified the viral accessory protein Vpu as an important determinant for accumulation of Gag and progeny virions in internal endosome-like compartments in HeLa H1 and HeLa cells (Harila et al., 2006; Neil et al., 2006). The results from these two studies indicated that a newly synthesized Gag was initially inserted into the plasma membrane. When Gag was coexpressed with Vpu, the protein remained at the plasma membrane and progeny virions assembled at the cell surface were efficiently released into the extracellular medium. In contrast, Gag expressed in the absence of Vpu produced progeny virions that remained tethered to the cell surface (Neil et al., 2006), and the plasma membrane-associated newly synthesized Gag, most likely in the form of a fully assembled virion, was efficiently retargeted from the cell surface to an internal endosome-like compartment by an as-yet-uncharacterized uptake mechanism (Harila et al., 2006; Neil et al., 2006). However, the initial insertion of newly synthesized Gag into the plasma membrane is a controversial issue. Biarsenical/tetracycline labeling and cell imaging studies have favored either direct insertion of Pr55^{Gag} into the plasma membrane in HeLa cells (Rudner et al., 2005), or provided evidence for trafficking of newly synthesized Gag to the plasma membrane via an endosomal intermediate (Perlman and Resh, 2006). Furthermore, cell fractionation studies have indicated that a newly synthesized Gag in 293T cells is first inserted into the plasma membrane (Finzi et al., 2007), or that the protein simultaneously reaches the plasma membrane and endosomal membranes (Grigorov et al., 2006). Studies using Gag-green fluorescent protein (Gag-GFP) molecules have suggested that internalization from the plasma membrane accounts for intracellular Gag in 293T cells and macrophages (Jouvenet et al., 2006).

In the present study, we have investigated the endocytic phenotype of Gag in HeLa H1 cells. Our results indicated that the endocytosis of Gag in Δ Vpu-background was not blocked by an inhibitor of macropinocytosis [5-(*N*-ethyl-*N*-isopropyl)amiloride], or by RNA interference (RNAi)-mediated knock-down of clathrin heavy chain (CHC). The uptake was sensitive to the cholesterol-sequestering drug filipin, but the effect of filipin was most likely indirect and due to negative impact of the drug on Gag multimerization and virus assembly. Notably, the targeting phenotype of Gag in Δ Vpu-background was found to be different in confluent versus subconfluent cell cultures, since newly synthesized Gag was simultaneously detected at the plasma membrane and internal membranes in subconfluent cells

whereas targeting to the plasma membrane in confluent cells clearly preceded the appearance of Gag in internal membranes.

Results

Impact of inhibitors of clathrin-mediated endocytosis and macropinocytosis on the uptake of cell surface-associated Gag

Our previous results in HeLa H1 cells indicated that a newly synthesized Pr55^{Gag} was inserted into the plasma membrane, but when expressed in the absence of Vpu, the protein subsequently shifted from the cell surface to an internal endosome-like compartment (Harila et al., 2006). Mammalian cells have several endocytic routes which can be classified into three main uptake modes: clathrin-mediated endocytosis, macropinocytosis, and cholesterol-dependent (raft-mediated) uptake (Marsh and Helenius, 2006; Pelkmans and Helenius, 2003). We used inhibitors of these three uptake modes to probe the mechanism of Gag endocytosis in HeLa H1 cells.

RNAi-mediated knock-down of CHC was used to probe whether the uptake of cell surface-associated Gag in Δ Vpu-virus background occurred by clathrin-mediated endocytosis. HeLa H1 cells were transfected with CHC-specific siRNAs or mock-transfected (i.e. treated only with the transfection reagent), and the following day infected with vesicular stomatitis virus (VSV) G-protein-pseudotyped NL4-3(Gag/ Δ Vpu), a recombinant virus carrying a genome with a defective Pol region and a defective *vpu* gene. Western blot analysis of cell lysates after 24 h postinfection demonstrated that the CHC siRNA effectively reduced intracellular levels of CHC (Fig. 1A). Biotin-tagged transferrin (TF) was used as a probe to measure clathrin-dependent endocytosis in the infected CHC knock-down cells. TF was initially bound to cells at cold, and the cells were then shifted to 37 °C for 0, 5 or 15 min to allow endocytosis. After incubation at 37 °C, cells were placed on ice, and cell surface-associated TF was removed by trypsin. The amount of trypsin insensitive, endocytosed TF was determined by Western blot analyses of cell extracts. Both mock- and siRNA-transfected cells bound ~equal amounts of TF (Fig. 1B; tot), and the TF at the cell surface was efficiently removed by trypsin (the 0 min time point in Fig. 1B). After 5 min of warming, TF had become fully resistant to trypsin in mock-transfected control cells, whereas the majority of TF (~70%) in CHC siRNA-treated cells remained susceptible to trypsin even after 15 min at 37 °C. This indicated that clathrin-dependent endocytosis was compromised in the CHC siRNA-treated cells. To determine the effect of CHC knock-down on endocytosis of Pr55^{Gag}, siRNA- or mock-transfected cells were infected with NL4-3(Gag/ Δ Vpu), and at 24 h postinfection cells were metabolically labeled with [³⁵S]methionine for 30 min and chased for 10 min or 180 min. Intact cells were then coated with cationic silica beads at +4 °C prior to homogenization, and crude cell extracts were fractionated on a Nycodenz step gradient. The gradient consisted of a 70% Nycodenz cushion and a 60% sample loading zone overlaid with 50% Nycodenz and buffer solutions. This fractionation method efficiently separates the plasma membrane from internal membranes, since

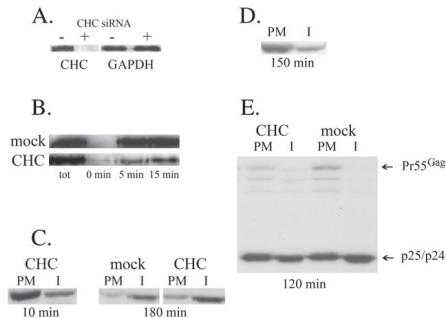


Fig. 1. RNAi-mediated knock-down of CHC does not inhibit uptake of cell surface-associated Pr55^{Gag}, but reduces the uptake of Gag when Gag and Gag-Pol are coexpressed. (A) The CHC-siRNA used effectively reduced the intracellular levels of CHC. HeLa H1 cells were transfected with CHC-specific siRNA (+) or mock-transfected (-). CHC levels in postnuclear supernatants were probed by Western blotting, and GAPDH was used as a control to confirm that equal amounts of siRNA- and mock-transfected samples were analyzed. (B) Clathrin-mediated endocytosis was inhibited in the siRNA-transfected cells. HeLa H1 cells were transfected with CHC-specific siRNA or mock-transfected prior to infection with NL4-3(Gag/ΔVpu), and clathrin-mediated endocytosis was probed by measuring the uptake of biotin-tagged transferrin. Transferrin was bound to cells at 4 °C, and internalized at 37 °C for the indicated times. Cell surface-associated transferrin was removed by trypsin prior to homogenization, and the amount of endocytosed, trypsin-resistant transferrin in cell extracts was determined by Western blotting. Tot sample was not trypsin-treated and it indicates the amount of transferrin originally bound to cells. (C) CHC knock-down does not inhibit endocytosis of Pr55^{Gag} expressed in the absence of Vpu. HeLa H1 cells were transfected with CHC-specific siRNA or mock-transfected prior to infection with NL4-3(Gag/ΔVpu). Cells were metabolically labeled with [³⁵S]methionine for 30 min and chased for the indicated times. After the chase, intact cells were coated with cationic silica beads at 4 °C prior to homogenization, and crude cell extracts were fractionated on a Nycodenz step gradient to separate the plasma membrane (PM) from internal membranes (I). Pr55^{Gag} proteins were immunoprecipitated from the PM and I fractions, and immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis. The 10 min CHC sample and the 180 min chase samples are from different experiments. (D) Intracellular localization of Pr55^{Gag} coexpressed with Vpu is not affected by the knock-down of CHC. HeLa H1 cells were transfected with CHC-specific siRNA and infected with NL4-3(Gag). Cells were metabolically labeled for 30 min and chased for 150 min prior to silica coating and fractionation. (E) Analysis of the effect of CHC knock-down on uptake of Gag when Gag and Gag-Pol were coexpressed [NL4-3(ΔVpu/ΔEnv)-infected cells]. Cells were metabolically labeled for 30 min and chased for 120 min prior to silica coating and fractionation.

due to the bound silica beads, the plasma membrane-derived vesicles and membrane sheets pellet through the 70% Nycodenz cushion during ultracentrifugation, whereas internal membranes float to the 50% Nycodenz-buffer interphase (for controls, see Harila et al., 2006). Distribution of labeled Pr55^{Gag} between the plasma membrane (PM) and internal membrane (I) fractions was determined by immunoprecipitation and by gel electrophoresis analysis of the immunoprecipitates. As shown in Fig. 1C, the CHC siRNAs did not block redistribution of labeled Pr55^{Gag} from the cell surface to internal membranes. After 10 min of chase, the majority of labeled Pr55^{Gag} was in the PM fraction in CHC siRNA-transfected cells, whereas the labeled Pr55^{Gag} had shifted to I fraction in the siRNA-transfected cells

as efficiently as in mock-transfected control cells after 180 min of chase. Thus, clathrin-mediated endocytosis is not involved in uptake of plasma membrane-associated Pr55^{Gag} in NL4-3(Gag/ΔVpu)-infected HeLa H1 cells. Lowering intracellular levels of CHC did not affect intracellular localization of Pr55^{Gag} coexpressed with Vpu, since the majority of labeled Pr55^{Gag} in NL4-3(Gag)-infected cells was found in the PM fraction even after 150 min of chase (Fig. 1D).

The effect of CHC knock-down was also tested in the context of Gag and Gag-Pol coexpression. CHC siRNA- or mock-transfected HeLa H1 cells were infected with NL4-3(ΔVpu/ΔEnv), a virus carrying defective *vpu* and *env* genes, and at 21 h postinfection cells were metabolically labeled with [³⁵S]methionine for 30 min and chased for 120 min. As shown in Fig. 1E, only trace amounts of labeled, unprocessed Pr55^{Gag} remained in mock-transfected and siRNA-transfected cells after the 120-min chase. Approximately ~60% of p24 CA was internal in mock-treated cells, whereas the internal p24 comprised ~49% of total cell-associated CA in CHC siRNA-treated cells. This small difference in the intracellular distribution of CA was reproducible.

The results in Figs. 1C and E suggested that the uptake of plasma membrane-associated Gag in NL4-3(Gag/ΔVpu)-infected cells was clathrin-independent, but in NL4-3(ΔVpu/ΔEnv)-infected cells, a subpopulation of Gag might be internalized by clathrin-mediated endocytosis. Alternatively, as there is the strong possibility that Gag is endocytosed as a fully assembled virion (Neil et al., 2006), the results in Figs. 1C and E might reflect differential impact of CHC knock-down on virus assembly in these two cells. To distinguish between these possibilities, we analyzed production of VLPs from NL4-3(Gag/ΔVpu)- and NL4-3(ΔVpu/ΔEnv)-infected cells containing normal or low intracellular levels of CHC. The NL4-3(Gag/ΔVpu)- or NL4-3(ΔVpu/ΔEnv)-infected cells were metabolically labeled with [³⁵S]methionine for 180 min and 240 min, respectively, and VLPs released into the culture supernatant, or VLPs tethered to the cell surface (= VLPs released after trypsin treatment of cells) were purified by pelleting through a 20% sucrose cushion. The amount of labeled Gag in the pellets was determined by gel electrophoresis. As shown in Fig. 2A, only trace amounts of labeled Pr55^{Gag} were released into extracellular VLPs from NL4-3(Gag/ΔVpu)-infected cells, but

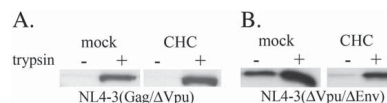


Fig. 2. Effect of CHC-specific siRNAs on virus assembly. Extracellular VLPs (-), or VLPs tethered to the cell surface and released by trypsin treatment of cells (+), were pelleted through a sucrose cushion and analyzed by SDS-polyacrylamide gel electrophoresis. (A) VLPs from cells that expressed unprocessed Pr55^{Gag} in the absence of Vpu [NL4-3(Gag/ΔVpu)-infection]. Cells were metabolically labeled for 180 min. (B) VLPs from cells that expressed Gag and Gag-Pol, but no Vpu [NL4-3(ΔVpu/ΔEnv)-infected cells]. Cells were metabolically labeled for 240 min. Only the CA form is shown, since the VLPs did not contain significant amounts of unprocessed Pr55^{Gag} or processing intermediates of Gag.

mock- and CHC siRNA-transfected cells contained similar amounts of labeled Pr55^{Gag} VLPs tethered to the cell surface. Thus, the knock-down of CHC apparently did not affect virus assembly in NL4-3(Gag/ΔVpu)-infected cells. In NL4-3(ΔVpu/ΔEnv)-infection, small amounts of labeled CA were detected in extracellular VLPs produced from control mock-transfected cells, but the majority of labeled CA was found in VLPs tethered to the cell surface (Fig. 2B). Knock-down of CHC reduced incorporation of labeled CA into both the extracellular VLPs and into VLPs that were tethered to the cell surface. Quantitations from two different experiments suggested that total VLP production was ~3-fold higher in mock-transfected control cells than in CHC siRNA-transfected cells. However, this could be an overestimation, since the VLP assay did not take into account internal (or internalized) VLPs. Since the mock-transfected and CHC siRNA-transfected cells had similar amounts of membrane-associated Gag and since there was no indications that low intracellular levels of CHC caused any Gag processing defects (Fig. 1E), the CHC siRNAs apparently did not reduce membrane targeting or higher-order multimerization of newly synthesized Gag in NL4-3(ΔVpu/ΔEnv)-infected cells, but produced an as-yet-undefined moderate late assembly defect in these cells. This late assembly defect might explain why CHC siRNAs gave slightly different results in NL4-3(Gag/ΔVpu)- and NL4-3(ΔVpu/ΔEnv)-infected cells in the cell fractionation experiments.

We next tested whether the endocytic uptake of Gag in ΔVpu-virus background occurred by macropinocytosis. If macropinocytosis was involved, virus infection would need to stimulate this uptake mechanism, since macropinocytosis is not a constitutive feature of HeLa cells. We measured the uptake of fluorescein-conjugated dextran into noninfected, NL4-3(Gag)- or NL4-3(Gag/ΔVpu)-infected HeLa H1 cells in order to determine whether macropinocytosis was upregulated in infected HeLa H1 cells. Flow cytometric analyses indicated similar low levels of macropinocytosis for all three cell populations (data not shown). The effect of 5-(N-ethyl-N-isopropyl) amiloride, a potent inhibitor of induced macropinocytosis (Meier et al., 2002; West et al., 1989), was tested in Fig. 3. NL4-3(Gag/ΔVpu)-infected cells were pulsed for 30 min, and chased for 180 min in the presence or absence of 100 μM amiloride. As shown in Fig. 3, amiloride did not block the shift of labeled Pr55^{Gag} from the PM to the I fraction in NL4-3(Gag/ΔVpu)-infected cells. Thus, the uptake of Gag in the ΔVpu-virus background is unlikely to occur by a macropinocytosis-like activity.

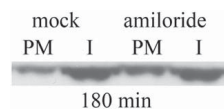


Fig. 3. The inhibitor of induced macropinocytosis, 5-(N-ethyl-N-isopropyl) amiloride, did not block shift of newly synthesized Pr55^{Gag} from the plasma membrane to internal membranes in NL4-3(Gag/ΔVpu)-infected HeLa H1 cells. Infected cells were pulsed for 30 min, and chased for 180 min in the presence or absence of amiloride prior to silica coating and fractionation.

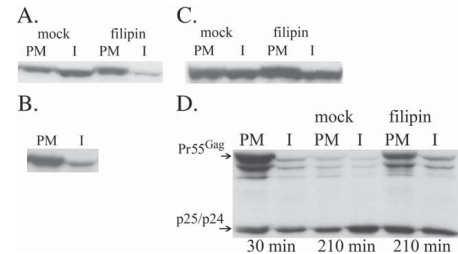


Fig. 4. Filipin reduces retargeting of newly synthesized Gag from the cell surface to internal membranes in infected HeLa H1 cells. (A) Cells expressing unprocessed Pr55^{Gag} in the absence of Vpu [NL4-3(Gag/ΔVpu)-infection] were metabolically labeled for 30 min, and chased for 210 min in the presence or absence of filipin prior to silica coating and fractionation. (B) Filipin does not affect intracellular localization of Pr55^{Gag} coexpressed with Vpu. NL4-3(Gag)-infected cells were pulsed for 30 min and chased in the presence of filipin for 210 min. (C) Analysis of NL4-3(Gag/ΔVpu)-infected cells when filipin was added after 30 min of chase. Cells were pulsed for 30 min and chased for 30 min before addition of filipin. The chase in the presence or absence of filipin was continued for further 180 min. (D) Similar analysis from NL4-3(ΔVpu/ΔEnv)-infected HeLa H1 cells, which express Gag together with Gag-Pol, and no Vpu. Filipin was added to the chase medium after 30 min of chase, and chase was continued for further 180 min. The 30 min sample indicates intracellular distribution of labeled Gag at the time of filipin addition.

Effect of filipin on the uptake of cell surface-associated Gag

The possible dependence of Pr55^{Gag} endocytosis on cholesterol was tested by filipin, an antibiotic commonly used to disrupt cholesterol-dependent raft-like microdomains at the cell surface. NL4-3(Gag/ΔVpu)-infected HeLa H1 cells were metabolically labeled with [³⁵S]methionine for 30 min and chased for 210 min in the presence or absence of filipin (3 μg/ml). This amount of filipin did not induce extensive shedding of microvesicles (a common side-effect of removal of cell surface cholesterol), but the filipin concentration was sufficient to inhibit cholesterol-dependent endocytic processes, since the clathrin-mediated uptake of transferrin was inhibited in filipin-treated cells (data not shown; Subtil et al., 1999). As shown in Fig. 4A, filipin treatment considerably reduced the amount of labeled Pr55^{Gag} in I fraction after 210 min of chase: quantitations from two separate experiments indicated that approximately 61% of labeled Pr55^{Gag} was detected in the I fraction in untreated cells whereas only ~19% of labeled Pr55^{Gag} was in internal membranes in filipin-treated cells. Filipin treatment did not change intracellular localization of Pr55^{Gag} that was coexpressed with Vpu [NL4-3(Gag)-infection], since, as in untreated cells (Harila et al., 2006) the majority of labeled Pr55^{Gag} in filipin-treated cells were plasma membrane-associated after 210 min of chase (Fig. 4B).

The results in Fig. 4A suggested that the endocytic uptake of Pr55^{Gag} could occur by a cholesterol-dependent mechanism. However, cholesterol is also required for efficient membrane binding and higher-order multimerization of HIV-1 Gag (Ono et al., 2007). Since there are strong indications that Gag is endocytosed as a fully assembled virion (Neil et al., 2006), the observed effect of filipin could be due to inability of plasma

membrane-associated Gag proteins to efficiently multimerize in the presence of the drug. To test this possibility, we added filipin after 30 min of chase, i.e. at the time when maximal membrane insertion, as well as at least some higher-order multimerization of labeled Pr55^{Gag} has already occurred (Ono et al., 2007; Tritel and Resh, 2000). As shown in Fig. 4C, filipin added after 30 min of chase reduced the amount of labeled Pr55^{Gag} in I fraction at the 210 min chase point, but the reduction was less than if filipin was present from the beginning of chase. In the experiment shown in Fig. 4C, ~37% and 49% of labeled Pr55^{Gag} was in internal membranes in filipin-treated and untreated cells, respectively. However, the efficiency with which filipin inhibited the uptake of Pr55^{Gag} varied between different experiments; the relative amount of labeled Pr55^{Gag} in the I fraction was 12%–32% lower in filipin-treated cells than in control untreated cells. To further probe the possibility that filipin exerted its effect primarily through inhibiting multimerization of Gag, we also tested the impact of filipin in the context of Gag and Gag-Pol coexpression, since processing of Pr55^{Gag} offers a convenient indirect marker for efficient multimerization of Gag (Kaplan et al., 1994). HeLa H1 cells were infected with NL4-3(Δ Vpu/ Δ Env)-virus, pulsed for 30 min, and filipin was added after 30 min of chase, and the chase was continued for further 180 min. At the time of filipin addition (Fig. 4D; 30 min), the PM fraction contained large amounts of labeled Pr55^{Gag}, whereas the small amounts of processed CA forms were ~equally distributed between the PM and I fractions. After 210 min of chase, only trace amounts of unprocessed Pr55^{Gag} were present in untreated control cells, and 68% of labeled CA was in the I fraction. In contrast, the filipin-treated cells contained significant amounts of plasma membrane-associated, unprocessed Pr55^{Gag} at the 210 min chase point, thus suggesting that filipin affected proper higher-order multimerization of Gag. Approximately 47% of total cell-associated labeled CA was in internal membranes after 210 min of chase. However, in another experiment, CA in filipin-treated cells distributed to the I fraction as efficiently as in untreated control cells (data not shown). Thus, the effect of filipin varied between different experiments, but overall, filipin had a relatively minor effect on intracellular distribution of CA. Therefore, a significant part of the apparent reduction in Gag uptake in the filipin-treated cells might be attributable to the inhibitory effects of filipin on higher-order oligomerization of Gag.

The apparent targeting phenotype of newly synthesized Gag is different in confluent and subconfluent HeLa H1 cultures

As mentioned in the Introduction, attempts to analyze intracellular targeting of newly synthesized HIV-1 Gag have produced contradictory results. The results in our previous publication (Harila et al., 2006), as well as for example Figs. 1C and 4D above, clearly indicate that a newly synthesized Gag is initially inserted into the plasma membrane in HeLa H1 cells. These results were from confluent HeLa H1 cells, but the trafficking phenotype of newly synthesized Gag turned out to be different when we repeated the targeting analyses in subconfluent HeLa H1 cell cultures (~30–40% confluency). Fig. 5A

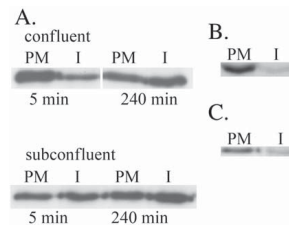


Fig. 5. Analysis of intracellular targeting of newly synthesized Pr55^{Gag} in subconfluent HeLa H1 cell cultures. (A) Confluent or subconfluent cells expressing unprocessed Pr55^{Gag} in the absence of Vpu [HXB2D-(Gag)-infection]. Cells were metabolically labeled for 30 min and chased for the indicated times prior to silica coating and fractionation. In contrast to confluent cells, newly synthesized Pr55^{Gag} was simultaneously detected in PM and I fractions in the subconfluent cell cultures. (B) Cells coexpressing Vpu and unprocessed Pr55^{Gag} [NL4-3(Gag)-infection] were analyzed after a 30 min pulse and a 5 min chase. Newly synthesized Pr55^{Gag} coexpressed with Vpu was primarily targeted to the plasma membrane in subconfluent cells. (C) Cells expressing an endocytosis-defective Pr55^{Gag}/HA [HXB2D-(GagHA)-infected cells]. Cells were analyzed after a 30 min pulse and a 5 min chase. No Vpu was expressed in the HXB2D-(GagHA)-infected cells.

shows analysis of confluent and subconfluent HeLa H1 cells infected with HXB2D-(Gag), a recombinant virus with a defective Pol region, a defective *vpu* gene, and a truncated *nef* gene. The infected cells were metabolically labeled with [³⁵S] methionine for 30 min, and chased for 5 min or 240 min. In confluent cells, the majority of newly synthesized Pr55^{Gag} was at the plasma membrane after 5 min of chase, but in subconfluent cells, labeled Pr55^{Gag} was simultaneously detected in PM and I fractions. Whereas there was a clear increase of labeled Pr55^{Gag} in the I fraction in confluent cells after 240 min of chase, the amount of labeled Pr55^{Gag} in I fraction in subconfluent cells increased only slightly during a longer chase (~60% was internal after 240 min of chase). When expressed in the presence of Vpu [NL4-3(Gag)-genome], the majority of newly synthesized Pr55^{Gag} was in the PM fraction in both subconfluent (Fig. 5B; a 30 min pulse and a 5 min chase) and in confluent cell cultures (Harila et al., 2006). The simultaneous detection of Pr55^{Gag} in the PM and I fraction in HXB2D-(Gag)-infected subconfluent cells could mean that membrane insertion of newly synthesized Pr55^{Gag} expressed in the absence of Vpu was random in subconfluent cultures, or, alternatively, that uptake of plasma membrane-associated Pr55^{Gag} occurred very rapidly in these cells. To distinguish between these possibilities, we tested targeting of Pr55^{Gag}/HA in subconfluent cells. Pr55^{Gag}/HA contains a carboxyterminal HA tag, and this Gag variant is not endocytosed after insertion into the plasma membrane when expressed in the absence of Vpu (Harila et al., 2006). Fig. 5C shows analysis of HXB2D-(GagHA)-infected subconfluent HeLa H1 cells after a 30 min pulse and a 5 min chase. The majority of newly synthesized Pr55^{Gag}/HA was detected in the PM fraction. Similar distribution was observed after 240 min of chase (data not shown). Taken together, these results indicate that cell confluency can have an impact on the

targeting phenotype of newly synthesized Gag when the protein is expressed in the absence of Vpu.

Discussion

We have previously shown that a newly synthesized HIV-1 Gag is inserted into the plasma membrane in infected HeLa H1 cells, but the final steady-state localization of the protein is influenced by Vpu; if coexpressed with Vpu, the majority of Gag stays at the cell surface, but in the absence of Vpu, a significant fraction of the cell surface-associated Gag proteins is subsequently endocytosed into an endosome-like compartment (Harila et al., 2006). Initial analysis of this Vpu-regulated uptake indicated that the endocytosis of Gag required interaction of Gag with the host protein Tsg101 (Harila et al., 2006; see also Neil et al., 2006). In the present study, we have further studied the endocytic phenotype of Gag in HeLa H1 cells. As discussed below, our interpretation of the results is that the uptake of Gag in Δ Vpu-virus background is clathrin-independent and indirectly cholesterol-dependent, and cell density influences kinetics of the uptake. This interpretation is based on the assumption that the plasma membrane-associated Gag is endocytosed as a fully assembled virion. Neil et al. (2006) demonstrated that viruses produced in the absence of Vpu have surface components that tether the nascent virions to the cell surface after budding, and they suggested that the endosomal Gag in Δ Vpu-background arises from uptake of these tethered virions. This suggestion was based on the observations that (1) endosomal particles were mature in morphology, (2) coexpression of Gag/Gag-Pol with a mutant Rab5a, which reduced the amount of endosomal Gag, resulted in accumulation of fully assembled virions at the cell surface, and (3) Gag-mutants unable to complete budding were only detected at the plasma membrane. It is difficult to firmly establish whether Gag in HeLa H1 cells is endocytosed as a fully assembled virion, or as a smaller assembly intermediate. However, observations that the uptake of Gag in HeLa H1 cells can be reduced by depletion of Tsg101, by cycloheximide (data not shown), or by addition of a HA tag to the carboxyterminus of Pr55^{Gag} (Harila et al., 2006), are consistent with fully assembled virions being the targets of the uptake mechanism, since all of these conditions interfere with virus assembly (data not shown; Garrus et al., 2001; Martin-Serrano et al., 2001).

We used RNAi-mediated knock-down of CHC to determine whether the endocytosis of Gag occurred by a clathrin-dependent or a clathrin-independent mechanism. The results from these knock-down experiments indicated that the CHC-specific siRNA used did not inhibit uptake of Pr55^{Gag} in NL4-3 (Gag/ Δ Vpu)-infected cells, although the endocytosis of transferrin was measurably inhibited in these knock-down cells. Overall, the uptake of Pr55^{Gag} in CHC knock-down cells occurred with similar kinetics and efficiency as uptake of Gag in mock-transfected cells (i.e. in cells treated only with the transfection reagent), or in untreated HeLa H1 cells (data not shown). However, the CHC-specific siRNA produced a relatively minor, but reproducible reduction in the uptake of Gag (p24) in NL4-3(Δ Vpu/ Δ Env)-infected cells. These results

could mean that the uptake of unprocessed Gag differs from that of Gag expressed together with Gag-Pol. However, if plasma membrane-associated Gag is endocytosed as a virion, the discordant results from NL4-3(Gag/ Δ Vpu)- and NL4-3(Δ Vpu/ Δ Env)-infected cells could be due to the fact that virus assembly in NL4-3(Gag/ Δ Vpu)-infected cells appeared to be insensitive to knock-down of CHC, whereas low intracellular levels of CHC clearly reduced virus formation in NL4-3(Δ Vpu/ Δ Env)-infected cells. Therefore, our interpretation of the experimental data is that the uptake of Gag is clathrin-independent. This conclusion is in agreement with a recent report demonstrating that endocytosis of Gag in 293T cells is not blocked by chlorpromazine, a drug that inhibits clathrin-mediated endocytosis (Finzi et al., 2007). At present it is unclear why the knock-down of CHC reduced virus assembly in NL4-3(Δ Vpu/ Δ Env)-infected cells, but apparently some very late step in the assembly was affected since processing of Pr55^{Gag} was not impaired by the CHC siRNA.

Our results from filipin-treated cells demonstrate that interfering with plasma membrane cholesterol reduces uptake of cell surface-associated Gag in Δ Vpu-virus background. HIV-1 Gag is considered to be a raft-associated protein, and raft-like microdomains at the plasma membrane have been suggested to function as platforms for virus assembly (Ding et al., 2003; Halwani et al., 2003; Holm et al., 2003; Lindwasser and Resh, 2001; Nguyen and Hildreth, 2000; Ono and Freed, 2001; Zheng et al., 2001). The observed effects of filipin could mean that the uptake of Gag occurs by a cholesterol-dependent, raft-mediated mechanism. However, filipin efficiently reduced the uptake only when added at the beginning of chase. Adding filipin after 30 min of chase produced a less pronounced inhibition. Furthermore, filipin had a variable and a relatively minor effect on intracellular distribution of CA. These observations could indicate that the uptake mechanism of Gag is only indirectly dependent on cholesterol. It has been shown that cholesterol is required for efficient membrane binding and higher-order oligomerization of HIV-1 Gag (Ono et al., 2007), and if virions tethered to the cell surface are the form in which Gag is endocytosed, then anything that interferes with virus assembly affects the uptake of Gag as well. Filipin in our experiments most likely reduced the overall virus assembly, since processing of Pr55^{Gag} was impaired in the NL4-3(Δ Vpu/ Δ Env)-infected cells. Thus, the inhibitory effect of filipin on Gag uptake might reflect reduced virus assembly rather than the uptake mechanism being cholesterol-dependent.

The uptake of Gag in our experimental setting was not inhibited by 5-(*N*-ethyl-*N*-isopropyl) amiloride, an inhibitor of induced macropinocytosis. Thus, none of the three inhibitors (filipin, CHC siRNA, amiloride) directed against cholesterol-dependent uptake, clathrin-mediated endocytosis, or macropinocytosis produced a clear-cut inhibition of Gag uptake in the Δ Vpu-virus background. This raises the possibility that the Gag uptake could occur by the poorly characterized clathrin- and lipid raft-independent uptake pathways (Pelkmans and Helenius, 2003). The uptake of Gag is unlikely to be caveolae-mediated, since (1) intracellular accumulation of Δ Vpu-viruses is also seen in T cells which do not express caveolin (Klimkait et al., 1990),

and (2) although HeLa H1 cells express caveolin-1, caveolae structures are rare in these cells, and genistein, an inhibitor of caveolae-mediated endocytosis (Pelkmans and Helenius, 2003), does not affect endocytosis of Gag (data not shown). Our further observations suggest that interaction of the MA domain of Gag with AP-3 δ subunit, or the Y84G substitution in the MA domain, does not play a key role in the Vpu-regulated endocytosis of Gag in HeLa H1 cells (data not shown), although both the AP-3 δ subunit and the Y84G substitution have previously been shown to affect targeting of HIV-1 Gag to endosomal compartments (Dong et al., 2005; Ono and Freed, 2004). We have not been able to test dependence of the Gag uptake on actin dynamics, since treatment of infected cells with for example jaspalakinolide, latrunculin B, or cytochalasin D disrupts the cells during silica coating (data not shown).

Our interpretation that uptake of Gag is mediated by a clathrin-independent mechanism is seemingly contradictory to the report that dominant negative forms of dynamin, EPS-15, and Rab5a, all known inhibitors of clathrin-mediated endocytosis, prevent endocytosis of Gag in HeLa cells (Neil et al., 2006). However, a dominant negative approach and RNAi do not always give the same results, as exemplified by the observations that dominant-negative CHMP5, but not depletion of endogenous CHMP5, inhibits HIV-1 release (Martin-Serrano et al., 2001; Ward et al., 2005). A dominant-negative mutant can inhibit a cellular function indirectly, for example, by perturbing more than one cellular pathway due to inactivation of essential factors that operate at more than one cellular site. Another possibility is that the uptake mechanism of Gag is cell type-dependent. Assuming that Gag is endocytosed as a fully-assembled virion, our results from e.g. NL4-3(Δ Vpu/ Δ Env)-infections indicate that the endocytosis process is directed by cellular factors present in the viral envelope, not the viral Env protein. The HIV-1 envelope contains several different cellular proteins (Chertova et al., 2006; Hammarstedt and Garoff, 2004), and subtle cell type-dependent changes in the composition of the virion envelope could specify the efficiency of Δ Vpu-virus uptake, and perhaps also the mechanism of the uptake.

Our previous (Harila et al., 2006) and present results indicate that the Vpu-regulated endocytosis of Gag is a key determinant for the steady-state localization of Gag and VLPs in HeLa H1 cells. Vpu most likely controls targeting of Gag in infected T cells as well, since deletion of *vpu* gene results in accumulation of virions into intracellular compartments of these cells (Klimkait et al., 1990). In contrast, concentration of Gag and progeny virions into the internally sequestered plasma membrane domains in macrophages is Vpu-independent (Deneka et al., 2007; Pelchen-Matthews et al., 2003; Welsch et al., 2007). However, the intracellular virus-containing vacuoles of macrophages and T cells or HeLa H1 cells are different, since the limiting membrane of Jurkat and HeLa H1 virus-containing vacuoles is not stained with the cell-impermeant dye ruthenium red (data not shown), and the vacuoles thus belong to the endosomal network. In general, intracellular trafficking routes that control cell surface versus endosomal localization of Gag and VLPs have been the subject of intense debates. Only few serious attempts have been made to characterize the intracellular

transport of a newly synthesized Gag (Finzi et al., 2007; Grigorov et al., 2006; Harila et al., 2006; Jouvenet et al., 2006; Neil et al., 2006; Perlman and Resh, 2006; Rudner et al., 2005), and results from these studies are conflicting. Cell fractionation analyses by us (Harila et al., 2006) and Finzi et al. (2007), as well as FIAsh/ReAsh imaging studies by Rudner et al. (2005) or fluorescence microscopy analyses of cells expressing Gag-GFP (Jouvenet et al., 2006; Neil et al., 2006), indicated that a newly synthesized Gag in HeLa H1, HeLa, 293 T cells, or macrophages was initially inserted into the plasma membrane. In contrast, FIAsh/ReAsh cell imaging studies by Perlman and Resh (2006) implied a 'perinuclear' and endosomal transport intermediates for cell surface Gag in HeLa, Cos-1, and Jurkat T cells. Cell fractionation studies by Grigorov et al. (2006) in turn demonstrated that newly synthesized Gag proteins in 293 T cells were simultaneously detected at the plasma membrane and on endosomal membranes. One explanation for these contradictory results can be the different expression systems used, since the above-mentioned studies utilized either proviral expression of Gag or codon-optimized plasmid-driven expression of Gag. At least three proteins of HIV-1, Nef, Gag, and Vpu, can potentially modulate membrane trafficking pathways of the host cell through interactions with cellular partners (Batonick et al., 2005; Costa et al., 2006; Dong et al., 2005; Madrid et al., 2005; Varthakavi et al., 2006), and thus intracellular targeting of Gag could be different in virus-infected cells and in cells in which a plasmid-driven expression system is used for synthesis of Gag. Even the multiplicity of infection and/or the time point postinfection could impact the apparent targeting phenotype of Gag. Our present study demonstrates that another important factor to consider in targeting analyses of Gag is the confluency of cell cultures; in confluent HeLa H1 cells, newly synthesized Gag was first detected at the plasma membrane in both Vpu+ and Δ Vpu-virus backgrounds, whereas in subconfluent HeLa H1 cultures, Gag expressed in the absence of Vpu appeared simultaneously in both the plasma membrane and internal membrane fractions. The molecular basis of this phenomenon is unclear at present. It is difficult to determine whether the newly synthesized internal Gag population in subconfluent cells represents Gag proteins directly inserted into the internal membranes, or proteins (rapidly) endocytosed from the cell surface. We favor the latter alternative, because (1) the majority of newly synthesized Gag coexpressed with Vpu fractionated with the plasma membrane fraction, and (2) newly synthesized Pr55^{Gag}/HA, which is not endocytosed when expressed in the absence of Vpu, was detected at the plasma membrane in subconfluent cultures as well. Overall, the results from the subconfluent cells stress the need to monitor targeting of Gag with assays that can detect rapid, dynamic changes in intracellular localization of Gag.

Materials and methods

Cell culture, viruses, and plasmid constructs

HeLa H1 and 293T cells were cultured as previously described (Harila et al., 2006; Holm et al., 2003). Production

of VSV G-protein-pseudotyped infectious recombinant HIV-1, as well as plasmid constructs for NL4-3(Gag/ΔVpu), NL4-3(Gag), HXB2D-Gag, and HXB2D-GagHA has been previously described (Harila et al., 2006). Plasmid pNL4-3(ΔVpu/ΔEnv), a derivative of pNL4-3 (Adachi et al., 1986), contains defective *vpu* and *env* genes, and a truncated *vpr* gene. The plasmid was constructed by replacing the 6459-bp *Apal*–*Bam*HI fragment of pNL4-3 with the equivalent *Apal*–*Bam*HI fragment of pHXB2D-(ΔVpu/ΔEnv) (Harila et al., 2006).

Subcellular fractionations

HeLa H1 cells on 10-cm plates were infected with VSV-G-pseudotyped recombinant HIV-1 (~1–5 infectious units/cell), and at ~21 h [NL4-3(ΔVpu/ΔEnv)-virus] or ~24 h [NL4-3(Gag/ΔVpu)-virus], postinfection cells were metabolically labeled with [³⁵S]methionine for 30 min and chased for various times as previously described (Holm et al., 2003). After the chase, cells were brought into suspension by incubation in phosphate-buffered saline (PBS) containing 0.02% EDTA (PBS-EDTA), intact cells were coated at 4 °C with cationic silica beads, and cell extracts were fractionated on a Nycodenz step gradient as previously described (Harila et al., 2006). Gag proteins were immunoprecipitated from sodium dodecyl sulfate (SDS)-solubilized plasma membrane and internal membrane fractions with mouse anti-HIV-1 CA monoclonal antibody 38:9 (Hinkula et al., 1990), or with a mixture of the 38:9 antibody and a mouse monoclonal anti-p24 antibody 32/5.17.76 (Abcam, Ltd.). The HA-tagged Gag was immunoprecipitated with a rabbit polyclonal anti-HA antibody (Sigma).

In filipin treatment, the chase medium contained 3 μg of filipin complex (Sigma) per ml. In 5-(*N*-ethyl-*N*-isopropyl) amiloride treatment, 100 μM of the drug (Alexis Biochemicals) was included into the chase medium. To measure macropinocytosis, cells were first preincubated in minimum essential medium containing 0.2% BSA (MEM-BSA) for 30 min, and then shifted to MEM-BSA containing 1 mg per ml of lysine-fixable fluorescein isothiocyanate-conjugated dextran (70,000 Da, Molecular Probes) for 10 min. After extensive washing, cells were incubated in MEM-BSA for further 5 min. To strip plasma membrane-associated dextran, cells were placed on ice and incubated for 5 min in a pH 5.5 buffer containing 100 mM sodium acetate and 50 mM sodium chloride. After washes with ice-cold PBS, cells were detached from plates by incubation in PBS-EDTA on ice for 20 min, fixed with 2% paraformaldehyde, and analyzed by a fluorescence-activated cell sorter.

VLPs

To analyze the efficiency of VLP production, NL4-3(Gag/ΔVpu)-infected HeLa H1 cells were metabolically labeled with [³⁵S]methionine for 180 min, and NL4-3(ΔVpu/ΔEnv)-infected cells were metabolically labeled for 240 min. Cell culture supernatants were collected, filtered (0.45 μm), layered on to a 20% sucrose cushion (w/v, in 25 mM HEPES pH 7.4, 150 mM NaCl, 2 mM MgCl₂), and centrifuged at 100,000×g for 60 min. Pellets were analyzed by gel electrophoresis. These

pellets represented constitutively released extracellular particles. To analyze the amount of VLPs tethered to the cell surface, cells were washed once with PBS after harvesting culture supernatants, and incubated with trypsin (0.5 mg/ml) at 37 °C for 10 min. The reaction was stopped by adding culture medium containing 7% fetal bovine serum, cells were pelleted and culture supernatants were filtered, and VLPs were pelleted through sucrose as described above.

RNAi

Short interfering RNAs (siRNAs) directed against the clathrin heavy chain (CCUGCGGUCUGGAGUCAACdTdT; 40 pmol per 10-cm plate; Hinrichsen et al., 2003) were transfected into HeLa H1 cells by using siLentFect (BioRad) in accordance with the manufacturer's instructions. Mock-transfected cells were treated with siLentFect alone. At about 22 h posttransfection, the cells were infected and analyzed about 21–24 h postinfection. Intracellular levels of clathrin heavy chain in mock- and siRNA-treated cells were determined from postnuclear supernatants of cell extracts by Western blotting with a polyclonal goat anti-clathrin heavy chain antibody (C-20; Santa Cruz Biotechnology, Inc.). The polyclonal anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (FL-335; Santa Cruz Biotechnology, Inc.) was used as a control.

To analyze the effect of CHC knock-down on clathrin-mediated endocytosis, NL4-3(Gag/ΔVpu)-infected HeLa H1 cells, mock- or siRNA-treated, were washed once with PBS (with MgCl₂ and CaCl₂) containing 0.2% bovine serum albumin (PBS-BSA), and cells were incubated for 30 min on ice in PBS-BSA containing 500 ng/ml human biotin-tagged transferrin (Sigma). After washing the unbound transferrin away, minimum essential medium containing 0.2% BSA was added to cells and cells were shifted to 37 °C for 0 min, 5 min, or 15 min. Cells were then placed on ice, treated with acid wash buffer (0.2 M sodium acetate buffer pH 4.5, 0.5 M NaCl) for 2 min, rinsed with the acid wash buffer, and washed with excess PBS-BSA and PBS. Cell surface-associated transferrin was removed by trypsin treatment (20 min on ice). Trypsin was inactivated by culture medium containing 7% fetal bovine serum, cells were homogenized, and postnuclear supernatants of cell extracts were prepared as previously described (Harila et al., 2006). The amount of trypsin-resistant transferrin–biotin in postnuclear supernatants was determined by Western blotting using streptavidin–peroxidase polymer (Sigma).

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