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ENVELOPMENT OF RETROVIRUS AND VACCINIA VIRUS

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This thesis describes envelopment of retrovirus and vaccinia virus. Retrovirus envelopment, i.e. budding, occurs from the plasma membrane (PM) of infected cells, in an environment that is abundant with cellular proteins. In general, the viral budding process has been assumed to displace cellular membrane proteins with viral proteins, thus producing viral particles that are free from cellular components. Although this appears to be true for tightly organised viruses such as alphavirus, a number of host proteins have been reported to be incorporated in retroviral particles.

Our aim was to study host protein incorporation on a general basis in Moloney-Murine Leukaemia virus Gag particles. Therefore, we developed a method by which it was possible to directly and quantitatively compare the protein composition of the PM used for budding, with the viral envelope. Our results demonstrated that most cellular proteins present in PM were recovered in the retrovirus membrane in almost similar concentrations. This indicated that the lateral associations of Gag proteins, while forming the internal shell, did not exclude any significant amount of host proteins.

Since the presence of host proteins in retrovirus particles was never studied in depth until now, the presence of a certain host protein in the particles was thought to be specific. Our results demonstrated that there is no specific sorting of host proteins during budding. In fact, retroviruses randomly included host proteins that were located in the area of budding. Structural studies of the matrix protein of Gag revealed a lattice of loosely packaged hexameric rings. This structure appears to give plenty of room to accommodate foreign proteins, as long as they do not exert significant sterical hindrance for the formation of the Gag shell.

We have also demonstrated an interaction *in vivo* between the Gag and Env protein, since the Env protein was five times more concentrated in viral particles, when compared to the PM. This amount was the same for the wild type retroviral particles. The Env-Gag interaction had no effect on the incorporation of host proteins in the viral particles.

One of the host proteins present in the Gag particles was identified by immunolabeling to be actin. By studying Gag particles in immunoelectron microscopy, we found that actin was located underneath the membrane, on top of the Gag-shell. We do not think that actin is a structural component of the particles, but rather we hypothesise that actin is an accidental stowaway from scissioning and release of Gag particles from the PM.

Intracellular mature virus (IMV) of Vaccinia virus (VV) is thought to be wrapped by a double membrane derived from the intermediate compartment between ER and Golgi. The membranes are modified by the insertion of VV membrane proteins to form two tightly apposed membranes, which line up as rigid crescents around the virosome. The virosome contains material, which is engulfed by the membrane crescents forming a spherical, immature virus, which matures into the brick-shaped IMV. The IMV membrane protein p21 (gene product of A17L) is essential for formation of the viral crescents, since in its absence, the precursor membranes do not form the rigid crescents.

By using immuno-electron microscopy and biochemical methods, we demonstrated that p21 is located on the surface of IMV, in the outer membrane, in addition to the internal membrane. Its localisation and topology makes it possible for p21 to form disulphide-linked homodimers between both leaflets of the double membrane. Thus, p21 might function as the merging force to flatten the compartment membranes and form the tightly apposed double membrane of IMV.

Key words: Retrovirus, Vaccinia virus, Envelopment, Host proteins, Gag-particles, IMV, A17L
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To my parents, Sigrid and Krister

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LIST OF ORIGINAL REFERENCES

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

I. Minimal exclusion of plasma membrane proteins during retroviral envelope formation. Hammarstedt*, M., K. Wallengren*, K. Winther Pedersen, N. Roos and H. Garoff. *Proc. Natl. Acad. Sci. USA* 2000, 97:7527-7532.

II. Localization of actin in Moloney murine leukemia virus by immunoelectron microscopy. Nermut, M. V., K. Wallengren and J. Pager. *Virology* 1999, 260:23-34.

III. The A17L gene product of Vaccinia virus is exposed on the surface of IMV. Wallengren, K., C. Risco, J. Krijnse-Locker, M. Esteban and D. Rodríguez. *Virology* 2001, in press.

* Shared first authorship

ABBREVIATIONS

aa	amino acid
AIDS	acquired immunodeficiency syndrome
ATP	adenosine triphosphate
BHK	baby hamster kidney cells
C	capsid protein of SFV
CA	capsid protein of retrovirus
CBB	Coomassie brilliant blue
Da	Dalton
ds	double stranded
EEV	extracellular enveloped virus
EIAV	equine infectious anaemia virus
EM	electron microscopy
Env	envelope protein
ER	endoplasmic reticulum
ERGIC	intermediate compartment between ER and Golgi
Gag	group specific antigen
HIV	human immunodeficiency virus
HTLV	human T-cell leukaemia virus
I	Gag-Gag interaction domain
IMV	internal mature virus
IN	integrase
IV	immature virus
kb	kilo bases
L	late assembly domain
M	membrane association domain
MA	matrix protein
MLV	murine leukaemia virus
M-PMV	Mason-Pfizer monkey virus
NC	nucleocapsid
NP	nucleoprotein of influenza
NP-40	Nonidet P-40
ORF	open reading frame
PM	plasma membrane
Pol	polymerase protein
PR	retroviral protease
RSV	Rous sarcoma virus
RT	reverse transcriptase
SD	standard deviation
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SFV	Semliki Forest virus
SU	surface domain
TM	transmembrane domain
VV	vaccinia virus
WHO	World Health Organisation
wt	wild-type

1 INTRODUCTION

Viruses are non-living organisms that depend on the host cell's replication machinery for propagation. Although some viruses carry a few specific viral enzymes that are indispensable and unique to their selected way of replication, they completely lack the complex machinery that allows genetic material to be copied and translated into proteins. Therefore, in order to "live", they are obligate parasites. For this reason, they have developed intricate mechanisms of breaking into cells and high-jacking the replication and translation machinery of the cell in order to copy their own genes and produce viral protein. Thus, the sole purpose of a virus particle is simply to protect and spread its viral genome.

In general, a virus particle consists of a cage constructed by proteins, which encloses the genetic material of the virus (Figure 1a). The outside of the cage, or capsid, is covered with projections that enable the virus to find and enter new cells to infect. Often these projections consist of separate spike proteins.

Once the viral proteins are produced inside the cell, they have the capacity to self-assemble and form new virus particles. The capsid proteins interact horizontally to form the cage and also recognize and bind the viral genome so that it can be packaged inside the forming particle. Additionally, the capsid proteins interact vertically with the spike proteins, thus forming the exterior layer of the virus particle. After the virus exits the cell, it is transported, via body fluid or aerosols, to new host cells, which it infects and the replication cycle of the virus is repeated.

1.1 VIRAL ENVELOPE FORMATION

Some viruses use a membrane in order to exit and enter cells. These viruses are called enveloped viruses and include for example the retrovirus and poxvirus families. The viral membrane is formed in the context of a cell membrane, e.g. the plasma membrane (PM), which is modified by viral spikes. The process of envelopment is called budding and occurs as the virus particles are released from the cell. When enveloped viruses enter a new cell, they use the spike to catalyse the fusion between the viral and the cellular membrane. The virus is then relieved of its envelope and the viral capsid is released into the cytoplasm. Therefore, the viral envelope could be envisaged as a coat that the virus puts on when it goes out and then takes off when it enters a new cell.

Two models for viral budding are depicted in Figure 2. Either all of the viral components assemble at the membrane concomitantly with budding, or capsids pre-assemble in the cytoplasm, and then migrate to the membrane where they bud. In either case, the individual components first need to be directed to the site of assembly, which could occur by active help from motors coupled to microtubules or filaments, or by receiving receptors at the site of budding. Next, the complete virus needs to assemble. This involves gathering of all the individual components such as the spike protein, capsid protein and genome, which interact cooperatively and start forming the viral particle. The efficiency and accuracy of assembly is probably dependent on both the strength of the intra-molecular interactions and on the concentration of the individual components at the site of assembly. To complicate the picture, viral assembly takes place in an environment that is crowded with cellular proteins in addition to the viral proteins. Not only are host proteins floating around everywhere in the cytoplasm, but they also create rigid cytoskeletal networks that traverse the cell, while other proteins generously occupy the membrane. In the middle of this heavily populated area, several additional assembly related questions arise: how does the virus collect the specific proteins needed? What happens with the cellular components? How do the viral proteins interact with each other and with proteins that belong to the host cell? How do the viral proteins modify the cellular membrane in order to

serve the purposes of the virus? More specifically: how are the envelopes formed?

In this thesis, I have tried to answer some of these questions in two different virus models: primarily in the Moloney murine leukaemia virus but also in the vaccinia virus.

1.1.1 Retrovirus and poxvirus

Moloney murine leukaemia virus (Mo-MLV) and vaccinia virus (VV) are non-related and each belongs to a different family of virus. Mo-MLV is a member of the retrovirus family, which includes the human immunodeficiency virus (HIV) that has attracted attention for being the causative agent of AIDS. Because Mo-MLV is a murine virus, it is non-pathogenic in humans, and for this reason, it is currently being used as a vector in gene therapy trials.

VV belongs to the poxvirus family, most famous for the smallpox virus Variola. In 1980, the WHO declared Variola to have been eradicated. This was accomplished thanks to the first vaccine ever to be developed. In the 18th century, an English medical doctor, Edward Jenner, noted that milk maids, working in close vicinity of infected cows, were resistant to smallpox. Hence it was discovered that cowpox could be used to protect humans against smallpox (Jenner, 1798). The modern vaccine is based on the closely related VV, which is not pathogenic in humans. VV is today considered a laboratory virus and is still used for engineering vaccines against other infectious agents.

Both retrovirus and poxvirus are animal viruses, and both carry a membrane envelope, but that is where the similarities end. Retroviruses are small, round particles with a diameter of approximately 100 nm, whereas the brick shaped VV, with a length of 200-400 nm, is the largest known animal virus. The DNA genome of VV encodes for approximately 200 proteins, of which only half are incorporated into the virus particle. On the other hand, simple retroviruses like Mo-MLV are comprised of only three viral gene products encoded by the RNA molecule that accounts for the genetic material of this virus. In addition, during the life cycle of VV, two forms of infectious virus, with an intermediate non-infectious form, are produced. Each form is enclosed by a different number of membrane envelopes, and at its maximum the virus is thought to have as many as four membrane bilayers. In contrast, the retrovirus, as most other viruses, is satisfied with one envelope.

It is not far fetched to assume that the retrovirus and the poxvirus assemble and recruit their envelopes in completely different manners. The retrovirus is enveloped by a single layer of host cell PM when exiting the cell. On the other hand, VV is believed to achieve a double membrane derived from internal, cellular compartments, during the first step of morphogenesis. The origin of the VV membrane was questioned for a long time because of the difficulty of determining whether it actually was a double or a single membrane that surrounded the immature virus particle. The double membrane enwrapment process gives rise to the infectious, internal mature virus called IMV, which represent the majority of the infectious progeny. Some IMV, though, become enveloped by an additional two membranes (Ichihashi *et al*., 1971; Payne and Kristenson, 1979) derived from the Golgi apparatus, which renders them with a total sum of four membranes. The outermost membrane then fuses with the cellular PM, in order to externalise the virus. Leaving one layer of membrane behind, the membrane fusion process results in a triple-membrane enveloped virus named EEV (externally enveloped virus).

In order for any enveloped virus to be able to accomplish the different steps in maturation, the viral membranes used for budding will have to be modified by viral proteins. It is obvious that the membrane recruitment process holds a central role in the assembly and maturation process of these viruses.

1.2 RETROVIRUS

The natural host of Mo-MLV is the mouse, in which the virus causes leukaemia. The life cycle of retroviruses includes a step in which the viral genome is inserted into the chromosomes of the host cell, thus altering the genetic code, which, in the worst scenario, could give rise to uncontrolled growth of the cell.

1.2.1 The retrovirus particle

The simple retroviral genome has three open reading frames, *env*, *gag* and *pol*, which encode the proteins necessary to make an infectious virus particle (Figure 1). The Env protein makes up the viral spike, which is located on the

exterior of the particle. The spike allows the virus to bind to receptors on cells, and thereby mediates entry of the virus into the new host cell by catalysing a membrane fusion event. The *gag* gene encodes a 65-kDa polyprotein called Gag, which is a precursor for the structural components of the viral core. These viral components are the matrix protein (MA), the capsid (CA) and the nucleocapsid (NC) protein, which are linked together in this order in the Gag precursor.

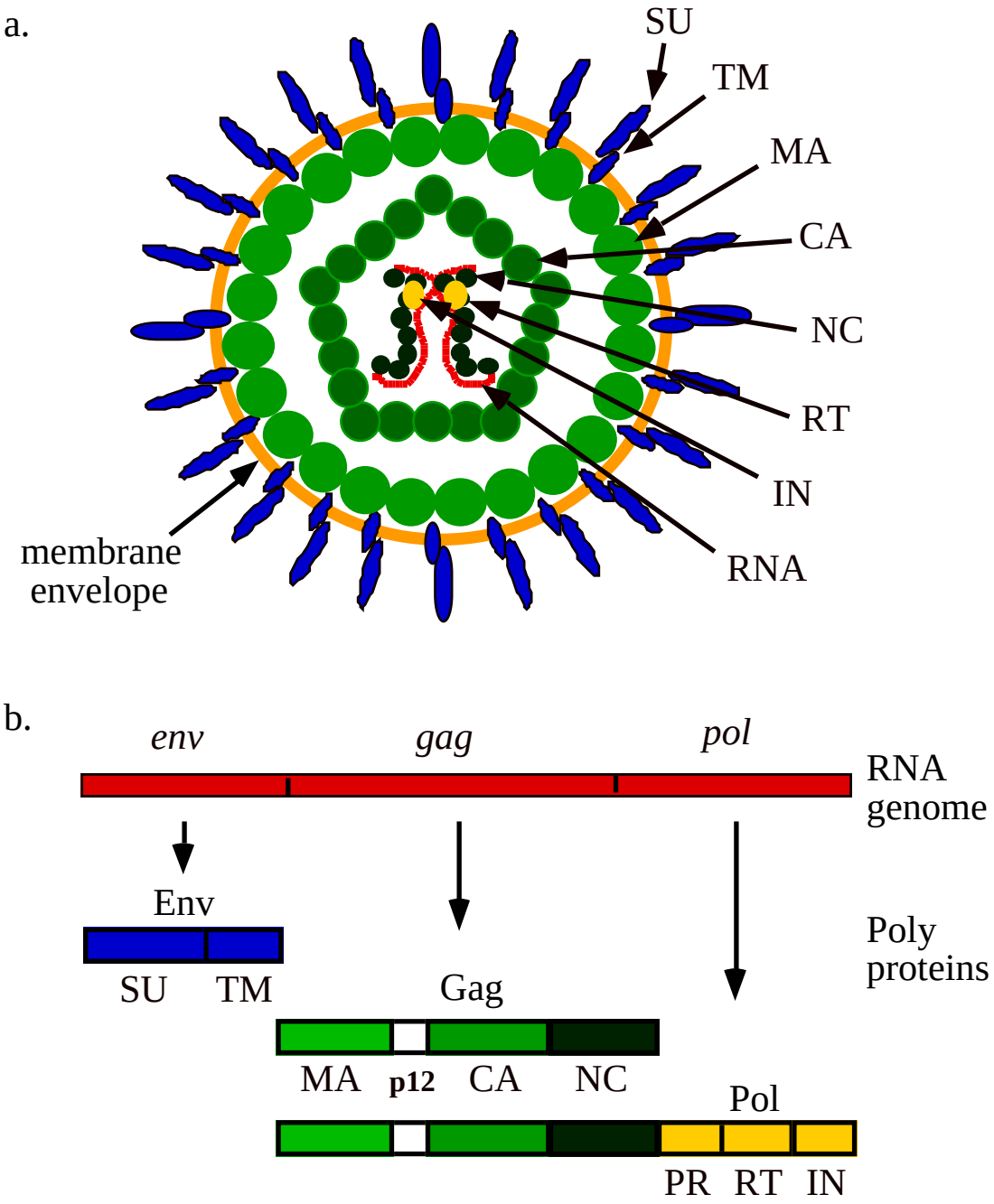

Figure 1: Schematic diagram of a retrovirus (MLV). a. Structure of a retroviral particle. **b.** Retroviral genes and gene products.

The precursor is not cleaved until the virus particle is formed, thus omitting the need for targeting signals of each individual protein, and at the same time ensuring incorporation of the components in equimolar amounts. Cleavage is performed by the viral protease (PR), which is encoded within the Pol precursor. The *pol* gene encodes the viral enzymes essential for the progression of replication. In addition to PR, these enzymes are the reverse transcriptase (RT) and integrase (IN), which are important in converting the viral RNA genome into a functional DNA strand that becomes integrated into the host genome (Levy, 1993).

1.2.2 Overview of the life cycle of retrovirus

Assembly of MLV retrovirus particles starts when the Gag and Gag-Pol precursors gather at the PM together with the Env protein (Figure 2; C type assembly). The Env proteins are transported in membrane vesicles to the PM where they are inserted in the lipid bilayer, while the Gag and Gag-Pol proteins arrive at the cytoplasmic side of the membrane. The N-terminal part of the Gag precursor (MA) interacts with the membrane, while the C-terminal part (NC) binds the viral RNA to bring it along within the virus particle. The individual Gag proteins also interact with one another and thus start forming the capsid at the PM. As the Gag and Env proteins concentrate, the membrane, harbouring the viral spike, is curved around the forming Gag shell. When the membrane covered protein shell is completed, the particle is still connected to the cell surface by a thin stalk of membrane, which eventually pinches off and sets the enveloped particle free. Upon release, the Gag precursor is cleaved into its mature components and the typical inner core, as seen in electron micrographs, condenses around the viral genome and renders the virus infectious.

The next step in the life cycle begins with the binding of Env to a host cell receptor. This interaction triggers the viral envelope to fuse with the cellular membrane in order to release the viral nucleocapsid in the cytoplasm of the new host cell. After uncoating is initiated, the viral RNA genome is transcribed by RT into a double-stranded DNA, which is transported to the nucleus in the form of a pre-integration complex. The IN then ensures proper integration of the viral genome into the host chromosome, where it is

transcribed by cellular polysomes and a new replication cycle is initiated (Levy, 1993).

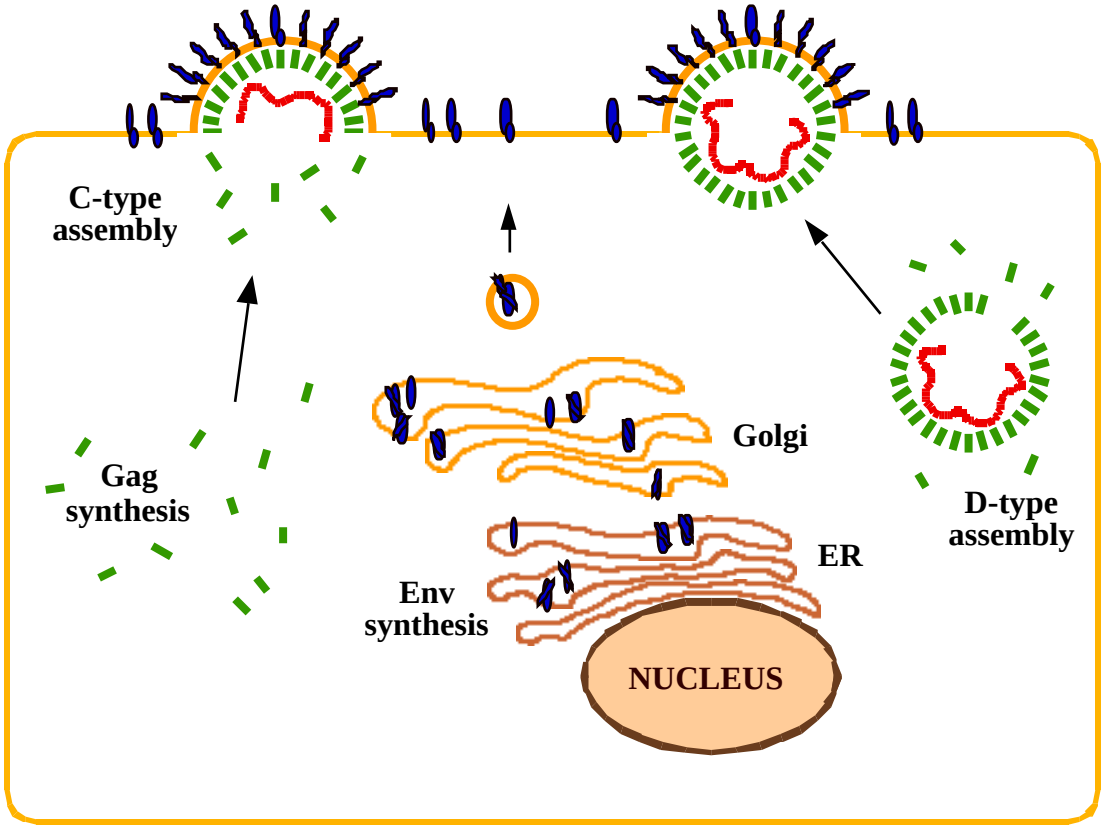

Figure 2: Overview of retrovirus production. Gag is synthesised on free ribosomes in the cytoplasm, while Env is synthesised in ER. Env is processed in Golgi and transported to the PM in transport vesicles. The figure shows two types of Gag assembly: C-type assembly at the PM concomitantly with budding, and B- and D-type pre-assembly of particles in the cytoplasm and subsequent budding at the PM.

1.2.3 The Env protein

The Env polyprotein is translated by membrane bound ribosomes and is subsequently glycosylated in the endoplasmic reticulum (ER) where it also oligomerises (Hunter and Swanström, 1990). The glycoprotein complex is then transported to the PM via the secretory pathway (reviewed in Einfeld, 1996). In Golgi, the attached oligosaccharides are processed and the glycoprotein is proteolytically cleaved into two parts by a cellular protease. Thus, the mature glycoprotein complex consists of two subunits: the surface (SU) domain and the transmembrane (TM) domain (Figure 1a). The external SU subunit determines receptor specificity while the membrane-spanning TM subunit is responsible for the fusogenic process during entry. The TM subunit

anchors the SU unit to the virus surface via a disulphide bond and together they form a homotrimer, which is situated on the surface of viral particles.

1.2.4 The Gag polyprotein

The Gag polyprotein encodes the structural components of the viral core and has been shown to form a virus like particle in the absence of other viral proteins (Delchambre *et al*., 1989; Gheysen *et al*., 1989; Morikawa *et al*., 1991; Rhee *et al.*, 1990; Wills *et al.*, 1989). Gag is synthesised as a polyprotein, which is not cleaved until the viral particle has formed. Upon cleavage, a major rearrangement occurs inside the virus particle when the mature viral core forms. The order of the individual proteins in the precursor, MA-CA-NC, reflects their position in the mature virus from the membrane to the core, with the NC located innermost (Figure 1a). After proteolytical processing, the MA protein, which accounts for the membrane binding properties of the precursor, remains intimately associated with the inner face of the viral membrane where it appears to form a discrete layer just beneath the envelope (Gelderblom *et al*., 1987). When the CA protein is liberated, it condenses into the viral core, which houses the ribonucleoprotein complex and the replicative enzymes. In lentivirus, the Gag polyproteins undergo the most prominent rearrangement, since the core forms a triangular cone, which is attached to the envelope in one end. In other retroviruses, the core appears more like a spherical shell. The nucleoprotein resides inside the CA core. The NC is thought to be responsible for specific packaging of the viral genome (Linial and Miller, 1990). The RNA is packaged as a hydrogen-bonded dimer and contains a packaging signal in the 5’ untranslated region near the dimer linkage site (Coffin, 1982). The NC has a conserved region that binds to the negative backbone of RNA, and entirely coats the two identical copies of RNA (Hunter, 1994; Wills and Craven, 1991).

There is little sequence homology between retroviruses, but great functional homology, which is reflected, in the three-dimensional structure of the proteins. The derivation of the name “Gag”, which stands for group-specific antigen, represents the obvious antigenic similarities. However, the MA, CA and NC proteins are often separated by short peptide sequences of unknown function, which constitute the major differences among the Gag proteins of different retroviruses. Most notable is a spacer peptide located in

between the MA and CA protein, called p12 in MLV, which is present in all retroviruses except for the lentivirus in which the corresponding peptide, p6, is located at the C-terminal end of the NC. These proteins are remarkably rich in proline and glycine, which is consistent with the possibility that this part of the Gag protein is not folded into a defined structure (Fuller *et al.*, 1997). Electron micrographs of immature MLV Gag particles reveal p12 as a less electron dense ring separating the MA and CA proteins. It is speculated that this spacer peptide could serve a function in release of particles from the PM (Yuan *et al.*, 2000; Yuan *et al.*, 1999).

1.2.4.1 Biosynthesis of Gag

The Gag polyprotein is translated from an unspliced RNA on free ribosomes in the cytoplasm (Johnson *et al.*, 1994). Pol is translated as a Gag-Pol fusion protein following read-through of a stop codon. In HIV, Rous sarcoma virus (RSV) and Mason-Pfizer monkey virus (M-PMV) this occurs through a ribosomal –1 frameshift mechanism (Jacks *et al.*, 1988; Jacks and Varmus, 1985; Sonigo *et al.*, 1986), while MLV uses a suppressor tRNA to read past the stop codon (Yoshinaka *et al.*, 1985). Read-through occurs with approximately 5% efficiency to regulate the level of enzymatic proteins incorporated into the virions (Wilson *et al.*, 1988). In this manner, two versions of the MLV Gag product are produced, namely Gag and Gag-Pol, with the latter being a product of the frame shift event. During translation, the Gag protein is modified by the addition of a 14-carbon myristic acid on the N-terminal glycine, once the initiator methionine is removed (Henderson *et al.*, 1983; Schultz *et al.*, 1988; Towler *et al.*, 1988).

1.2.4.2 Transport of Gag protein

After synthesis in the cytoplasm, the MLV Gag protein has to arrive at the PM in order to assemble. This can occur either through free diffusion in the cytoplasm, or Gag can be actively transported to the PM with help of the cytoskeleton. The cytoskeleton traverses the cell with a three-dimensional network consisting of microtubules and actin filaments. The latter also underlie the entire PM. One important function of the cytoskeleton is to mediate intracellular vesicle transport by making use of myosin motors for movement along actin filaments, and dynein motors in the case of microtubule.

Both MLV and HIV Gag proteins have been shown to associate with cytoskeletal elements (Edbauer and Naso, 1983; Ott *et al*., 1996), which has led to the suggestion that the cytoskeleton mediates transport of Gag protein to the PM (Goto *et al*., 1998; Kim *et al*., 1998; Liu *et al*., 1999; Ott, 1997; Rey *et al*., 1996). Experiments, in which the actin filaments have been disrupted by treatment of cells with cytochalasin D, have shown 40% reduced release of HIV-1 particles (Sasaki *et al*., 1995) and 70-80% decrease in MLV particle release (Luftig and Lupo, 1994). However, no quantitative or conclusive analysis of the involvement of the cytoskeleton in Gag transport has been performed. A recent study did not find a convincing association of Gag-GFP (green fluorescent protein) fusion protein with actin filaments *in situ* (Perrin-Tricaud *et al*., 1999). Therefore, it remains unclear how Gag arrives at the PM, nor is it known whether Gag arrives in the form of individual molecules or as small oligomeric complexes (Wills and Craven, 1991).

1.2.5 Morphogenic classes of virus assembly

The retroviruses are divided into C-, and B- and D-type viruses based on where assembly and envelopment occurs (Figure 2). In C-type viruses such as Mo-MLV, both assembly and budding occur at the PM, as described above, whereas in B- and D-type viruses, the Gag shells assemble at internal assembly sites inside the cytoplasm (reviewed in Wills and Craven, 1991). Not until the particles are completely assembled into immature cores, called A-type particles, are they transported to the PM, where they are enveloped and released from the cell. D-type budding, including transport of pre-assembled M-PMV to the PM, has been shown to require ATP (Weldon *et al*., 1998).

The avian and mammalian leukaemia/sarcoma viruses (e.g. RSV, MLV, and avian leukosis virus) are classed as C-type viruses, but also the human T-cell leukaemia virus (HTLV) and lentivirus (e.g. HIV) assemble their cores in a similar fashion. Viruses belonging to the second morphogenic class of viruses include the D-type M-PMV, the related simian retroviruses (SRV 1-5) and members of the spumavirus family. There are no major differences between B- and D-type assembly. D-type assembly, though, has been better characterized. The prototype B-type virus is mouse mammary tumour virus.

1.2.5.1 Gag assembly

Despite the different sites of assembly in C-, and B- and D-type viruses, it is believed that the assembly process is a fairly uniform event in all retroviruses. Evidence for this is that a single amino acid (aa) substitution in MA (R55W) was found to redirect the morphogenic pathway of M-PMV Gag from D-type to C-type (Rhee and Hunter, 1990). Similarly, assembly away from the membrane has been reported when C-type Gag is overexpressed in insect cells (Delchambre *et al.*, 1989; Royer *et al.*, 1991), indicating that any retrovirus core could assemble independent of membrane association provided its local concentration is sufficiently high (Campbell and Vogt, 1995; Klikova *et al.*, 1995). However, the efficient assembly of C-type retrovirus may be facilitated by an interaction with host cell membranes or membrane-associated factors, and thus occur at lower concentrations of Gag.

1.2.6 Interactions during retrovirus assembly

Assembly of wild-type (wt) retrovirus involves several types of interaction between the co-operating components. These include interactions sideways within each protein lattice, as well as cross connections between the different layers of the particle, i.e. horizontal binding between the Gag proteins, and vertical connections between the Env and Gag layers. Interactions, which could serve a concentrating or scaffolding function, include Gag binding to the membrane, as well as to the RNA genome. Probably all these different types of interactions are required in concert to make the assembly process efficient and accurate.

The different interactions involved have been dissected, and it has been shown that the Gag protein can form enveloped virus like particles without the need for any other viral proteins. This implies that the driving force for budding lies within the lateral interactions between the individual Gag molecules. Since Gag particles constitute the simplest form of self-assembly, they have been extensively studied in order to understand the budding process of retrovirus.

1.2.6.1 Membrane binding of Gag

A series of basic residues, located in the first 50 aa residues of the MA protein, are exposed on a platform that is formed when the MA protein trimerises

with itself (Hill *et al.*, 1996). The positive charges are neutralised by interacting with the acidic inner side of the PM. This ionic interaction co-operates with the hydrophobic myristic acid, which is positioned just above the platform, and anchors the protein in the lipid bilayer. Together, the myristic acid and the basic aa residues account for the membrane binding properties of the protein (Rein *et al*., 1986; Zhou *et al.*, 1994). However, membrane affinity alone may not be sufficient for concentrating the Gag protein at the PM, because virus budding would otherwise occur elsewhere in the cell, especially in the ER, which constitutes the major membranous element of the cell, while PM only accounts for 3-5% (Bolender, 1974; Weibel *et al.*, 1969). Either Gag has a receptor on the cytoplasmic side of the PM, or Gag is directed to the PM through active transport. Transport could be mediated by either transport vesicles, the cytoskeletal network, the involvement of myosin motors, or simply by free diffusion. The mechanism by which Gag reaches the PM has not been resolved yet. At the PM the Gag polyprotein oligomerises into larger structures and, thus, initiates the assembly and budding process. It is still unclear whether the Gag polyprotein reaches the PM as individual monomers or as preformed small oligomers.

1.2.6.2 Functional domains of Gag

Mutations have been performed on the Gag protein to identify domains that may be functional during virus assembly. Such assembly domains do not necessarily have to reside within the boundaries of the individual mature proteins, but instead they may span cleavage sites. In this manner the assembly domains could be functional when required during particle assembly, and be silenced after the virus has matured and needs to adopt the reverse ability in order to dissociate the particle following infection.

Three assembly domains have been identified and designed M, I and L to reflect their specific function during assembly: (M) Membrane association, (I) Gag-Gag Interaction and (L) Late budding release (Craven and Parent, 1996). The M domain is located in the N-terminal section of the Gag protein, and includes the stretch of basic aa residues and the myristic acid, which together confer the membrane binding properties of the protein (Bennett *et al.*, 1993; Wills and Craven, 1991; Zhou *et al.*, 1994). The I domain is essential for production of Gag particles with correct density and size (Sakalian *et al*., 1994; Weldon and Wills, 1993), and it is believed to represent the major site of

interaction between Gag molecules. The domain spans over the C-terminal last quarter of CA into the first half of the NC region, which is responsible for RNA encapsidation (Craven and Parent, 1996; Parent *et al.*, 1995). This indicates the possibility for RNA to act as a scaffold upon which the interacting Gag proteins are densely packaged during assembly (Sakalian *et al*., 1994). RNA scaffolding might be associated with the I domain function. The L domain overlaps with the spacer peptides p9 in Equine infectious anaemia virus (EIAV), p2b in RSV, p6 in HIV-1 and p12 in MLV. The L domain is functionally interchangeable between retroviruses, as well as positionally independent (Parent *et al.*, 1995). Mutations in the L domain result in budding particles, which remain attached to the PM (Wills *et al.*, 1994), indicating the participation for this domain in pinching off and final release. The mechanism of release can merely be speculated upon at this point, but is thought to include the participation of host proteins, since recent results indicate a function for ubiquitin (Patnaik *et al.*, 2000; Schubert *et al.*, 2000; Strack *et al.*, 2000).

1.2.6.3 Env incorporation

The facts that the spike is not required to drive budding, as it is for other viruses, e.g. alphaviruses (Lopez *et al.*, 1994; Suomalainen *et al.*, 1992), and that Gag can bud alone, raises the question of how the homologous Env protein is incorporated into the particles. Is there a specific interaction between Env and Gag that is responsible for bringing the spike into the particle? Is Env only randomly included in the budding particles, or is Gag-Env complementation assured by co-localisation at specific sites used for assembly?

Many mutational analyses have been made to try to elucidate this issue, however, results show that even the tailless form of the Env protein of RSV, HIV and Simian immunodeficiency virus is incorporated into the viral particles to the same extent as the wt Env (Gabuzda *et al.*, 1992; Perez *et al.*, 1987; Wilk *et al*., 1992; Zingler and Littman, 1993). The only positive evidence for a Gag interacting domain in Env has been presented by Cosson (1996). By using immobilised HIV Env tail-mutants, coupled to glutathione-S-transferase (GST)-beads, he managed to show a direct and specific interaction with HIV MA. The interacting region was narrowed down to the C-terminal 67 aa residues of Env. Unfortunately, these results have not gained popular

support. However, some mutations or deletions in the MA region have lead to particles with little or no incorporation of Env, indicating the presence of an Env binding region in MA (Dorfman *et al.*, 1994; Freed and Martin, 1995; Lee and Linial, 1994; Rhee and Hunter, 1990; Yu *et al.*, 1992).

Another way of demonstrating a Gag-Env interaction has been by studying budding in polarised cells. When HIV-1 Gag alone was expressed in epithelial MDCK cells, budding occurred from both the basolateral and apical membranes (Owens *et al.*, 1991), but when co-expressing Env, budding was limited to the basolateral membrane. Similarly, experiments performed in primary dorsal root ganglion cells, showed that in the absence of Env, Gag particles assembled in both axons and dendrites, while in the presence of Env, budding occurred only at the somatodendritic region, which is the equivalent of the basolateral side in fibroblasts (Weclewicz *et al.*, 1998). These results indicate that Env is not randomly included in the particles, but that there indeed does exist an interaction between Env and Gag. Such an interaction might be too weak to be detected *in vitro*, but could function efficiently enough *in vivo*. However, no conclusive study has been done to determine the Env-Gag interaction.

1.2.6.4 Interactions with host cell proteins

For a long time, the consensus was that viral envelope formation involved stringent protein sorting, leading to displacement of cellular proteins with viral glycoproteins. Indeed, analysis of purified Sindbis virus showed no host protein contamination (Strauss, 1978). Host protein exclusion is probably true for the Alphavirus family in which the tight interactions formed between the envelope proteins themselves and between the envelope- and capsid proteins probably force out host proteins (Cheng *et al.*, 1995). The interactions are specific and tolerate no interference of foreign proteins for the budding process to be successful. In other viruses, though, the formation of pseudotyped viruses demonstrated that some virus particles could incorporate heterologous viral glycoproteins. Phenotypic mixing could occur either in co-infected cells or as a result of co-expression of heterologous genes, as exemplified by HIV particles carrying HTLV spikes (Landau *et al.*, 1991), vesicular stomatitis virus with an MLV coat (Zavada, 1972) and by RSV capsids with influenza heamagglutinin in the envelope (Dong *et al.*, 1992). The “pseudotypic paradox” argues that, since the viral glycoproteins could be

interchanged and the host proteins excluded, the viral glycoproteins would have a common denominator causing them to be incorporated into viral particles. This model suggests a degenerate interaction between the spike and the matrix protein, which would cause sorting out of cellular glycoproteins. A second, postulated model is that the viral envelope is cleared from cellular glycoproteins because of the connection of the latter with the underlying cytoskeleton. This would lead to active exclusion of host proteins but would allow mobile Env proteins to freely diffuse into the assembly site and ultimately be included in the particle. Yet, a third possibility is that sorting of proteins does not take place during retrovirus budding. This would mean that any protein present at the assembly site could be included in the forming particle, not distinguishing between viral and cellular proteins. So far it has not been possible to determine which of the three models applies to retrovirus.

Since the pseudotypic paradox was first proposed, many cellular proteins have been reported to be incorporated into viruses. More than 20 different host proteins have been identified in the HIV-1 envelope. These are for example the cellular self-recognising proteins MHC class I and II, adhesion molecules such as ICAM-1 and -3, LFA-1, -2 and –3, and CD44 (Arthur *et al.*, 1992; Bastiani *et al.*, 1997; Hoxie *et al.*, 1987; Orentas and Hildreth, 1993). However, it was unclear if these proteins were associated with the viral particles or if they derived from contaminating cellular vesicles.

For a long time, it was difficult to purify retrovirus particles especially since infected cells were known to release non-virus related vesicles. These PM-derived microvesicles co-purify with retroviral particles in isolation procedures involving density or velocity separation in sucrose gradients (Bess *et al.*, 1997; Gluschankof *et al*., 1997). Analysis of the resulting material has therefore been misleading. Nevertheless, true incorporation of host proteins into viral particles has been proven by gold immunolabelling host proteins on the surface of particles analysed by electron microscopy (EM) (Gelderblom *et al*., 1987; Henderson *et al.*, 1987) and by the ability to neutralise retrovirus infection with antibodies to host membrane proteins (Arthur *et al.*, 1995; Chan *et al*., 1995). Since the common belief was that host proteins generally were excluded, any inclusion of a foreign protein in virus particles was therefore thought to be intentional and questions were immediately raised regarding

the reasons why viruses would incorporate a specific host protein. Consequently, when MHC class II was found to be incorporated into HIV particles, it was suggested that the virus specifically uses MHC class II as a mechanism to evade the immune system by T-cell activation or apoptosis (Esser *et al.*, 2001; Rossio *et al.*, 1995). Furthermore, Thy-1 was reported to be specifically incorporated into MLV and was implicated as a co-receptor for infection (Calafat *et al.*, 1983). ICAM-1 in HIV-1 was suggested to enhance viral infectivity (Fortin *et al.*, 1997). The reports of specific incorporation of host proteins augmented, and concomitantly the explanations got more imaginative.

In spite of the fact that some host proteins have been shown to be included in retrovirus particles, the major question regarding the mechanism remains the same. Are host cell plasma membrane proteins passively included into particles or not during retrovirus assembly? In particular one would like to compare the surface concentration of the proteins on the PM surface with the equivalent of the virus surface in order to answer this question.

1.3 POXVIRUS

Regarding the poxvirus, which are rather different from retrovirus, the questions concerning viral envelope formation take on different problems. Compared to MLV, much less is understood about the complex and elusive vaccinia virus, which entices researchers with its complicated and intriguing form of existence.

1.3.1 Overview of biogenesis of vaccinia virus

Vaccinia virus is the largest known animal virus, and it carries a single 190 kb chain of dsDNA, in addition to its own set of transcriptional factors and enzymes. It has a high degree of independence from the cell, but still it is dependent on host cell factors for transcription (Rosales *et al.*, 1994; Thornton *et al.*, 1996). Upon infection, vaccinia initiates early mRNA synthesis in the cytoplasm, giving rise to a large variety of enzymes such as intermediate transcription factors, DNA and RNA polymerase, as well as cellular growth factors and products necessary for resistance to the cellular defence system,

i.e. interferon resistance (Smith *et al.*, 1997). Vaccinia replicates entirely in the cytoplasm of the cell, where it sets up replicational factories in the perinuclear space (Cairns, 1960; Kates and McAuslan, 1967; Munyon *et al.*, 1967). DNA replication occurs through concatemer intermediates (Archard, 1979), in chorus with intermediate RNA transcription of late transcription factors. Consequently, late transcription gives rise to the approximately 100 structural proteins that make up the virion. The current hypothesis suggests that viral morphogenesis starts by the recruitment of membranes from the intermediate compartment between ER and Golgi (ERGIC) to the virosome (Figure 3) (Sodeik *et al.*, 1993). The virosome is an electron dense mass, which can be seen inside the viral factories, and is where assembly is thought to occur. The previous hypothesis claimed that a single membrane is synthesised *de novo* within the viral factory (Dales and Mosbach, 1968), and this theory was recently recalled after an EM study in which the authors could only distinguish a single membrane (Hollinshead *et al.*, 1999). However, according to the current model of VV membrane biogenesis, the ERGIC derived membranes are modified by the insertion of viral proteins, and these viral membranes line up around the virosome where they form rigid crescents in close contact with the electron dense material of the virosome (Moss, 1996). Through a process that is understood poorly, the crescents engulf part of the electron dense material from the virosome, encloses it, and forms spherical particles called immature virus (IV) (Dales and Pogo, 1981; Dales and Siminovitch, 1961; Ichihashi *et al.*, 1971). The IV contain a linear DNA genome, as well as the rest of the components necessary for starting a new infectious cycle.

Formation of the enveloped IV is the beginning of the maturation process for the virus, which goes from IMV (intracellular mature virus), to IEV (intracellular enveloped virus), and ultimately to EEV (extracellular enveloped virus). Maturation of IV involves proteolytical processing, which transforms the spherical particle into the brick shaped IMV, representing the first infectious form of the virus (Katz and Moss, 1970; Moss and Rosenblum, 1973; Vanslyke *et al.*, 1993). IMV is then transported to the Golgi compartment, where the virus acquires two additional membranes, resulting in the formation of IEV, which is non-infectious. IEV is then able to induce actin polymerisation in order to be transported to the PM (Cudmore *et al.*,

1995). At the PM, the outermost membrane of IEV fuses with the PM, which causes externalisation of the resulting EEV, which contains one lipid bilayer less than IEV. EEV account for a minor part (5% for the Western Reserve Strain) of the virus progeny (Blasco and Moss, 1992; Garon *et al.*, 1978). A small fraction of extracellular virus particles remain associated to the PM of the host cell. These viruses are called CEV (cell associated enveloped virus) and are responsible for cell-to-cell transfer, while EEV are in charge of long-range transmission (Blasco and Moss, 1992). IMV by far constitutes the major infectious progeny, and stays in the cytoplasm until cell lysis.

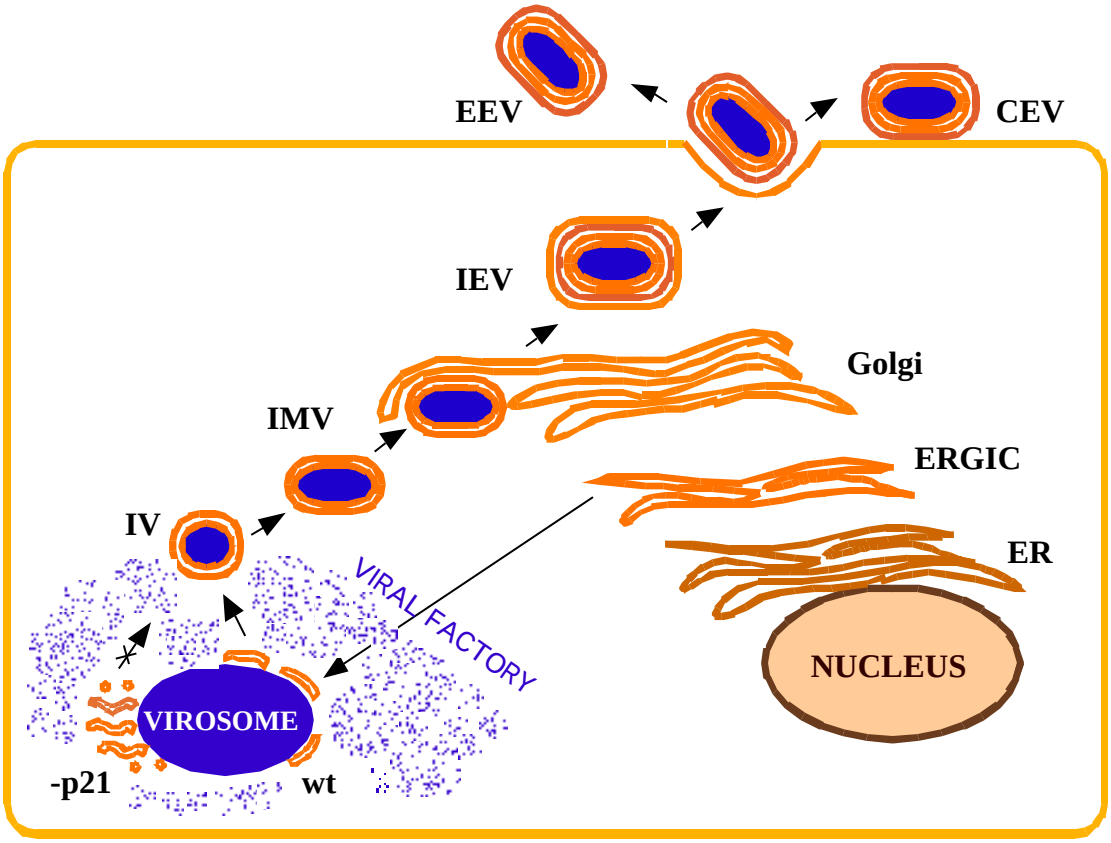

Figure 3: Overview of Vaccinia virus production. Vaccinia replicates in the viral factory, in which electron dense material gather to form the virosome where assembly of IV occurs. Double membranes are recruited from ERGIC, which, in the wild-type (wt) case, form crescents that line up around the virosome. The crescents engulf material from the virosome and form IV, which then mature into IMV. Some IMV are wrapped by Golgi membranes to form the four-membrane enveloped IEV. IEV are externalised following membrane fusion of the outer membrane with the PM, and thus releases EEV. Some particles remain attached to the surface of the cell and are called CEV.

In the absence of the viral protein p21, membrane morphogenesis is arrested at a step in which membrane tubules and vesicles gather around the virosome, but no crescents are formed, and consequently no virus particles.

1.3.2 Proteins involved in envelope morphogenesis of IMV

Proteins involved in the assembly process of VV have been identified by using recombinant VV in which the gene of interest is controlled by the Lac-operon of E. Coli. Thus, expression is inducible by the addition of IPTG (Rodríguez and Smith, 1990b). Cells infected with these recombinants are studied by EM to reveal the phenotypic effect of a given protein on VV morphogenesis. This strategy has uncovered proteins essential for virus morphogenesis, and conclusions have been drawn regarding their function in the virus.

Based on sequence analysis, VV has been predicted to encode 263 potential proteins of 65 aa or more. The open reading frames (ORF) have been named after the 16 fragments generated after HindIII cleavage, designated by the letters A to P in decreasing order of size. The names then include the number of ORF of that fragment (from left to right) followed by the sense in which it is transcribed, L (left) or R (right), for example A17L (Moss and Flexner, 1987; Rosel *et al.*, 1986).

Gene products that have been shown to be essential for IMV envelope formation are an internal scaffolding protein of 65 kD and two membrane proteins, p21 and p15, derived from the ORFs A17L and A14L, respectively. The latter two, together with a third membrane protein p8, encoded by A13L, with a still unknown function, constitute the major components of the IMV membrane (Salmons *et al.*, 1997). The inner side of the envelope is thought to be scaffolded by the p65 protein (Sodeik *et al.*, 1994), while the outer IMV membrane is heavily coated by protein (Risco et al., unpublished). The outer membrane also hosts the surface protein p14, the gene product of A27L, which is necessary for the second envelopment stage in which IMV is transformed into IEV (Rodríguez and Smith, 1990a). p14 interacts with p21, and thus takes part in the membrane protein complex made up by p15 and p21. A more detailed description of each of the proteins involved will now follow.

1.3.2.1 p21 (A17L)

The 21-kDa gene product, encoded by the ORF A17L, was first identified as a product that co-precipitated with the A27L gene product p14 (Rodríguez *et al*., 1993). p21 has been shown to form homodimers that interact with a trimer of p14. p21 is co-translationally inserted into ER and has been found to be an

integral membrane protein of IMV. It contains two transmembrane domains that are long enough to span the membrane twice. Hence, p21 has been predicted to adopt a topology in which it traverses the membrane four times, exposing both of its end terminals on the cytoplasmic side (Figure 4a) (Krijnse-Locker *et al.*, 1996; Rodríguez *et al.*, 1993). However, more recent reports support a two-transmembrane spanning model (Figure 4b) (Betakova and Moss, 2000; Betakova *et al.*, 1999a). The two suggested topologies predispose p21 to quite different possibilities in interacting with other proteins and with itself. Hence it is of fundamental importance to know the topology of the protein in order to set up models for its participation and function in envelopment.

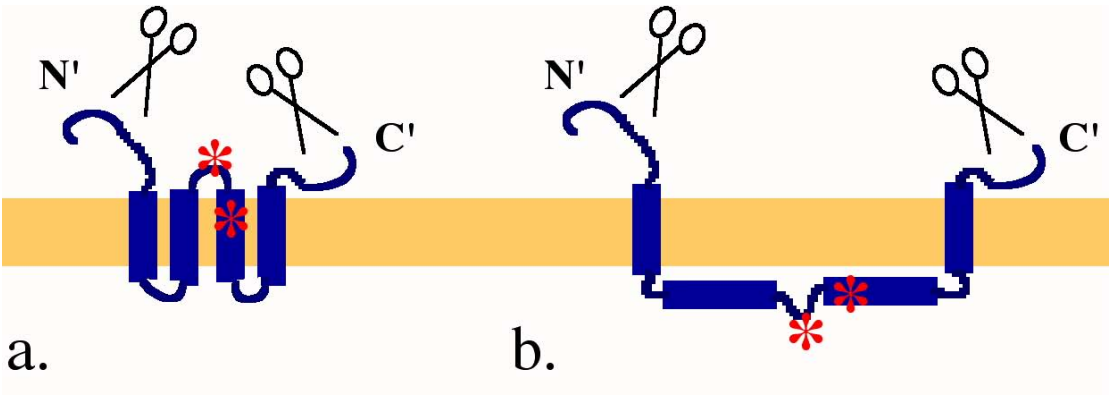

Figure 4: Topology of p21 in the IMV membrane. a. The four-transmembrane model **b.** The two-transmembrane model. Scissors indicate cleavage of the N- and C-terminal tails. Red stars indicate cystein residues in position 101 and 121.

p21 has been found to associate with the membrane crescents that line up around the virosome at early stages of infection. Immunogold labelling of A17L in infected cells analysed by EM, demonstrate that the protein is located on the inner, concave side of the crescents, suggesting that it is present on the inner membrane (Krijnse-Locker *et al.*, 1996). Despite its supposedly interior localisation, p21 has been shown to interact with p14, which is located on the outer surface of IMV. Thus, the suggested model is that p21 serves to anchor p14 to the viral envelope, since the latter lacks a transmembrane domain (Rodríguez *et al.*, 1993). The contradicting facts that p21 is situated on the inner membrane, but interacts with the p14 protein on the outer side, suggests that p21 localisation should be re-examined.

A17L is essential for IMV morphogenesis. In its absence morphogenesis is blocked at a stage where numerous tubules and vesicles collect at the periphery of the virosome, but the characteristic crescents are not formed (Figure 3) (Rodríguez *et al.*, 1995; Rodríguez *et al.*, 1997; Wolffe *et al.*, 1996). It has therefore been suggested that p21 takes part in shaping the membranes into the rigid viral crescents required for IV formation. It has been suggested that the initiation of membrane morphogenesis is regulated by phosphorylation, since membrane morphogenesis in the absence of the F10 protein kinase, is arrested at a stage prior to the formation of viral crescents (Traktman *et al.*, 1995; Wang and Shuman, 1995). Both p21 and p15 are heavily phosphorylated, and are the targets of the F10 protein kinase (Betakova *et al.*, 1999b; Traktman *et al.*, 2000).

1.3.2.2 p14 (A27L)

p14, which is encoded by A27L, is a multifunctional protein that has been implicated in viral attachment and fusion, as well as in the formation of enveloped virions (Chung *et al.*, 1998; Rodríguez *et al.*, 1987; Rodríguez and Smith, 1990a). Antibodies directed against p14 have a neutralising effect on infectivity (Gong *et al.*, 1990; Rodríguez *et al.*, 1987) and in the absence of p14, mature IMV particles accumulate at the periphery of the virosome, but do not wrap to form IEV (Rodríguez and Smith, 1990b; Sanderson *et al.*, 2000). p14 is synthesised on free ribosomes and not until post-translationally, does it co-localise with the membrane crescents and the IV membranes. p14 is located on surface of IMV where it forms a homotrimer linked by disulphide bonds (Sodeik *et al.*, 1995; Vázquez *et al.*, 1998). However, it lacks a transmembrane domain and it is therefore suggested that it is attached to the membrane by binding to p21 (Rodríguez *et al.*, 1996; Rodríguez *et al.*, 1993). In the absence of p21, EM micrographs reveal that p14 is scattered in the cytoplasm of the infected cell. The p14/p21 interaction takes place early during infection, because p14 can be seen to associate to the crescents already when forming around the virosome.

1.3.2.3 p15 (A14L)

p15 is an integral membrane protein of IMV and has been found to form a complex with p14 and p21 (Rodríguez *et al.*, 1997). p15 interacts with p21 at an early stage in morphogenesis when the viral crescents are formed. EM

examination of VV infected cells, not expressing p15, reveals membranous elements that look like unfinished crescents scattered around the virosomes (Rodríguez *et al.*, 1997). p15 is essential for VV morphogenesis and has been suggested to be necessary for both correct assembly of the viral crescents, as well as for attaching them to the surface of the virosome (Rodríguez *et al.*, 1998; Traktman *et al.*, 2000). p15 is myristylated and phosphorylated and appears mostly as disulphide linked dimers. The mechanism by which it would confer control over the membranes is not understood, but could very well be in conjunction with p21.

1.3.2.4 p65 (D13L)

A protein not to be forgotten when discussing membrane morphogenesis of IMV, is the 65-kDa protein encoded by D13L. p65 has been shown to be the target for the antibiotic rifampicin, which reversibly blocks formation of the crescents formed around the virosomes. The block in morphogenesis induced by rifampicin is the same caused by the absence of p65, suggesting that p65 is the target of the drug. p65 is an integral protein of the IMV and has been suggested to function as an internal scaffolding protein (Sodeik *et al.*, 1994) analogous to the matrix proteins of other viruses. It is non-glycosylated and may self assemble in a highly co-operative manner. p65 localises to the inner, concave surface of the membrane crescents as well as in IV. The aa sequence of p65 lacks an obvious membrane anchor or signal sequence for membrane targeting, suggesting that it is a cytosolic protein (Baldick and Moss, 1987; Tartaglia *et al.*, 1986). In spite of this, p65 is bound to the viral membranes, suggesting the presence of a yet unidentified membrane receptor, which targets p65 to the intermediate compartment. In the wt scenario, the convex side of the crescent shaped membranes are covered with characteristic spicules that are lost in the absence of D13L. Lack of p65 also gives rise to irregular membranes forming around the virosomes, suggesting that p65 orients the crescent membrane to take on the typical semi-circular shape eventually leading to IV formation. Unlike the retroviral Gag proteins, it is unlikely that p65 performs this task by itself, and to begin with it would be interesting to identify its membrane-binding counterparts.

1.3.3 The envelope of internal mature virus

For a long time, the origin of the envelope of IMV has been debated, as well as the number of membrane envelopes that wrap the IMV. The original hypothesis suggested that IMV is enveloped by one single membrane, which is synthesised *de novo* in viral factories (Dales and Mosbach, 1968). This theory originates from the difference in lipid composition of viral and cellular membranes, and the lack of continuity between the two. In addition, EM micrographs only revealed one lipid bilayer. This hypothesis was recently proposed again based on observations in EM, which were interpreted as showing one lipid membrane of 5 nm, covered with an 8 nm thick protein coat (Hollinshead *et al*., 1999). In comparison, the examined cells clearly revealed the tightly apposed double membranes of cell-cell junctions as two separate units of membrane. Hence, again it was suggested that IMV only contains one membrane. The single membrane was proposed to be synthesised *de novo* in the viral factories, since they did not show continuity with the cellular membranes. *De novo* synthesis of membranes is not known to occur as a phenomenon within cellular biology, which is why the hypothesis is very unconventional.

In the more easily embraced hypothesis, the viral membranes are suggested to derive from the intermediate compartment between ER and Golgi (Sodeik *et al*., 1993). This theory is based on immuno-EM analyses, which demonstrated that viral membrane proteins decorate the ERGIC membranes, in conjunction with labelling of ERGIC cellular proteins. The ERGIC markers, however, were not detectable in virus structures (Sodeik *et al*., 1993). Additionally, treatment with reducing agents, or protease, gave rise to separated membrane layers, as seen by EM. Unpublished data by Risco *et al.* provide evidence that support the idea of a double membrane. In these studies, freeze-substitution has allowed better structural preservation of the samples, revealing that the IV envelope and the membrane crescents consist of two lipid bilayers. The inner layer shows the characteristics of any cellular membrane, while the outer membrane appears to be thicker, because it is heavily modified by proteins. In conventional studies, the outer membrane has appeared as a diffuse zone, seeming to be exclusively proteinaceous. Hence the theory of one lipid bylayer covered with a protein coat. Based on the recent results, it seems possible to conclude that the IMV has two units of

membrane that are tightly apposed to one another, and heavily modified by protein.

1.3.3.1 Membrane morphogenesis of vaccinia virus

The viral membrane proteins, which modify ERGIC, are synthesised in the ER, and probably contain a retention signal that prevents them from proceeding along the default pathway to the PM. Hence they accumulate in the ERGIC, which they consequently transform into the viral crescents used for virus formation. In the vicinity of the virosome, the double membrane is organised into rigid crescents that line the virosome. By engulfing material from the virosome, the membrane crescents form spherical immature particles, i.e. IV, that mature into IMV following proteolytical processing. The double membrane has been suggested not to fuse with itself to form a continuous membrane, but instead incompletely surrounds the particle. This hypothesis is derived from experiments in which internal IMV antigens became exposed after treatment of IMV with reducing agents (Roos *et al.*, 1996). Thus, overlapping membrane could be held together by protein disulphide bridges, since reducing agents, as well as protease digestion of permeabilised IMV, clearly show how the membranes separate from each other (Roos *et al.*, 1996).

The next step involving enwrapment of destined IMV particles occurs at the trans Golgi compartment (Schmelz *et al.*, 1994) and requires, among other IMV proteins, the presence of p14 on the surface of IMV (Rodríguez and Smith, 1990a). EM pictures demonstrating the envelopment process of IMV particles, reveal no budding features similar to retrovirus. Instead, the second double membrane seems to be achieved by yet another engulfing process, whose mechanism remains to be resolved.

2 AIMS

Aims of the thesis:

- To study the fate of host proteins during retrovirus assembly
- To elucidate a possible function for actin during retrovirus budding
- To determine topology and localisation of p21 (A17L) in vaccinia IMV

3 METHODS

3.1 PRODUCTION OF GAG PARTICLES

Production of virus particles from wt MLV producing cell lines is very low and thus make them difficult to use when studying questions relating to assembly. With regards to additional questions about host protein sorting during budding, they are practically impossible to use because of the low virus yield. It is therefore necessary to turn to heterologous expression systems in which higher quantities of particles can be obtained. We decided to use the Semliki Forest virus (SFV) expression system for our purpose since it allows efficient expression of genes in fibroblasts.

Since retroviral Gag particles have the ability to self assemble in the absence of other viral proteins, they constitute the simplest form of virus assembly. Therefore, they are attractive to study in order to understand assembly and budding, and they are easy to produce since they only require expression of the Gag protein in cells. Hence, I have chosen to study Gag particles produced by the SFV expression system to answer the question of host protein sorting during budding.

3.1.1 The SFV expression system

The SFV expression system is based on the positive stranded RNA genome of SFV into which the gene of interest is inserted in place of the structural SFV proteins (Liljeström and Garoff, 1991). The SFV genome contains regions for

its non-structural polymerase protein subunits and structural capsid and membrane (spikes) protein subunits. The non-structural polymerase proteins are responsible for converting the positive stranded RNA of incoming virus into negative full length RNA from which new positive stranded RNA is made. In addition, the negative stranded mRNA is used for synthesis of the viral subgenome, a 26S mRNA from an internal promoter. The latter is used for translation of the viral structural proteins. Thanks to the C-encoding region, which functions as a translational enhancer, the structural proteins are produced in high amounts. In the genetically engineered SFV expression vector, the 26S coding sequence is exchanged for foreign genes. Two expression vectors have been constructed with and without the translational enhancing C-protein. This allows expression to be varied 10-20 times (Sjöberg *et al.*, 1994), which permits us to assess the effects of overexpression when studying host protein sorting during retrovirus budding. An additional advantage of the SFV expression system is the shut-off of host protein synthesis induced by the SFV replicon. This makes it possible to study protein synthesis on a clear background.

The SFV expression vector can be transcribed *in vitro* and the RNA directly used for expression after electroporation of baby hamster kidney (BHK) cells. In the cell, the SFV expression vector RNA will replicate and produce subgenomes for translation of the foreign protein. Alternatively, the expression vector RNA can first be packaged into recombinant SFV particles and then these RNAs can be used for expression analysis after SFV mediated infection of the target cell. Such recombinant SFV particles are produced in BHK cells by co-transfecting the SFV expression vector RNA and a helper RNA, which carries the genes for the structural proteins of SFV. As only the expression vector RNA contains a viral RNA packaging signal, only these RNAs, and no helper RNA, will be incorporated into the recombinant SFV particles. The generated recombinant SFV particles will be one-time infectious particles, since they will have the capacity to infect, but will be unable to produce progeny because of the lack of viral, structural genes. The heterologous protein gene, however, will be expressed in high amounts concomitantly with extensive host protein shut-off.

3.1.2 Purification of Gag particles

The main problem of studying retroviral particles is to achieve virus particle preparations that are free from cellular contaminants. This is especially important when examining host protein incorporation in particles. The standard method is to isolate particles on sucrose gradients where they sediment to their isopycnic density. The problem is that cells are known to release non-virus related vesicles, so-called microvesicles, which co-migrate with the viral particles because of their similar density. We tried several types of sucrose gradient centrifugation to isolate particles, either by pelletation on sucrose cushions, by separating them according to their density in isopycnic sucrose gradients, or by size in velocity sucrose gradients. None of the methods worked satisfactorily because of cellular, co-sedimenting vesicles, or too broad sedimentation of Gag particles. The solution to the problem was to separate the particles in iodixanol gradients. Iodixanol is a more isotonic solution than sucrose, which preserves the physical properties of the migrating Gag particles and therefore results in good separation based on size (Bess *et al.*, 1997; Gluschankof *et al.*, 1997; Raposo *et al.*, 1996).

Since purity of the Gag particle preparation was crucial to our analysis, we included two control cultures in the experiments. One was cells infected with wt SFV and the other cells infected with recombinant SFV expressing the non-budding nucleoprotein (NP) of influenza virus. Media from these cultures were exposed to gradient centrifugation in parallel with Gag particle purification. SDS-PAGE analysis of the resulting fractions from the control cultures did not reveal the presence of cellular proteins in the region corresponding to the fractions utilised for Gag particle extraction. Additionally, we analysed the isolated material by EM in search of contaminating microvesicles in the preparations. The absence of cellular material in the control cultures, as determined both by SDS-PAGE and EM, indicated that the purification procedure was suitable to use in our studies. Thus, any cellular material encountered in preparations of MLV Gag particles, had to be specifically associated to the particles.

3.1.2.1 Host protein incorporation in Gag particles

As we were interested in studying host protein incorporation in MLV Gag particles on a quantitative and qualitative basis, we had to develop a method that would allow us to quantitatively compare the host protein density of the

cellular PM with that of Gag particles (membranes). This would require comparing equal surface areas of PM with Gag particle membrane. As a common denominator of membrane area, we made use of radioactively labelled lipids, which we could quantify. Based on lipid quantifications, we were able to equalise the membrane content of isolated PM and Gag particles, and thus directly compare the protein concentrations of the two by SDS-PAGE.

3.1.2.2 Purification of plasma membrane

In order to analyse the host cell membrane that donates the viral envelope, we tried several methods to isolate the PM. The conventional method for isolating cellular membranes is by separating them according to their different densities on sucrose gradients. However, it is particularly difficult to isolate PM since it spreads over a wide range of densities (1.136 -1.164 g/ml), including over the densities of the Golgi membranes (1.13-1.15 g/ml) (Suomalainen *et al*., 1996). Most PM preparations are therefore inevitably contaminated by other cellular membranes. After having tried conventional density purification, we tried more unconventional methods for isolating PM. These included binding of colloidal gold particles to the surface of cells in order to increase the density of the PM, making it possible to separate from other membranes either by density or velocity gradient centrifugation.

However, these methods were abandoned in favour of a method in which it was possible to specifically isolate PM, which was used for Gag budding. Isolation of these membranes also uses sucrose centrifugation, but Gag protein bound to the PM, in the process of budding, have a defined density, which makes these membranes possible to purify from other membranes. The use of membranes that are specifically involved in budding also invalidates the criticism of whether Gag buds from specific regions of the PM. This method was initially developed by Suomalainen *et al.* (1996).

3.1.2.3 Lipid analysis

We steady state labelled BHK cells with [^{32}P]orthophosphate for 40 hours prior to infecting with SFV recombinant particles carrying the Gag gene, in order to ensure complete labelling of cellular membranes. [^{32}P]labelled membranes were then isolated and lipids were extracted and quantified. Lipids were initially separated from contaminating [^{32}P]labelled nucleic acid

and phosphoproteins by extraction using the classical methanol/chloroform procedure. Then the lipids were analysed by chromatography and quantified using liquid scintillation. However, this procedure was complicated and prone to inaccuracy because of variation in the lipid extractions. Since the material used for protein analysis was provided by parallel experiments without [^{32}P]labelling, to reduce radiation when handling samples, it was critical to obtain an accurate and reproducible method for lipid quantification. A method, which turned out to be much simpler and precise, was the use of SDS-PAGE to separate lipids. Isolated [^{32}P]labelled membranes and particles were solubilised in hot SDS, creating SDS-phospholipid micelles, which migrated as a broad band of 18 kDa and could be separated from RNA and free orthophosphate on polyacrylamide gels. A phosphorimager was then used to quantify the amount of lipid directly from the gel.

3.1.2.4 Protein analysis

Since SFV efficiently shuts off host cell protein synthesis, it was necessary to steady state label BHK-21 cells with [^{35}S]methionine for extended amounts of time before infection. We found that 15 hours pre-incubation with [^{35}S]methionine was sufficient to get a good degree of membrane proteins labelled. The protein content of both isolated Gag particles and PM were analysed in parallel by SDS-PAGE, based on equal amounts of lipid. A wide range of different proteins feature in the PM, which makes their separation complicated. We tried to use two-dimensional SDS-PAGE in order to improve separation of the cellular proteins. However, this method involved non-quantitative protein extraction, and gave too low detection level, which altogether made it useless for our purpose. We also used Triton X-114 with which we managed to separate integral membrane proteins from peripheral membrane proteins, with the intention of characterising the host proteins.

3.1.2.5 Identification of actin in murine leukaemia virus Gag particles

Purified MLV Gag particles were analysed in duplicates by 10% SDS-PAGE. One half of the gel was incubated with Coomassie brilliant blue (CBB) while the other was prepared for transfer to nitrocellulose filter. The filter was reacted with monoclonal antibody to actin according to conventional immunoreactive methods. The antibody binding was visualised by horseradish peroxidase (Figure 5).

3.2 LOCALISATION OF PROTEINS IN VIRUS PARTICLES

3.2.1 Localisation by electron microscopy

A powerful tool available to determine localisation of proteins in virus particles is immunogold labelling followed by EM analysis. This method utilises antibodies coupled to colloidal gold to detect the protein of interest, and which can be visualised in negatively stained preparations by EM. Stereo pictures from EM supplement the determination of a protein's location.

An additional method to determine the localisation of proteins is to treat particles with detergent prior to EM analysis. For example, Nonidet P-40 (NP-40) strips the particles of their membrane and exposes the inner capsid. Thus, the desired protein can be visualised by immunolabelling and its localisation can be established on the surface of the delipidised particle.

3.2.2 Localisation by biochemical methods

Additionally, biochemical methods are available, which can be used to predict the localisation of proteins in virus particles. Such methods differentiate between proteins that are susceptible or resistant to modification, depending on whether their localisation exposes them or protects them from treatment. An example is to modify proteins by biotinylation, which enables protein precipitation by streptavidin coupled to agarose beads. Another method is to treat purified particles with protease (i.e. proteinase K or trypsin), which reveals a protein's localisation on the surface if it is susceptible to protease digestion. The crucial factor in such experiments is the use of intact particles, which is why great care should be taken in choosing purification methods, as well as positive and negative controls. I used conventional sucrose gradient centrifugation in order to purify intact IMV followed by either trypsin treatment at various concentrations or biotinylation in ice-cold conditions.

A challenge posed by VV in choosing analysing methods, is the relative lack of knowledge of the virus, both morphogenically and biochemically. In VV, neither the processing nor the topology of many proteins is known, which, in certain cases, has lead to wrong conclusions being drawn. An example is the A17L gene product that was determined to be located

exclusively on the inner surface of VV immature virions (IV) (Krijnse-Locker *et al*., 1996). The antibodies used in those studies were directed to parts of the p21 product that now are known to be cleaved off in the mature virus. For my experiments I used a set of polyclonal antibodies directed against peptides generated from p21. Before applying the antibodies in experiments, we characterised the products that they recognised and verified the processing previously suggested for p21. Hence, we were able to verify the proteolytic cleavage products as well as study the time course for processing. We then used the antibodies to detect proteins after protease or biotinylation treatments.

It should be stressed that the double envelope of IMV is not thought to be continuous. The overlapping ends, suggested to be held together by disulphide bonds, make the IMV envelope vulnerable to protein altering agents. It is likely that protease treatment destroys such protein “locks” and makes the interior of the particles accessible to the exterior environment. Considering this, protease treatment as a method for establishing protein localisation on the surface or inside IMV, could not be quantitative, although it could be indicative if using diluted concentrations and previously characterised control proteins. However, the ERGIC derived double membrane continuously encloses what was the lumen of the original donor compartment. Thus, proteins located in the inter-membrane space would be protected from e.g. protease treatment, and thereby conclusions could be drawn on the topology of membrane proteins of IMV following extended protease treatment.

4 RESULTS

4.1 CELLULAR PROTEINS IN RETROVIRUSES

Our aim was to bring clarity to the issue of host protein incorporation in retrovirus particles. Therefore, we developed a system in which we quantitatively could compare cellular membranes used for virus budding, with purified particles. Our results, presented in paper I, demonstrated that MLV Gag particles contain a wide range of host proteins. Host proteins specifically co-migrated with MLV particles, whereas SFV was devoid of cellular proteins. As a control for the purity of our preparations, we performed parallel experiments in which we analysed medium from cells expressing the non-budding influenza NP. No host proteins were detected in the fractions used for particle isolation, demonstrating that our purification method was free from cellular contaminants.

When we compared the protein content of Gag particles with isolated PM, we were able to find almost all host proteins from the membrane fraction, in the Gag particles. Exceptions were a few, high molecular weight proteins (between 186-kDa and 148-kDa), which were excluded from the particles. On the other hand, some proteins were enriched in the particles, suggesting that they were selectively brought into the particles. Host proteins enriched in the particles were most notably a protein of 24-kDa, and to a lesser degree a 67-kDa and a 148-kDa protein. Taken together, our results indicate that

retrovirus Gag particles possess, at most, limited capacity of excluding non-viral proteins, and therefore host proteins are randomly included in particles.

Because of the criticism that high levels of Gag budding might give rise to an artificial situation that would allow non-specific incorporation of host proteins into particles, we compared our results with those of an SFV expression vector with a ten fold less expression of Gag protein. Additionally, we analysed Gag particles produced at a very early time point of Gag expression, when it can be assumed that the system has not yet been affected by overexpression of the Gag gene. Both experiments gave the same result as previously, i.e. extensive host protein incorporation in Gag particles.

We also analysed Gag particles that were co-expressed with the homologous Env protein in order to see whether Env incorporation could confer sorting capacity to the assembly process. The Env protein was incorporated to an extent of one Env per five Gag molecules in the particles, which is comparable to the situation in wt retrovirus (Henderson *et al.*, 1984). In fact, the homologous Env protein was actively incorporated into the particles where it was three to four times more concentrated in comparison to the PM. This is the first proof of active incorporation of Env in retroviral particles. However, supplementing Gag particles with the homologous Env protein did not alter or reduce the amount of host protein incorporation. It is therefore our conclusion that retrovirus particles specifically bring in the homologous Env protein, but randomly include any membrane protein present at the site of budding.

4.1.1 Actin in Gag particles

When isolated Gag particles were analysed by SDS-PAGE, CBB staining revealed that the major component of the Gag particles, except for the Gag protein itself, is a protein of approximately 42 kDa. Several enveloped viruses have been reported to contain the cytoskeletal protein actin, e.g. RSV, HIV, and Sendai virus (Arthur *et al.*, 1992; Wang *et al.*, 1976). Since the molecular weight of actin corresponds to 42 kDa, we reacted purified Mo-MLV Gag particles, separated by SDS-PAGE, with anti-actin antibodies. Our results show that the anti-actin antibodies indeed recognised the 42-kDa band, indicating that actin is present in Mo-MLV Gag particles (Figure 5). The amount of actin varies in different Gag particle populations, which are

broadly distributed in sucrose gradients. Almost half of the mass of Gag, in some particles, was made up of actin. In purified particles, though, the amount of actin is approximately 1% of the Gag protein as determined by quantification of CBB stained protein by densitometry scans. This amount corresponds to approximately 20 actin molecules per Gag particle, which has been estimated to consist of 2000 copies of Gag (Strömberg *et al.*, 1974). When comparing the amount of the other cellular proteins to the quantity of actin in Gag particles, it can be concluded that each one is present in 0.1-10 copies per particle.

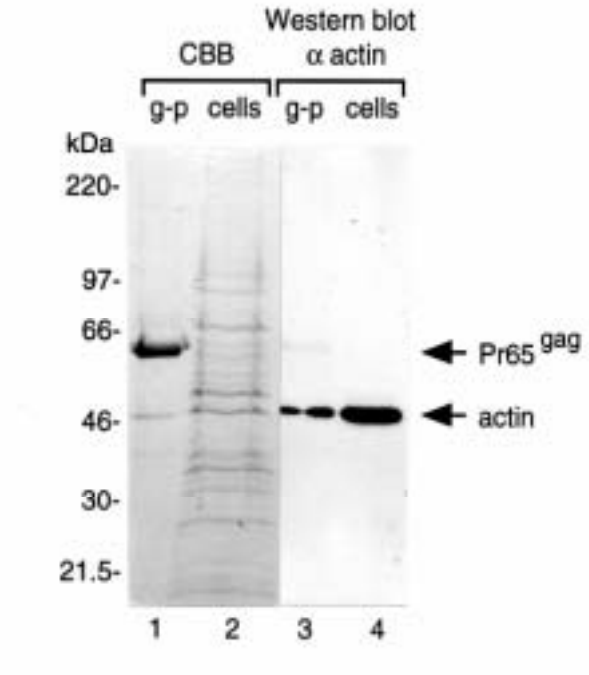

Figure 5: Identification of actin in Mo-MLV Gag particles. Duplicate samples of purified Gag particles (g-p) and lysed BHK-21 cells, analysed in parallel by SDS-PAGE. Samples in lanes 1 and 2, were stained with Coomassie brilliant blue (CBB). Lanes 3 and 4 were subjected to Western blot, and the nitrocellulose filter was reacted with monoclonal antibody against actin, and visualised by chemiluminiscence (ECL).

4.1.1.1 Localisation of actin

In paper II, we analysed the localisation of actin in Gag particles by EM. Immunogold labelling of actin on the surface of intact Gag particles was negative. However, after stripping purified Gag particles of their envelopes by treating them with NP-40, it was possible to gold immunolabel the actin protein on the surface of the delipidised particles. Stereo pictures clearly demonstrated localisation of colloidal gold on the surface of the non-enveloped particles. By extending treatment with NP-40, actin labelling was lost, while labelling for the internal MA, p12 and CA proteins was unaffected.

The distribution of actin labelling was 23 gold particles per Gag particle (SD 29, n=146). However, distribution was uneven with some particles rich in actin, and others negative for actin. Although, the Gag particles vary in size

between 80 and 130 nm, the amount of labelled actin did not correlate with the size of the particles, indicating that actin does not serve a structural role in the particles. The fact that CA was labelled by antibody could be explained if the network would be loosened after detergent treatment or even broken in places. In conclusion, actin is located in the particles underneath the membrane, on top of the Gag-shell to which it is loosely attached.

4.2 LOCALISATION OF p21 IN VACCINIA VIRUS

Deciphering the topology and localisation of the membrane protein p21 in IMV is crucial in order to understand and further elaborate on the protein interactions and function that apply to p21. By using EM and biochemical methods, we were able to determine that p21 is located on the surface of IMV particles where it also exposes its N-terminal. Our results, presented in paper III, are based on EM analysis of immunogold labelled, purified IMV, which showed abundant labelling of p21 on the surface of particles. This labelling was as abundant as labelling for the surface protein p14, while labelling for the internal core protein p39 (gene A4L) was negative. Additionally, p21 of intact IMVs was accessible to biotinylation and consequently biotinylated p21 precipitated with streptavidin after solubilisation of IMV. Our results do not exclude that p21 is located in the inner membrane of the IMV as well, and hence p21 is probably located in both, considering previous results.

4.2.1 Topology of p21

Protease treatment of particles demonstrated that the N-terminus of p21 is sensitive, while the C-terminus is resistant to degradation. Antibodies directed to the N-terminus of p21 have the capacity to neutralise infectivity of IMV, while C-terminal antibodies have no effect. Thus, we conclude that the N-terminus of p21 is exposed on the surface of IMV. On the other hand, the C-terminus seems to be embedded in the membrane or otherwise hidden from antibody recognition, for example by interacting with other proteins. Protease sensitivity of the p21 N-termius was absolute, indicating that p21 adopts the same topology in both IMV membranes of the IMV exposing the N-terminal tail to the outer side in both.

5 DISCUSSION

5.1 CELLULAR PROTEINS IN RETROVIRUSES

We have shown that numerous proteins of cellular origin are incorporated into MLV Gag particles (paper I). These host proteins are equally concentrated in particles and in PM, demonstrating that cellular proteins are randomly incorporated in particles during budding. The same holds true even when the homologous Env protein is included in particles, proving that its participation does not confer sorting capacity to the budding process. Therefore, retroviruses do not seem to have evolved a mechanism enabling them to exclude non-viral proteins.

5.1.1 Accommodation of foreign proteins in Gag particles

The capacity to incorporate foreign proteins in the viral envelope is primarily a matter of space. The alphavirus, which shows no incorporation of host proteins (paper I; Strauss, 1978), exhibits a tight icosahedral structure, as has been revealed by cryo-EM (Cheng *et al.*, 1995; Lescar *et al.*, 2001; Mancini *et al.*, 2000). Thus, it is easy to understand that the organisation of this virus accepts no interference or modification by foreign proteins. However, the retrovirus with its Gag shell appears different in this respect.

5.1.1.1 Structure of the Gag shell

The structure of the Gag shell should predominantly determine inclusion or exclusion of host cell proteins. However, the structure of the Gag shell is not yet clear, but it is evident that it is not an icosahedral structure (Yeager *et al.*, 1998). Primarily the structure of the MA lattice would dictate the accessibility of transmembrane proteins, since this Gag domain is located adjacent to the PM. X-ray analysis of MA crystals have revealed that the MA protein forms a trimer (Hill *et al.*, 1996). Previous studies demonstrated a fullerene-like model for the organisation of the Gag protein shell of HIV, with a ring-like arrangement of Gag molecules forming a hexagonal network (Nermut *et al.*, 1994). The trimers have been superimposed on the hexagonal network, thus presenting a model of a hexameric lattice of MA trimers (Forster *et al.*, 2000). This hexameric lattice creates a patchwork of large and small holes inside the hexameric rings and between the individual MA trimers, respectively (Figure 6). The large holes (~1500 $Å^2$) have a diameter of 4 nm (Forster *et al.*, 2000; Hill *et al.*, 1996), and according to the hypothesised model, the glycoprotein knobs are situated over the centre of these rings (Figure 6a). This would give a theoretical centre-to-centre knob spacing of 21 nm, which is close to the measured mean of 22 nm (Figure 6b) (Forster *et al.*, 2000). The glycoprotein heads have a dimension of 14 nm, which, at the closest site, would leave a space measuring 7 nm in between the spikes, and a total of seven completely unoccupied holes around each triangular facet carrying three glycoproteins (Figure 6a). This spacious arrangement of the viral spikes on the surface could comfortably house quite a number of cellular membrane proteins, and certainly the loose structure of the underlying Gag lattice would not impose any significant restriction that would lead to their exclusion. The only limitation is brought upon by the size of the hole in the Gag-ring, which could exclude transmembrane proteins with a large cytoplasmic tail. It is easy to visualise that the Gag particles could accommodate a wide variety of cellular proteins in its envelope, which could be a reasonable explanation for our present findings.

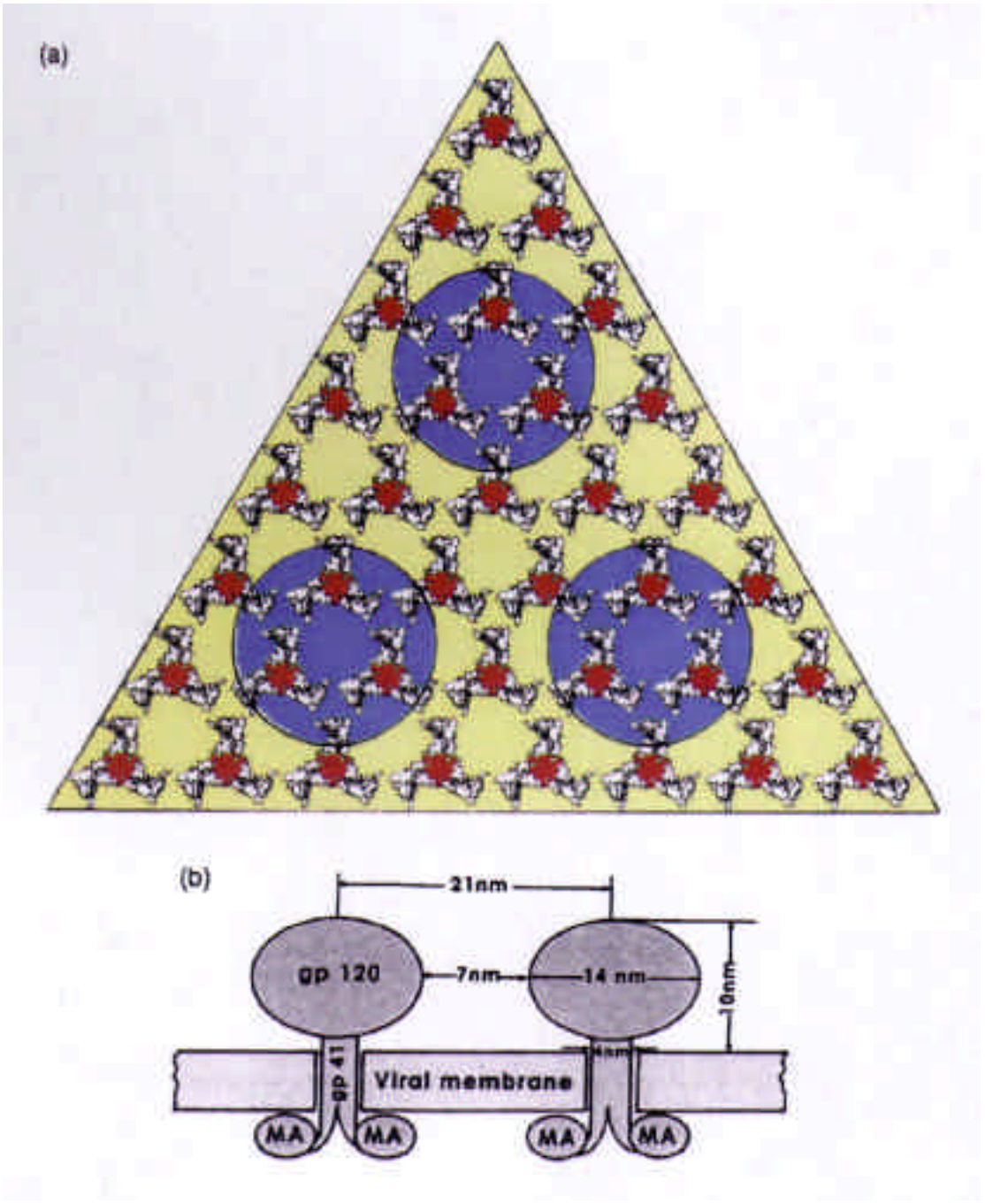

Figure 6: a. Model of the triangular face of the MA network in HIV. Homotrimers of MA form hexagonal rings together with MA proteins from adjacent trimers. The hexagonal rings form large holes of 4 nm. The viral spikes (HIV Env, gp120; blue) are distributed above the network according to measurements from EM images. **b.** MA trimers associating at the viral membrane with the viral spike. The diagram depicts the Env TM unit (gp41) inserted into the MA network. Diagram is approximately to scale. (From Forster et al., 2000. Reproduced with permission from the copyright holder).

5.1.2 Exclusion or inclusion of host cell proteins

Many cellular proteins have been reported to be present in retrovirus particles, and the explanations of why, are as many as there are reported proteins. The presence of individual cellular proteins in the particles is not surprising in the light of our results (paper I) and from the structure considered in the previous section. Our results do not rule out that individual proteins might serve a function in the particles, but the mechanism for how they end up in the particles does not seem be through preferential incorporation. Instead there seems to be a random incorporation of most proteins present at the site of budding.

However, according to our results, a few host membrane proteins were indeed excluded from the particles. These proteins were mainly large proteins over 150 kDa. Specific exclusion of proteins from particles is much easier to envision than specific incorporation. Exclusion could easily be an effect of large three-dimensional structures that sterically hinder incorporation. Some proteins that have been reported not to reside in retroviral particles are, for example, the HIV co-receptors CXCR4, CCR3 and CCR5 (Lallos *et al*., 1999). These chemokine receptors belong to the seven-transmembrane receptor family and, as indicated by their name, contain seven transmembrane segments. It would not be surprising to find that such a large membrane-occupying element would sterically hinder them from being incorporated into virus particles.

On the other hand, if a host protein would be specifically included in retrovirus particles, it would require a defined interaction with the viral proteins for it to be singled out and incorporated during budding. A cellular protein that has been considered to be specifically incorporated is CD86. This conclusion is drawn from experiments comparing host proteins in HIV particles and cellular microvesicles released from infected cells (Esser *et al.*, 2001). The results demonstrate that CD86 is present in virions, as well as in microvesicles, while another cellular protein, CD45, was present in microvesicles, but not in HIV particles, in spite of it being the most highly expressed protein on the cells examined. However, CD45 has an unusually large cytoplasmic domain, which consists of two tandem repeats of approximately 300 aa residues each. Most certainly, such a large, intracellular structure would have a negative impact on its incorporation in a viral

envelope. Microvesicles, on the other hand, are assumed to merely be membrane blebs, and therefore not have a specifically arranged array of proteins, comparable to that of virus particles, which could conduct restrictions on protein incorporation. Maybe it would be more appropriate to consider specific exclusion of proteins, rather than specific inclusion, in order to elucidate the mechanism of host protein incorporation.

A schematic diagram of some of the host proteins reported by others to be incorporated or excluded from HIV particles (Esser *et al.*, 2001) is presented in Figure 7.

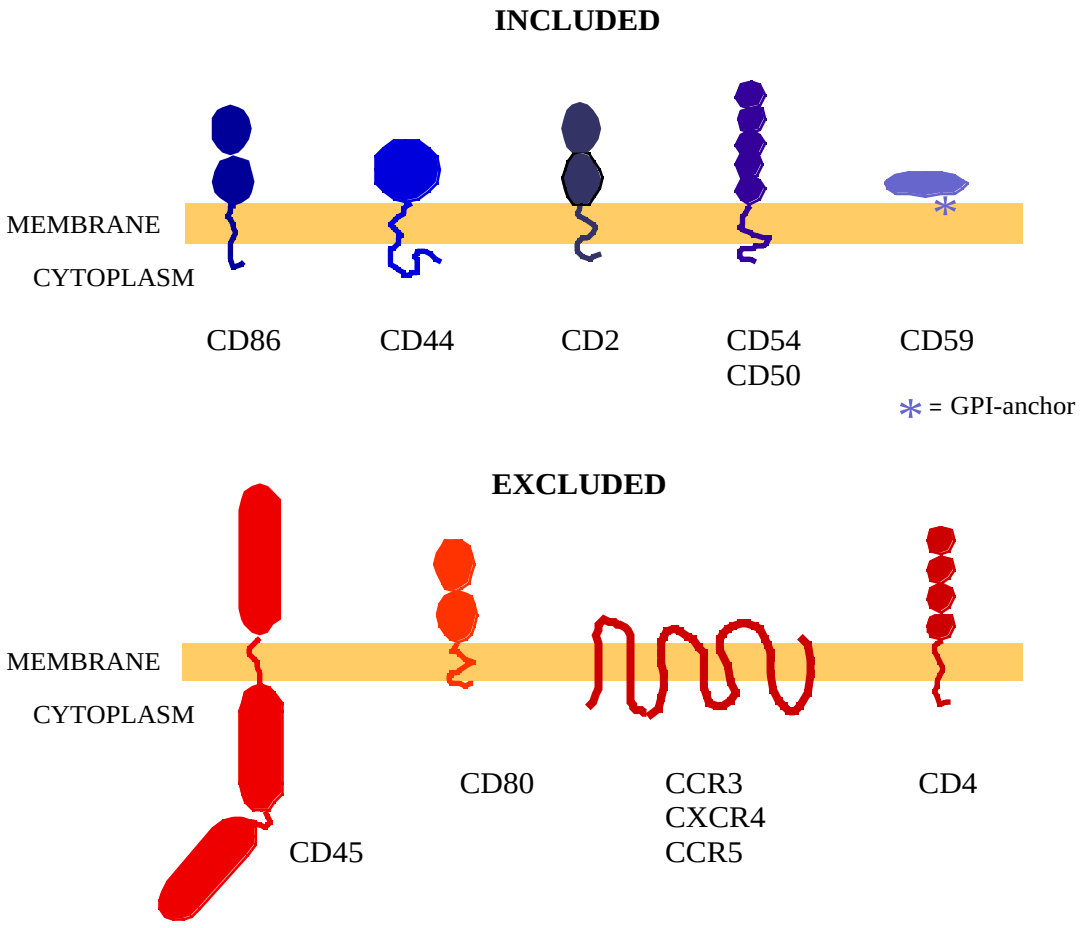

Figure 7: Cellular membrane proteins included or excluded from HIV-1 particles. Schematic diagram of proteins to give a sense of their two-dimensional structure and membrane topology. Data on protein structure from http://www.ncbi.nlm.nih.gov/PROW/guide

It should be noted that proteins with large intracellular or transmembrane domains are excluded from particles, while other proteins are included, which supports our hypothesis. Even so, some proteins, with an

apparent non-intrusive, two-dimensional structure, are excluded from particles. However, I have been unable to consider protein-protein interactions and the formation of large protein aggregates that might sterically hinder their incorporation.

5.1.2.1 Function of host proteins

Why have retroviruses evolved a structure that generally allows them to incorporate cellular proteins? An explanation might be that cellular proteins could act as co-receptors in order to enhance virus infection. Cellular proteins located on the PM are mainly involved in cell adhesion or cell signalling, and have corresponding receptors exposed on the surface of neighbouring cells. Including these proteins in virus particles would facilitate binding of the virus particle to cells prior to interacting with the corresponding Env receptor, needed to catalyse the entry process. Bound particles allow a two-dimensional screening for the cognate receptor, while unbound particles must seek their receptors in a three-dimensional space. These speculations are supported by results showing that stable virus adsorption to cells is independent of Env-receptor interaction and that certain cell types bind virus only poorly, despite the presence of the same levels of Env receptor (Pizzato *et al.*, 1999).

5.1.3 Env incorporation

The Env protein is necessary for the particles to be infectious, but how is it incorporated into retrovirus particles? It has been very difficult to define a domain that would account for an interaction between Env and Gag. Does the Env protein actually require a specific interaction considering that we have shown that membrane proteins are randomly incorporated?

Our results are the first to prove that the homologous Env protein is concentrated in the virus particles. In fact, Env is concentrated three to four times above the level expressed on the PM in Gag particles (paper I). The amount of Env incorporated in the Gag particles is equivalent to what is found in wt MLV particles. These results clearly show that there does exist an interaction *in vivo* between Env and Gag, which is responsible for recruiting the spike into the budding particles.

The accompanying question is; where in the cell does the Env-Gag interaction occur? For Env to be able to dictate the site of budding in epithelial and neuronal cells, the interaction would have to occur before Gag reaches the

non-indicated membrane. Thus, the interaction has to take place concurrently with Env transport. The cytoplasmic tail of Env should be exposed during vesicle transport, and thus available to interact with Gag. The Gag proteins could then be co-transported with Env by hitch-hiking on the outside of transport vesicles to the site of assembly. It would be interesting to examine whether heterologous spikes could direct budding of pseudotyped particles in polarised cells. However, Gag hitch-hiking with Env on the outside of transport vesicles to the PM, is probably not the prevailing mode of transport for Gag. This is because Gag on its own is specifically directed to the PM, and not to any other internal membranes, since Gag budding does not occur elsewhere within the cell.

Another possibility could be that the interaction with Env lowers the energy required for Gag to assemble at the PM. This could be the reason why Gag preferentially assembles in the presence of its homologous spike, and this could also explain how Env can dictate the site of budding. In the absence of Env, the membrane affinity of Gag seems to be sufficient to induce budding. Possibly Gag budding alone, requires higher concentrations of Gag in order for the Gag-Gag interactions to overcome the energy threshold required to initiate assembly. The theory that the Env-Gag interaction, is week, but facilitates budding by lowering the energy required for assembly, could explain the results obtained in this field so far.

5.2 INVOLVEMENT OF CYTOSKELETON IN GAG BUDDING

5.2.1 Actin in Gag particles

We found that actin represents the most prominent host protein included in MLV Gag particles (paper I, and data presented in this thesis (Figure 5)). According to our biochemical data from purified Gag particles, actin is present at levels of approximately 20 molecules per particle, although large variation exists between Gag populations. The low number of actin molecules, despite it being the best represented host protein in particles, as well as the random variation of actin concentration in particles, has led us to believe that actin has no structural function in the particles. Additionally, actin appears to be sorted out from particles during budding, since its

concentration in the PM is higher than in particles. From EM stereo pictures of immunogold labelled actin on membrane-stripped Gag particles, we were able to demonstrate that most actin was located on the surface of the Gag-shell, but underneath the membrane (paper II).

5.2.1.1 The actin cytoskeleton

How do our result of actin exclusion during budding relate to the situation *in situ*? What is the density of the actin network that the virus encounters during assembly? What does the cytoplasmic surface of a PM look like?

In the EM picture, kindly provided by M. V. Nermut, shown in Figure 8, the cytoplasmic side of the PM has been uncovered in an RSV-transformed fibroblast (Nermut *et al.*, 1991). The micrograph shows how the network, created by the actin cytoskeleton, covers the inner side of the membrane. Some actin filaments, though, might have been lost in the procedure, and the figure might therefore under-represent the density of the network. For orientation purposes, the size of a retrovirus particle is indicated in the picture. In contrast to the heavily occupied cytoplasmic side of the membrane, the outer cell surface appears smooth, showing only small globular projections, which could represent protein complexes. The picture gives a visual understanding of the cellular environment in which the virus assembles. Several filaments of actin appear to be in the way for virus assembly, and hence, as indicated by our results, they are probably pushed away during virus budding, unless the virus assembles in areas with a less dense actin network.

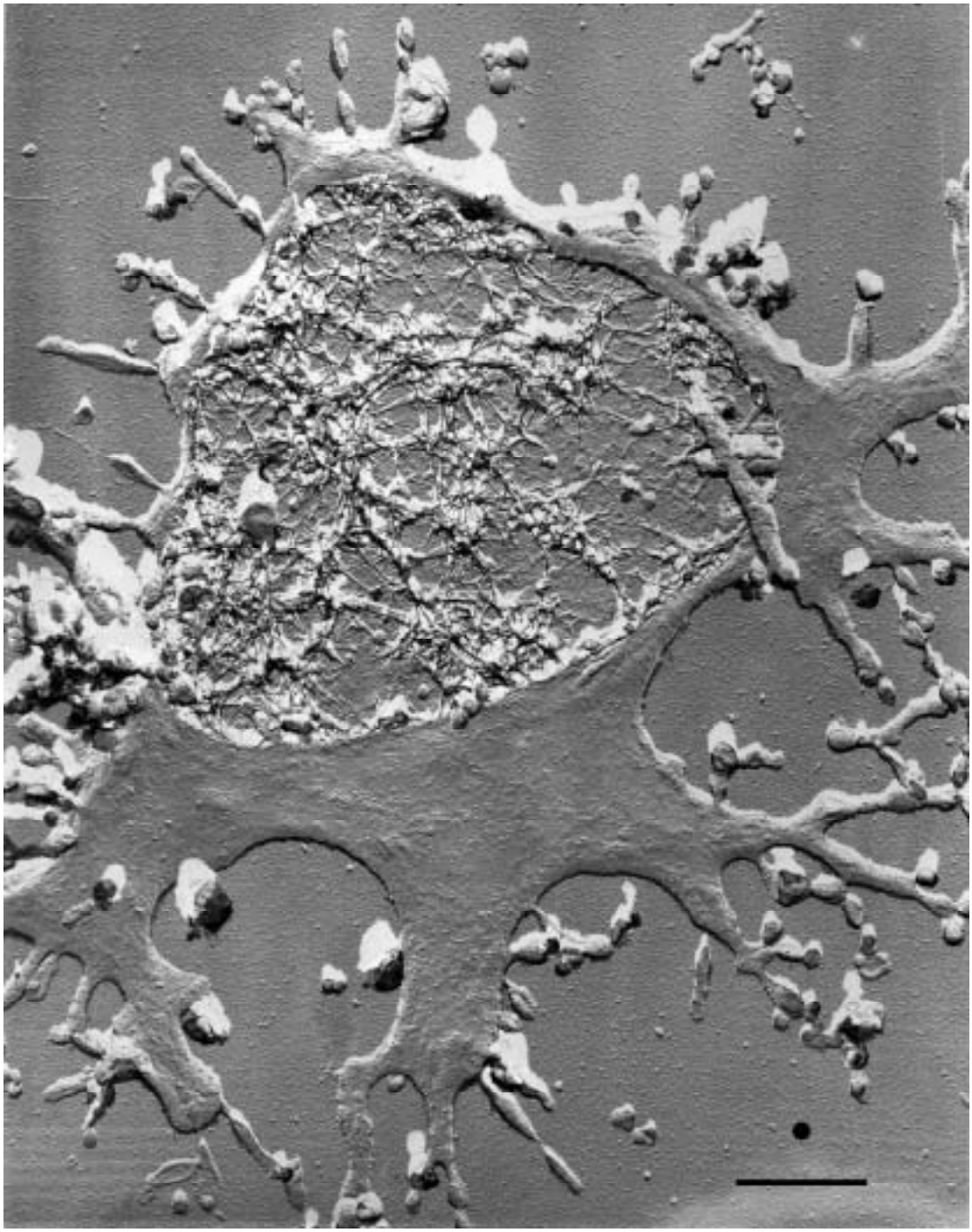

Figure 8: RSV transformed cells revealing the cytoplasmic side of PM. Rat-1 fibroblasts transformed by temperature sensitive LA-29 RSV mutant, cultured under permissive temperature (35ºC). Cells were cultured on glass cover-slips and squirted with Pipes buffer using a syringe to remove the bulk of the cell contents, and expose the cytoplasmic surface of the adherent PM. Freeze-dried and shadowed replicas of cells were observed by electron microscope. Black spot represents size of a retroviral particle (diameter 120 nm). Bar represents 500 nm. (Courtesy of M. V. Nermut)

A more detailed understanding of the actin network of the PM can be derived from erythrocytes, whose cytoskeleton has been thoroughly studied. The systematic arrangement of proteins in the erythrocyte cortex provides a simplified model for the actin-based cytoskeletal network that supports the PM in all other animal cells. The PM is supported by a two-dimensional network of spectrin tetramers that are connected at their ends by very short actin filaments. Spectrin is linked to the cytoplasmic tail of an abundant transmembrane carrier protein by means of ankyrin bridges (Figure 9; folded in picture). The actin filaments are very short, 37 nm rods, acting only as cross-linking elements between spectrin tetramers (Byers and Branton, 1985). An actin oligomer of the size of the rod would contain 13 actin monomers when arranged as F-actin. There are about 250 actin oligomers/μm^2 in intact erythrocyte membranes (Byers and Branton, 1985). The surface area of a Gag particle is approximately 0.05 μm^2, which indicates that a budding Gag particle encounters on average 150 actin molecules during the process of envelopment (Figure 9). When this number of actin molecules is compared to the approximately 20 actin molecules that we detected in the Gag particles, it agrees with our finding that actin is excluded from the particles.

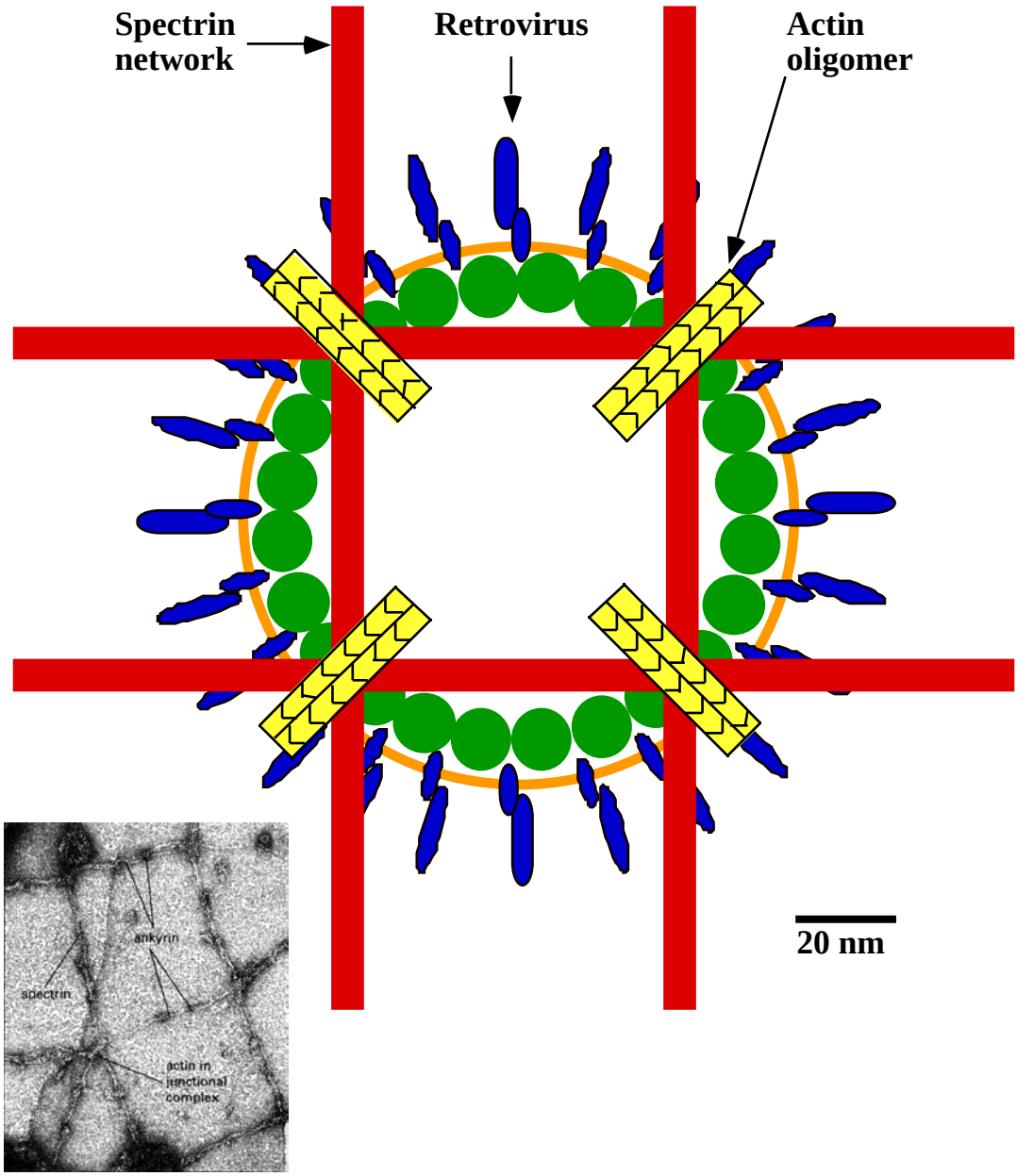

Figure 9: **Schematic diagram of membrane skeleton.** A network is formed by long spectrin tetramers joined at their ends by junctional complexes, consisting of actin oligomers organised in short rods (37x9 nm). Each oligomer contains 13 actin molecules when arranged as F-actin. For comparison, a retrovirus is shown in the background. Diagram is approximately to scale. Folded in, is an EM picture of the stretched out network of an erythrocyte, indicating spectrin filaments, ankyrin bridges and actin rods. Adopted from Byers and Branton (1985).

5.2.2 Function of actin

How come actin is found in Gag particles? Is it just fortuitously incorporated in the particles because of its location at the membrane, or is it a remnant of a cytoskeleton mediated event involved in assembly and budding, i.e. Gag transport or scissioning and release? The answers to these questions are unknown and I can merely speculate.

Actin has been suggested to participate in cytoskeleton-mediated transport of Gag to the PM, but there is no direct evidence of this. It cannot be excluded that Gag involved in C-type assembly might very well arrive at the PM through free diffusion. On the other hand, D-type assembly, including transport of pre-assembled M-PMV to the PM, requires ATP (Weldon *et al.*, 1998) suggesting that, in this case, transport is an active process. Another indication of the difference in D- and C-type transport of Gag to the PM, has been found using High Five insect cells, in which assembled D-type particles are trapped intracellularily and are not transported to the PM, while C-type PM association occurs normally at the PM (Parker and Hunter, 2000). Most probably transport of the large, internally pre-assembled particles requires active transport to the PM, while individual Gag molecules, or small Gag oligomers, could diffuse freely.

Interestingly, C-type budding in High Five cells is blocked, not during Gag transport, but at the stage of virus release, which can be seen by EM as particles that remain attached to the PM. This indicates a common factor, which could be the actin cytoskeleton, that participates in both transport of D-type particles to the PM, as well as in release of C-type particles. However, this factor is not involved in PM transport of C-type Gag.

5.2.2.1 Release of Gag particles

How could the cytoskeleton be involved in release of Gag particles?

Parallels have been drawn between models of virus particle assembly and the formation of cellular clathrin-coated vesicles during endocytosis. Endocytosis, however, occurs through “inverse budding”. By curving the PM inwards instead of outwards, as for the exiting virus particles, the enveloped, clathrin-coated vesicle is formed. The cytoskeleton has been suggested to participate in this process.

Apodaca has set forward a model for the formation of clathrin coated pits, in which actin forms a contractile ring in conjunction with myosin

motors (Apodaca, 2001). The contractile ring would assist in the formation of the vesicle by ensnaring the membrane at the stalk forming stage. The model is based on results from uninfected cells that are unable to release coated pits when treated with cytochalasin D, which disturbs the equilibrium between monomeric and polymeric actin (Gottlieb *et al.*, 1993). As seen by EM, the emerging coated vesicles remain connected to the membrane by long necks, and are unable to pinch off. An interesting connection to viral budding is the observation by Sasaki *et al.* that wortmannin, which is an inhibitor of myosin light chain kinase, inhibits the stalk formation necessary for release of HIV particles from cells (Sasaki *et al.*, 1995). The authors conclude that it is the myosin-actin interaction that is important in stalk formation and budding. This indicates that the model suggested for the formation of clathrin-coated pits could apply to retrovirus budding. It has been demonstrated that the Gag protein of several retroviruses interacts with KIF-4, which is a cellular motor protein active along the microtubules (Kim *et al.*, 1998). Thus, we suggest that actin might participate in a process together with myosin, or other motor proteins, in the final stages of budding, by forming the contractile ring, leading to stalk formation and final release of viral particles.

5.2.2.2 Late assembly domain of Gag and interaction with host-proteins

A mediator for binding actin to the Gag protein might be the late assembly domain (L), which is responsible for pinching off and release of Gag particles. A phenotype typical for the absence of the L domain is arrested budding particles connected with a long stalk to the PM (Göttlinger *et al.*, 1991). The function of the L domain is dependent on a proline rich sequence, which in MLV is present in two copies (Pager *et al.*, 1994). The sequence consists of a rigid stretch of aa residues, namely PPPY, which is conserved among RSV, MLV and M-PMV (Wills *et al.*, 1994; Yasuda and Hunter, 1998; Yuan *et al.*, 1999). The L domains of HIV and EIAV, utilises PTAPP and YXXL, respectively (Parent *et al.*, 1995; Puffer *et al.*, 1997). The proline rich sequence is also typical of actin binding proteins like profilin, and is recognised by so-called WW motifs (Chen and Sudol, 1995; Holt and Koffer, 2000).

The WW domain is a protein-protein interacting module composed of 35-40 aa residues with two conserved tryptophans (W) spaced 20-22 aa residues apart. The WW domain binds proline-rich or proline-containing ligands, and based on ligand specificity, the WW domains have been divided

into five subgroups (Bork, 2001). The WW subgroup I, which recognises the proline-rich sequence in the retroviral L domain, includes actin-binding proteins such as IQGA and dystrophin/utrophin. IQGA cross-links actin filaments (Bashour *et al.*, 1997; Erickson *et al.*, 1997; Fukata *et al.*, 1997) and dystrophin/utrophin is a transmembrane multi-subunit complex, which anchors actin filaments to the PM and creates a link between the cytoskeleton and the extracellular matrix (Winder, 1996). If the late assembly domain of Gag is recognised by WW domains of actin binding proteins, they could constitute the link between Gag and actin, and might therefore be responsible for conveying actin into the Gag particles.

Another protein that carries the WW motif I is the ubiquitin-ligase NEDD4, which is involved in targeting proteins destined for degradation (Staub *et al.*, 1997). Targeting is performed by the addition of a poly-ubiquitin tail to the selected protein, which is consequently degraded in the proteasome, while ubiquitin is recycled. Yet another ubiquitin-ligase function, which is more interesting in this respect, is the addition of mono-ubiquitin, which selects the protein for endocytosis. Ubiquitin is found throughout the cell and recycles between the proteasome and the PM inner surface. Free ubiquitin can be depleted from cells by treatment with proteasome inhibitors, preventing recycling of ubiquitin, which then accumulates in the proteasome. Experiments have shown that in the presence of these inhibitors, Gag budding is arrested at the stalk forming stage prior to release (Patnaik *et al.*, 2000). When overexpressing ubiquitin in the presence of inhibitor, budding is resumed, indicating that ubiquitin participates in late steps of virus budding.

Free ubiquitin, which is a small molecule of 76 aa residues, has been found in retroviruses at levels that are four times higher than in the surrounding cytoplasm. A small percentage (2-5%) of Gag proteins have also been found to be mono-ubiquitinated. Ubiquitin is added to lysines that are present in the close vicinity of the PPPY sequence of the L domain, which has been shown to be critical for ubiquitination of the Gag protein (Strack *et al.*, 2000). The mechanism of action of ubiquitin is unclear, but it has been suggested that ubiquitin could mediate membrane scissioning in budding by attracting the membrane remodelling machinery utilised during endocytosis (Vogt, 1998). Although the topology of endocytosis and virus budding differ, they both involve a membrane fusion event, which might be catalysed by

transmembrane elements independent of the direction of the forming vesicle. Evidence to support the relationship between the endocytosis machinery and viral budding, comes from the late assembly domain in EIAV, which has been shown to bind the AP-2 adapter protein involved in formation of clathrin-coated pits (Puffer *et al.*, 1998). AP-2 is located in the PM where it functions as a clathrin-associated adapter protein complex, which is believed to recruit other cellular proteins to the site of assembly to facilitate the membrane fission event.

It seems as if the final stage of retrovirus budding could engage a complex, cellular machinery that mediates contraction and closure (i.e. actin-myosin) of the budding vesicle as well as membrane fusion and release (e.g. ubiquitin) of the particle.

5.3 ENVELOPMENT OF RETROVIRUSES

Taken together, membrane envelopment of retroviruses involves a whole range of proteins in addition to the Gag protein itself. Although the Gag protein can perform budding without the other viral proteins, budding is such a complex process that it cannot be viewed upon as individual proteins performing simple interactions. Now that the process has been dissected and scrutinised by scientists for several years, we should not forget to pick up the pieces and analyse the outcome of the whole picture.

Most probably, the budding process is a matter of local protein concentrations and cascade reactions. Onset of budding could involve an initiating interaction, which energetically would favour the following interactions. Lowering the threshold for certain interactions, when the whole armada of proteins is present, could ensure that a correctly assembled and functional particle is the end product. For example, both PM and RNA could function as scaffolding for Gag polymerisation (Sakalian *et al.*, 1994). When one Gag protein has bound, it could nucleate the binding of additional Gag proteins. In the same way, a weak Gag-Env interaction, which would not hold to carry a Gag protein along the outside of transport vesicles to the PM, could grow stronger by the network formation occurring at the PM. According to the structural model presented by Forster *et al.* (2000), the tail of the Env trimer would extend into the centre of the hexameric Gag rings. Each Env

protein would then bind two Gag proteins, in an interaction that would be stabilised by the formation of the network itself. Thus, the trimeric Env spike could aid formation of the hexameric Gag ring. This might also be the reason why, in the presence of Env, particle budding is redirected to the basolateral membrane in polarised cells. The proper Env-Gag assembly, which would be energetically more favourable, would then recruit and consume all available Gag protein so that Gag budding would not have a chance to occur elsewhere.

In comparison, the budding chains of virus particles of L-domain mutants, demonstrate budding from one specific site. The phenotype of the PPPY deletion mutant of MLV reveals strings of Gag particles that are connected to one another with stalks of membrane, both horizontally and vertically (Yuan *et al.*, 2000). Particles fail to release from the membrane, and the next particle starts budding immediately underneath the former, thus creating a network of membrane-connected particles. This indicates that once budding has started, the same site is used for continued budding. Hence, once the nucleation process for assembly has been overcome, and all necessary viral and host factors have been recruited, it is simple for additional particle assembly and budding to follow the ready made track.

The number of host proteins encountered in retroviral particles, not only indicates the spacious structure required to accommodate them, but also tells the tale of the complex budding machinery provided by the cell. It has not been possible to depict specific roles for the host proteins inside the particles, but in accordance with our results, I believe that they are mostly fortuitous remnants of proteins taking part in the assembly process, as well as the occasional misfortunate protein, which simply has not been excluded.

5.4 VACCINIA VIRUS MEMBRANE MORPHOGENESIS

Turning to vaccinia virus, we are faced with other matters regarding envelopment such as the formation of the viral double membranes. First, we shall consider the basic understanding of the protein interactions involved in membrane morphogenesis.

5.4.1 p21 in internal mature virus

p21 has been determined to be necessary for correct assembly of the rigid membrane crescents that later form the virus particles (Rodríguez *et al.*, 1995; Rodríguez *et al.*, 1997; Wolffe *et al.*, 1996). Since p21 is synthesised and retained in the cellular membranes that are later used for virus assembly, and ends up being one of the predominant proteins of the IMV double membrane, it is not surprising that it holds an essential role in membrane morphogenesis. In order to speculate about the function of p21, it is fundamental to know the topology and localisation of the protein.

5.4.1.1 Localisation

We were able to show that p21 is exposed on the surface of IMV, and thus, is located on the outer of the IMV double membrane (paper III). A previous study had determined p21 to localise only to the inner membrane of IMV particles (Krijnse-Locker *et al*., 1996), but since p21 had been shown to interact with the surface protein p14, it was difficult to see how this would be in accordance with a purely internal localisation of p21. Our results conclude that p21 indeed is positioned on the outer membrane as well, where it is able to anchor p14, as has been suggested (Rodríguez *et al*., 1996; Rodríguez *et al*., 1993). Taking previous results into account, p21 is most probably located in both layers of the double membrane (Figure 10).

The results from Krijnse-Locker *et al.* (1996), which had positioned p21 only on the inner membrane of IMV, were based on immunogold labelling of infected cells analysed by EM. These results showed that p21 preferentially labelled the concave side of membrane crescents and the inner membrane of IV. The protein was not detected in IMV particles, which was explained by the masking of epitopes due to protein interactions. However, the antibodies used in the study by Krijnse-Locker *et al.*, recognised the end terminal domains of p21, which now are known to be cleaved off in the mature

particle. In our studies, we had access to antibodies that recognise both the cleaved off N-terminus as well as other antibodies recognising the internal parts of the protein, which are preserved in the processed form of p21.

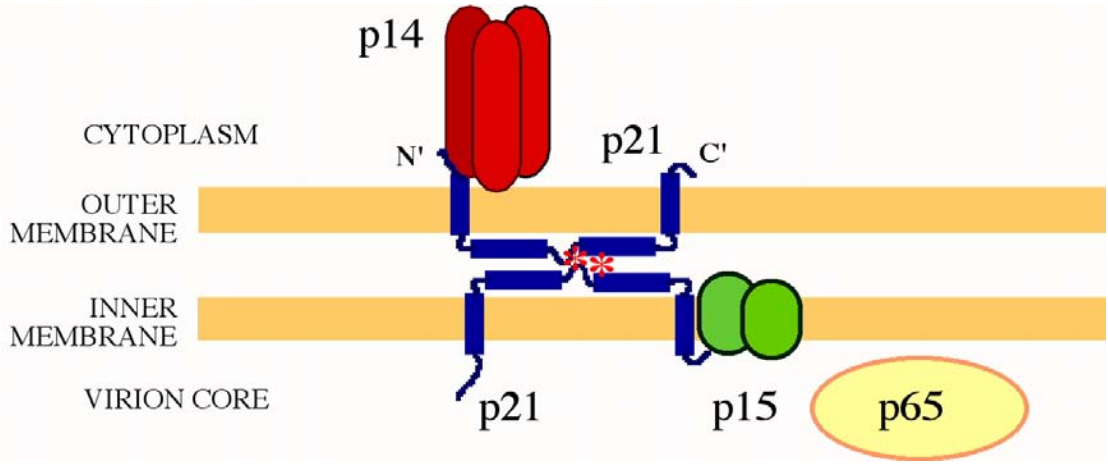

Figure 10: Structural proteins of Vaccinia virus IMV double membrane. p21 is located in both the inner and outer membrane of IMV, where it could dimerise and merge the two membranes. p21 anchors the homotrimer, p14, to the PM and interacts with the p15 homodimer. The core protein p65 acts as a scaffolding protein. Red stars indicate possible disulphide bonds.

5.4.1.2 Topology

p21 has previously been shown to adapt a topology in which both its end-termini face the cytoplasm (Krijnse-Locker *et al*., 1996; Wolffe *et al.*, 1996). Our results from protease digestions reveal no sub-populations of p21 that would be resistant to treatment (paper III). Hence, we conclude that the topology adapted by p21 is the same in both layers of the double membrane, but sitting inverse to each other, so that the terminal ends face the cytoplasm in both membrane units of the crescent (Figure 10). Susceptibility to protease treatment of p21 located in the inner membrane of IMV, may be explained by the sensitivity of the membrane to protein modifying agents, which renders the envelope leaky, since it is thought not to consist of continuous lipid membrane.

The fact that both the N- and C-terminal ends face the same side of the membrane (Krijnse-Locker *et al*., 1996) indicates an even number of membrane spanning segments, which coincides with a topology with either two or four membrane spanning regions. Such a topology would leave a

shorter or longer inner segment to reside in what was the lumen of the membrane-donating compartment.

p21 is known to form a disulphide-linked homodimer. There is evidence for disulphide bond formation involving cysteine residues positioned at aa 101 and 121 (Betakova and Moss, 2000). The four transmembrane topology suggested for p21 would leave one of the potential disulphide donating cysteines inside the membrane (Figure 4), whereas the two-transmembrane model would reconcile a homodimer formation involving these residues. Our results from protease treated, intact IMV demonstrated that the interior domain of p21 (aa 99-117) connecting the TM domains three and four, is resistant to protease digestion. These results support the two-transmembrane topology, in which the interior loop of p21 would be protected inside the double membrane. Considering that p21 is situated in both membranes of the double envelope of IMV, disulphide bonds could form between p21 proteins situated in each of the two lipid bilayers. Hence, the p21 homodimer could form by utilising the two above mentioned cysteins (Figure 10).

5.4.1.3 Function

The double membrane that surrounds the IMV appears to be recruited from the ERGIC compartment, which is modified by the insertion of viral membrane proteins to form two tightly apposed membranes. The fact that p21 is located in both the inner and the outer membrane of IMV, and the fact that it is known to form disulphide linked homodimers, gives it the potential to form the connecting link that attracts the two membranes. A connecting bridge between one p21 protein located in the outer membrane and a p21 located in the inner membrane, could account for the merging force between the membranes. This hypothesis is supported by the observation that, in the absence of p21, the ERGIC membranes, believed to be the precursors of the IMV envelope, accumulate around the virosomes as elements that are approximately twice as wide as the viral crescents (30 nm) (Rodríguez *et al.*, 1997). This indicates that the flattening step required for the formation of the crescents has not occurred under these conditions. Additionally, it has been shown that disruption of disulphide bonds with reducing agents, gives rise to floppy membranes of IMV (Roos *et al.*, 1996), which would further support

the idea that the membranes are held tightly together by protein-protein interactions.

5.4.2 Envelopment of vaccinia virus

A model for formation of the double membrane of IMV involves, in the first step, viral modification of the ERGIC precursor membranes. Although not proven yet, it has been hypothesised that phosphorylation of a key substrate may initiate the extension of precursor membranes into crescents. This was suggested based on the phenotype of a temperature sensitive VV mutant defective in the viral F10 kinase. In cells infected with this mutant, morphogenesis was interrupted at a very early stage, before the formation of viral crescents (Traktman *et al.*, 1995; Wang and Shuman, 1995). Interestingly, this kinase is responsible for phosphorylation of both p21 and p15 envelope proteins. Many events in the cell are modulated by the phosphorylation state of key components, e.g. signal transduction and cell cycle progression. In this context it is intriguing to speculate that phosphorylation of the membrane proteins could be a manner of controlling the onset of membrane transformation.

Next, the precursor membranes of the ERGIC compartment need to be transformed into the tightly apposed double membrane that enwraps the IV and IMV. Interactions between viral proteins, in both leaflets of the membrane, could cause the compartment to collapse and compress its membranes into forming the tightly apposed double membrane. p21 could be a protein responsible for this process since it is located in both membranes, and is known to form a disulphide-linked homodimer. If enough p21 proteins interact throughout the membrane, they might be responsible for the compactness and rigidity that characterises the crescents. Since p15 also appears to be located in the inner membrane, and has been shown to interact with p21, p15 could be a potential binding partner for p21 in attracting the membranes. However, the crescent-like structures that assemble in the absence of p15 look rather fussy, but they do exhibit a normal thickness and general organisation (Rodríguez *et al.*, 1998; Traktman *et al.*, 2000), indicating that p15 does not participate in joining the double membrane. This leaves p21 to constitute the main attracting force for connecting the membranes. In the absence of p15 though, the membranes are frequently open or bent at their

edges. However, the characteristic phenotype in the absence of p15 is crescents that do not adhere to the virosome, rather indicating a function for p15 in attaching the membrane to the material of the virosome, possibly by binding to proteins on the viral core.

After the membranes are tightly connected, the 65 kD protein could participate in curving the membranes. Previous studies by EM have shown that the irregularly shaped membranes, formed in presence of the drug rifampicin, are tightly imposed, and within minutes after the drug was washed out, the arrested membranes converted into virus crescents by first acquiring the protein spikes on the outer surface, and then developing the curvature (Grimley *et al*., 1970). Since p65 is the target of rifampicin, these results suggest that the double membranes are already tightly connected to each other when p65 enters the scene. p65 could participate in curving the membrane by interacting with membrane proteins of the crescents.

5.4.2.1 Membrane enwrapment

The process in which the crescents enwrap the dense material of the virosomes, and form the spherical IV, is not understood. However, wrapping, as opposed to budding, is emerging as a second pathway of virus envelopment, since enwrapment by a membrane cisternae appears to be a general mechanism for envelopment of large DNA viruses, i.e. VV, herpes virus and African swine fever virus. The latter virus, in one step, acquires a double membrane from ER, or possibly ERGIC, concomitantly with viral core assembly (Andres *et al.*, 1997; Cobbold *et al.*, 2000).

It is not known whether the enwrapment process leads to the double membranes fusing with one another to form continuous membrane envelopes, or if they incompletely surround the particles and are held together by protein interactions. However, Golgi enwrapment of IMV during the formation of IEV, probably requires a fusion process to join the new double membrane and make them form two continuous layers of membrane. Theoretically, the membranes will have to form a continuous layer if the outermost Golgi derived membrane is to fuse with the PM in a conventional manner when externalising EEV.

Infection by IMV, which also at some stage will require des-envelopment, hints to be a very intriguing issue if the case proves true, that the double membranes of IMV do not form a continuous envelope, and hence

will not enable the virus envelope to fuse with the cellular membrane in a conventional manner. The envelopment process of VV holds many unanswered questions, which will be enticing to elucidate.

5.4.3 Involvement of the cytoskeleton

In order to proceed, the multi-step envelopment process of VV requires transport of internal virus particles between the different envelopment stations. The cytoskeleton has been determined to be involved in transporting the internal virus particles intra-cellularily (Cudmore *et al.*, 1995). Recently it was shown that the IMV surface protein p14 (A27L) is required for microtubuli mediated transport of IMV to Golgi (Sanderson *et al.*, 2000), but if p14 interacts directly with the cytoskeleton, is not known. p14 is also essential for Golgi enwrapment of IMV for IEV to form (Rodríguez and Smith, 1990b). IEV has the capacity to promote polymerisation of actin tails, which transport the IEV to the PM. When IEV contacts the PM, the outer membrane of IEV fuses and thus externalises the particle. The particles are then propelled outwards on long microvillar-like projections, protruding from the cell surface, and facilitate virus spread into neighbouring cells. Actin polymerisation is induced by viral proteins that recruit host proteins that are normally associated with the actin cytoskeleton (Goldberg and Theriot, 1995; Kocks *et al.*, 1995; Smith *et al.*, 1995). For example, VV contains a virally encoded profilin, which nucleates actin, and could take part in forming the actin fibres (Blasco *et al.*, 1991).

Little is known about the role of the cytoskeleton during VV infection, although it appears to play a crucial role in viral transport. The involvement of the cytoskeleton in the envelopment process has not been studied. However, the intra-cellular compartments are not renowned for being covered by an actin based network reminiscent of the PM, which is why wrapping by internal membranes probably is dependent on viral protein, and, possibly, other cellular proteins.

5.4.4 Host proteins included in vaccinia virus particles

5.4.4.1 Internal mature virus

The fate of host cell membrane proteins during VV morphogenesis has not been elucidated. Unpublished results from Risco *et al.* demonstrate that, above all, the outer membrane of IMV is heavily modified by protein. So far, the main membrane proteins encoded by VV have been reported to be the p21, p15 and p8 (A13L) proteins, of which the function of the latter is still unknown. Whether their internal protein interactions are extensive enough to exclude cellular membrane proteins is impossible to deduce without the corresponding crystal structures, and more knowledge has been gained regarding the underlying virion core.

Analysis of host protein incorporation in IMV include immunogold labelling of IMV particles analysed by EM, which turned out to be negative for the examined cellular ERGIC proteins (Sodeik *et al.*, 1993). The structure of these marker proteins has not been determined. It is too early to draw conclusions regarding host protein sorting during VV envelopment, however, I can speculate.

From a theoretical point of view, the tightly apposed IMV double membrane would leave little room for intervening proteins, especially if they carry a large, lumenal domain that could not fit between the tightly apposed membranes. This model favours an idea of host protein exclusion from IMVs. However, the membrane area required for IMV envelopment is ten times larger than that surrounding a retrovirus particle, indicating the difficulty of clearing the membranes from host proteins. A contributing factor to host protein clearance, though, could be VV induced shut-off of host protein synthesis, although that would not affect the presence, in ER or Golgi, of long-lived circulating cellular proteins that mediate vesicular transport. In the end, IMV might exclude host proteins that sterically hinder the formation of the tightly apposed double membrane, although random incorporation of host proteins with short lumenal tails could take place if the protein lattice of the underlying and surrounding viral proteins allow it.

In any case, a cellular protein that has been found in purified IMV preparations is a lipid-modified ubiquitin (Webb *et al*., 1999). The protein was detected by immunoblotting of the detergent phase following TX114 extraction of IMV. It cannot be excluded that ubiquitin could be a remnant of

cellular vesicle co-purification with IMV particles, not the least because ubiquitin was found to be present in all viruses tested (herpes simplex virus, baculovirus and African swine fever virus) (Webb *et al.*, 1999). However, the authors postulated the lipid-anchored ubiquitin to function in a novel mechanism for membrane attachment of proteins. If this would be the case, it could be a way for the anchor-less scaffolding protein p65 to attach to the viral membranes.

5.4.4.2 Extracellular enveloped virus

Cellular proteins derived from the wrapping membrane of Golgi were found in EEV, but not in IMV (Vanderplasschen *et al.*, 1998). The additional Golgi derived, two membranes of EEV, are clearly distinguishable by EM, indicating that they are not as tightly apposed as the IMV double membrane. This observation indicates that there is more lumenal space in the outer double membrane of EEV. Therefore, host proteins that exhibit a broader range of three-dimensional structures might be randomly included in the viral envelope. Therefore, it would not be surprising to find that EEV demonstrate the same ability of random host protein incorporation, as do retroviruses, while the structurally constrained IMV would adapt a more restrictive attitude.

The proteins examined in the above study were MHC class I, CD71 (transferrin receptor), CD46, CD55, CD59, and CD81 (Vanderplasschen *et al.*, 1998). The first three are trans-membrane proteins type I with short cytoplasmic tails, and therefore exhibit a non-offending secondary structure as regards to their incorporation into a virally restricted protein lattice. CD55 and CD59 are GPI-anchored surface proteins, while CD81 is a four-transmembrane signal transducer, albeit a small protein of 26 kDa. Thus, none of the examined proteins demonstrate a structure that necessarily would limit their incorporation into a loosely joined double membrane.

The host proteins in EEV were suggested to contribute to EEV resistance to the human complement immune system. Another possibility could be that they increase virus binding to cells, and thus enhance viral infection, as proposed for retroviruses. It could very well be that several types of virus include random host protein incorporation as a way to increase infectivity, but in order to draw such conclusions, other viruses remain to be thoroughly investigated.

6 CONCLUDING REMARKS

Our results concerning virus and host protein interactions aid in explaining the mechanisms of retrovirus envelope formation. Considering host protein incorporation to be a random process during retrovirus budding, opens up a new point of view regarding their function in viral infection. This means that the cellular functions of the included host proteins are adopted by the viral particle and could facilitate binding to, and infection of new target cells. In fact, random host protein incorporation might be adopted by many types of virus, including vaccinia, to enhance infection, as long as there is spatial allowance.

Additionally, our results have consequences for using retrovirus as vectors in gene therapy. A mechanism of random membrane-protein incorporation opens up future possibilities of incorporating heterologous proteins in order to generate specific cell targeting viral vectors. However, it also raises a point of concern that the foreign proteins in the vectors might provoke immunological complications when using them in therapy. Although morphogenesis of vaccinia virus appears to be completely different from that of retrovirus, the same concerns of host protein incorporation might apply particularly when considering the use of vaccinia in vaccine designs.

7 SUMMARY IN SWEDISH

Avhandlingen handlar om hur viruspartiklar bildas, och hur de interagerar med sin värdcell medan de sätts ihop. Jag har studerat retrovirus och poxvirus som sinsemellan har väldigt olika struktur och livscykel.

Det mest kända retroviruset är HIV (humant immunbristvirus) som orsakar AIDS. För att förstå hur virussammansättning går till, har jag fokuserat på det enklare retroviruset MLV (musleukemivirus). På grund av sin enkelhet och relativa harmlöshet, studeras MLV på laboratorier för att användas som bärare av gener för genöverföring hos människor, s.k. genterapi. Poxvirus innefattar vacciniavirus som, i slutet på 1700-talet, blev det första vaccinet någonsin, och tack vare det, kunde smittkoppor utrotas. Vacciniavirus fortsätter att vara intressant än idag för att tillverka vacciner mot andra infektiösa ämnen.

Virus orsakar sjukdom bl.a. genom att introducera virusgener i cellens kromosomer och kan därmed störa gener som kontrollerar celltillväxt, vilket i värsta fall ger upphov till cancer. Andra virus orsakar sjukdom genom att omvandla cellen till en virusfabrik så att cellen inte längre kan utföra sin specifika funktion, eller så dödas den.

Till skillnad från bakterier, som kan föröka sig själva genom delning, är virus icke-levande partiklar eftersom de saknar egna system för att kopiera gener och tillverka proteiner för att bilda nya viruspartiklar. För att virus ska kunna föröka sig är de därför helt beroende av celler som kan utföra dessa uppgifter åt dem. Virus är i princip små kapslar av protein som innesluter virusgener, och har en diameter på i storleksordningen en 10 000-dels mm. Utsidan av en viruspartikel är försedd med s.k. taggproteiner, vilka gör det möjligt för virus att ta sig in i celler, d.v.s. infektera dem. En del virus har ett hölje av fettmolekyler, d.v.s. ett membran svept runt

kapseln och i vilket ”taggarna” sitter som nedstuckna. Membranet har viruset stulit från värdcellen som tillverkade viruset, och både retrovirus och vacciniavirus är membranhöljda.

Hos retrovirus kommer höljet från cellens yttermembran som helt omsluter cellen. MLV sätts ihop vid yttermembranet där proteinkomponenterna samlas och interagerar sinsemellan så att en ny, sfärisk viruspartikel bildas. Samtidigt böjs cellmembranet runt partikeln som till slut blir helt insvept. Membranet snörs av och återförsluts, liksom en såpbubbla som lämnar öglan, och frisätter på så vis en membranhöljd viruspartikel. Ett virus infekterar genom att dess taggar hittar och binder viruset till ytan av en ny cell. Virushöljet tas av genom att membranet smälter samman med den nya värdcellens yttermembran, och därmed släpps den inre kapseln med virusgenerna lös inuti cellen. På så vis kan membranet liknas vid en ytterrock som viruspartikeln tar på sig när den går ut ur cellen, och av sig när den går in i nästa cell.

Bildning av virus kräver deltagande av cellulära proteiner, men man har trott att cellproteiner sorteras ut från viruspartikeln medan den sätts ihop, så att den till slut enbart består av virusproteiner. Jag har emellertid visat att cellulära proteiner slumpvis följer med in i retroviruspartiklar. Detta är förmodligen bra för viruset därför att cellulära ytproteiner kan ge viruset ökade möjligheter att binda till nya celler och därigenom ökar virusets infektionsförmåga. Detta eftersom cellulära ytproteiner ofta är involverade i att binda samman celler. Vidare har jag visat att ett av de cellulära proteinerna som kommer in i retroviruspartiklar är aktin som bildar i aktinfiber och som bland annat ingår i våra muskelfiber. Dessa fiber bildar normalt ett nätverk genom hela cellen och längs insidan av dess yttermembran och fungerar därför som cellens skelett. Aktinfibrerna kan vara involverade i processen att svepa cellens yttermembran runt viruspartikeln, och framför allt under avknoppningen av den nya viruspartikeln från cellen.

Vacciniavirus har till skillnad från retrovirus flera lager av membranhöljen. Under dess livscykel produceras partiklar, som har från två och upp till fyra olika membranhöljen. Höljena fås från cellens interna organeller (”organ”) som också är inneslutna i membran. Jag har visat var ett specifikt membranprotein är placerat i viruset och föreslår på vilket sätt proteinet kan vara viktigt för att forma och hålla ihop de olika membranen i viruspartikeln.

Mina resultat bidrar till att öka förståelsen för hur virus bildas, vilket t.ex. underlättar utvecklandet av mediciner som kan skydda oss mot virusburna sjukdomar. Det gör det också möjligt att använda virus i terapeutiska syften och därmed bota eller förebygga andra åkommor. Till exempel kan ofarliga retrovirus överföra en “frisk” gen till en person med lungsjukdomen cystisk fibros, eller så kan vacciniaviruset användas till att utveckla vaccin mot t.ex. HIV.

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