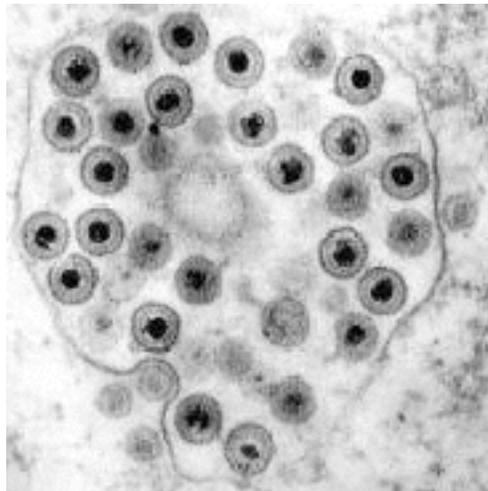


INHIBITION OF APOPTOSIS BY THE U_s3 PROTEIN KINASE OF HERPES SIMPLEX VIRUS 1

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Stockholm
Fall 2004

**Microbiology and Tumor Biology Center
Karolinska Institute
Stockholm, Sweden**

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Front cover: Herpes simplex virus, the causative agent of fever blisters. Thin section of virus particles as they leave the nucleus of an infected cell. Magnification of approximately x40,000. (Micrograph from F. A. Murphy, School of Veterinary Medicine, University of California, Davis).

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To my family and My DEAREST friends:
Victoria, Lisette, Ulrika, Elio, Kristina, and
Anna B

INHIBITION OF APOPTOSIS BY THE U_s3 PROTEIN KINASE OF HERPES SIMPLEX VIRUS 1

Anna Cartier

Doctoral thesis from the Microbiology and Tumor Biology Center
Karolinska Institute, Stockholm, Sweden

The Herpes Simplex Virus 1 (HSV-1) U_s3 protein kinase is part of the anti-apoptotic arsenal that salvage HSV-1-infected cells from damage caused by different stimuli. The U_s3 gene encodes a protein kinase that phosphorylates serine/threonine residues within a specific arginine-rich consensus sequence. U_s3 was shown to block apoptosis induced by the virus infection itself. The aim of this thesis was to study the mechanisms by which U_s3 blocks apoptosis induced by cytolytic lymphocytes and exogenous stimuli.

U_s3 was shown to protect HSV-1-infected cells from lysis by MHC class I-restricted CD8T cells by acting downstream of antigen presentation. Expression of U_s3 was associated with inhibition of CD8T cell-induced activation of caspases-3, -8 and -9 and a significant reduction in the cleavage of the pro-apoptotic protein Bid. U_s3 selectively targeted the processing of Bid since recombinant human granzyme B (GrB) failed to cleave Bid in cytosolic extracts from U_s3 positive cells and recombinant human Bid served as substrate for U_s3 phosphorylation *in vitro*.

The role of U_s3 in infected cells against cytotoxicity mediated by NK and LAK cells was also tested since these cells share common mechanisms in inducing death of virus-infected cells. In contrast to their lower sensitivity to CD8T cell lysis, HSV-1-infected cells were lysed by NK or LAK cells as efficiently as the uninfected controls. Both MHC-restricted and non-restricted cytolytic effects were significantly dependent on the activity of GrB and were efficiently blocked by treatment with a GrB inhibitor. However, in contrast to a significant induction of Bid cleavage mediated by CD8T cells, Bid was not cleaved when targets were co-cultured with LAK or NK cells suggesting that these effectors induce lysis by a mechanism that does not involve cleavage of Bid.

The ability of U_s3 to block apoptosis induced by exogenous stimuli was also studied. We used a set of stable HSV-1 U_s3 transfected cell lines with constitutive and inducible expression of the protein to show that U_s3 alone is sufficient in inhibiting apoptosis induced by virus infection, sorbitol and Fas antibody treatment. U_s3 blocked the activation of caspase-3 and caspase-9 in virus-infected cells. Bad was rapidly phosphorylated in cells expressing high levels of U_s3 following virus infection or induction from a tetracycline-regulated plasmid, and Bad served as substrate for U_s3 phosphorylation *in vitro*.

Taken together our data illustrate that modification of cellular pro-apoptotic proteins by U_s3 presents a new viral strategy to prolong survival of the infected host cells. The ability of NK and LAK cells to overcome the anti-apoptotic function exerted by U_s3 further demonstrates the intricate interplay between the virus and the immune system to control HSV-1 infection.

LIST OF PUBLICATIONS

The work of this thesis is based on the following papers and manuscript, which will be referred to by their Roman numerals in the text.

- I A Cartier, E Broberg, T Komai, M Henriksson and MG Masucci. **(2003)** The herpes simplex virus-1 U_s3 protein kinase blocks CD8T cell lysis by preventing the cleavage of Bid by granzyme B. *Cell Death and Differentiation*. **10**:1320-1328.
- II A Cartier and MG Masucci. Differential regulation of MHC class I restricted and non-restricted cytotoxicity by the U_s3 protein kinase of herpes simplex virus-1. *Submitted*
- III A Cartier*, T Komai* and MG Masucci. **(2003)** The U_s3 protein kinase of herpes simplex virus 1 blocks apoptosis and induces phosphorylation of the Bcl-2 family member Bad. *Experimental Cell Research*. **291**:242-250.

*These authors contributed equally to the work.

ABBREVIATIONS

AIF	apoptosis-inducing factor
Apaf-1	apoptotic protease activating factor-1
Bcl-2	B cell lymphoma-2
BH	Bcl-2 homology
Caspase	cysteiny l aspartate specific protease
CAD	caspase-activatable DNase
CARD	caspase-activating recruitment domain
CI-MPR	cation-independent mannose-6-phosphate receptor
CL	cytolytic lymphocyte
CTL	cytotoxic T cell
Cyt c	cytochrome c
DED	death effector domain
DD	death domain
DIABLO	direct inhibitor of apoptosis binding protein with low isoelectric point
DISC	death-inducing signaling complex
Endo G	endonuclease G
FADD	Fas-associated death domain
Fas L	Fas ligand
GAG	glycosaminoglycan
Gr B	granzyme B
HLA	human leucocyte antigen
HveA	herpes virus entry A
HBV	hepatitis B virus

HSV-1	herpes simplex virus type 1
ICAD	inhibitor of CAD
ICP	infected cell protein
KSHV	kaposi's sarcoma-associated herpesvirus
LAK	lymphokine activated killer cell
LAT	latency-associated transcript
LCL	lymphoblastoid cell line
M6P	mannose-6-phosphate
MHC	major histocompatibility complex
NK	natural killer
PARP	poly(ADP-ribose) polymerase
PFN	perforin
PI-9	proteinase inhibitor-9
PK	protein kinase
PNS	peripheral nervous system
PS	phosphatidylserine
SG	serglycin
Smac	second mitochondrial activator of caspases
TNF	tumor necrosis factor
U_L	unique long
U_S	unique short
U_{S3}	unique short 3
VP	virion protein
XIAP	x-linked IAP

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1. HERPES SIMPLEX VIRUS TYPE I

1.1. GENERAL INTRODUCTION

The herpes simplex virus type-1 (HSV-1) is the prototypic member of the *Alphaherpesviridae* family. HSV-1 is a common human pathogen responsible for causing orofacial infections (cold sores) and encephalitis [1]. It is thought that the virus transmits from person to person as cell-free virus. HSV-1 gains access to the living cells through the abrasions in the skin surface. During the productive phase of infection, vesicular lesions in the mucosal epithelia are formed. After the initial primary infection, the virus travels along the innervating neuronal axon in a retrograde manner to the neuronal cell body.

Once within the neuron, the virus enters the lytic cycle and can be transmitted across synapses. A further consequence of ganglionic infection is the establishment of lifelong latency where the virus enters a quiescent state in which the lytic genes are not expressed. During latency, the HSV-1 genome remains in the neuron as a circular, extra-chromosomal DNA [2, 3]. Latency-associated transcripts or LATs are the only abundant viral RNAs detected in the neurons. Proper signals such as physical stress, illness or exposure to ultraviolet light trigger the reactivation of the virus from latency and new progeny is produced. The viral capsid then moves down the neuronal axon via anterograde transport to the site of primary infection. Enveloped virus is then assembled for transmission to epithelial cells where it re-initiates a lytic life cycle and results in recurrent disease at or adjacent to the site of primary infection [4].

1.2. VIRION STRUCTURE

HSV-1 is a large enveloped virus of approximately 200 nm in diameter [5]. The virion is composed of four parts, which have the distinct structural characteristics of the herpesviruses (**Fig. 1A**) [6]. HSV-1 has an electron dense core containing the viral DNA tightly packaged in a toroid structure [7]. The core is surrounded by an icosahedral capsid shell and contains 162 capsomers. An amorphous layer containing additional viral proteins called tegument lies between the capsid and the envelope. The outer membrane envelope is a trilaminar lipid bilayer studded

with glycoprotein spikes, some of which are essential for viral entry into cells, and spread between cells [6, 7].

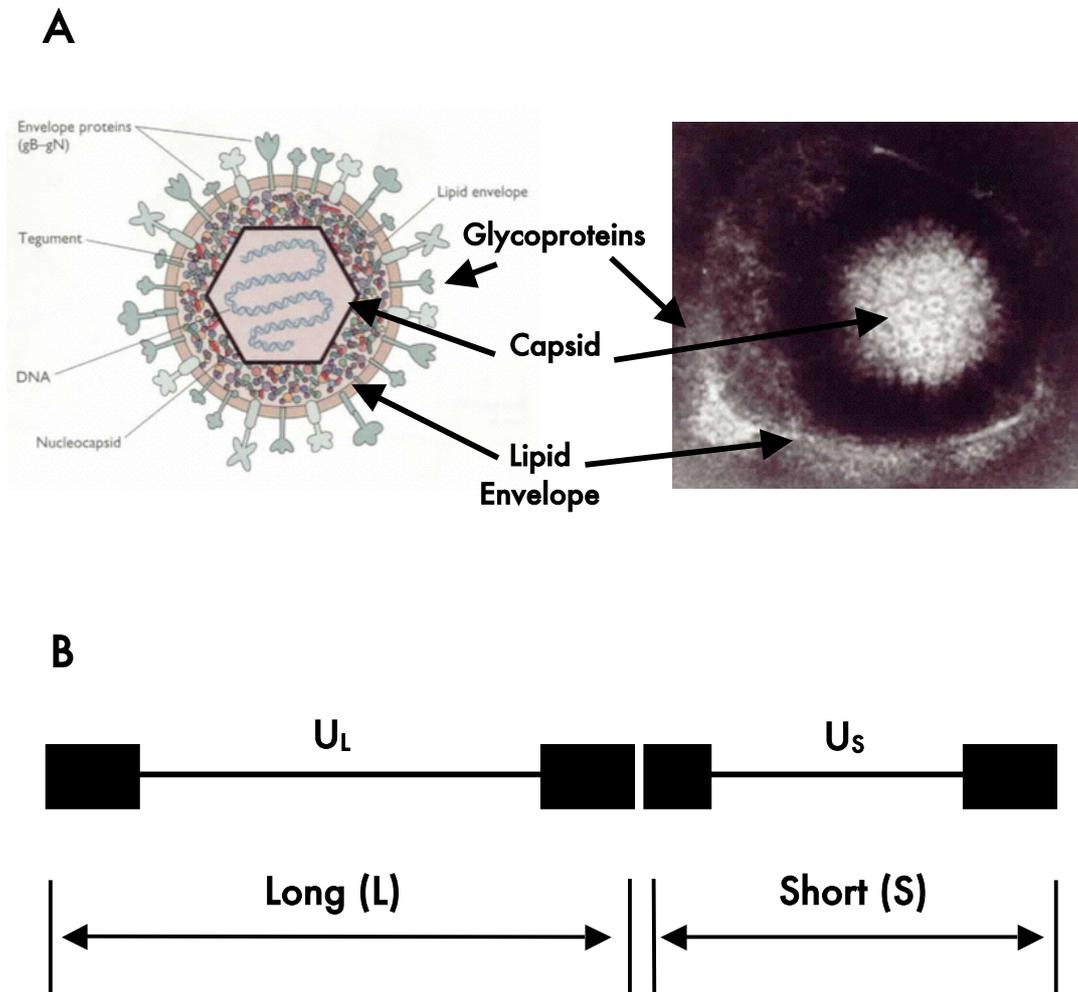


Figure 1. HSV-1 virion structure and genome organization. (A) The HSV-1 virion is composed of four major features: 1) the viral double-stranded DNA; 2) an icosahedral capsid shell; 3) a tegument, and 4) a lipid envelope studded with glycoproteins. An EM photograph (right) of the major structural components of the virion. **(B)** The HSV-1 genome is organized into two regions, long (L) and short (S). Each component contains a unique region (U) flanked by inverted repeat regions. ■ = Inverted Repeat.

1.3. GENOME ORGANIZATION

The HSV-1 contains a large, linear double-stranded DNA consisting of approximately 150,000 base pairs encoding at least 84 different polypeptides [8]. The genome is composed of two covalently linked segments, called Long (L) and

Short (S) based on their relative length. These segments are further divided into unique sequence regions, Unique Long (U_L) and Unique Short (U_S) flanked by inverted repeat sequences (**Fig. 1B**) [9]. In general, viral genes are named based on their position from left to right in the U_L or U_S region such as U_L1 or U_S3. Viral proteins are also named on the basis of their function for example Infected Cell Protein (ICP4) number or Virion Protein (VP16) number. Genes in the unique region are present in the genome as a single copy and genes that are coded by the repeat regions are present in two copies [9]. Each gene has its own promoter to direct transcription.

Three origins of replication are present within the HSV genome. These are named *oriL* and *oriS* after their location in the U_L or U_S region, respectively. *OriL* is present as a single copy in the U_L segment but the *oriS* is present in the repeat region of the U_S, and therefore found in two copies [10-13]. Both origins of replication are palindromic sequences and consist of AT-rich center region flanked by the inverted repeats. Either *oriL* or *oriS* is sufficient for viral replication [14, 15].

1.4. VIRUS ENTRY

Entry of the virus into the cells is mediated by a number of viral envelope glycoproteins and occurs in a step-wise manner. These glycoproteins include glycoprotein B (gB), gC, gD and the gH/gL heterodimer. The first step in the entry of HSV-1 is initiated by virus attachment to the host cell via an interaction between the virion gC and heparan sulfate moieties on the host cell [16]. This interaction is not an absolute requirement for virus entry since cells that lack heparan sulfate remain permissive to the virus [17], and gC is dispensable for viral growth and replication *in vitro* [16]. In the second step, virion attachment is further stabilized by the interaction of the gD protein with a cellular receptor referred to as Herpes virus entry A (HveA) [18]. The viral envelope then fuses with the host cell membrane in a mechanism that requires gD [19], gB [20], and the gH/gL heterodimer [21]. Following fusion, the viral capsid and tegument proteins are released in the cytoplasm where some viral proteins are transported to the nucleus (virion protein 16; VP16) and some other remain in the cytoplasm (virion host shut off; vhs) [22].

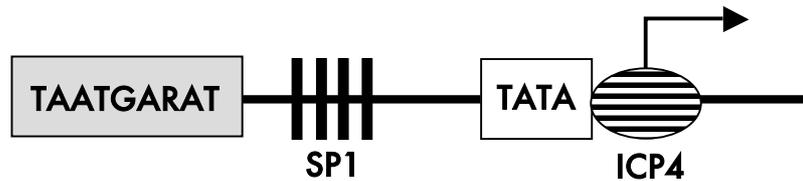
1.5. VIRAL GENE EXPRESSION

DNA viruses such as HSV-1 utilize the host cell machinery to express viral proteins necessary for viral replication and spread. Expression of HSV-1 genes in productively infected cells is regulated tightly in a sequential and coordinated fashion by viral-encoded and cellular nuclear factors [23]. The three groups of virus-specific polypeptides are designated as immediate early (α), early (β) and late (γ). The late genes are further classified into the early/late and leaky late or γ_1 genes and the true late γ_2 genes, respectively.

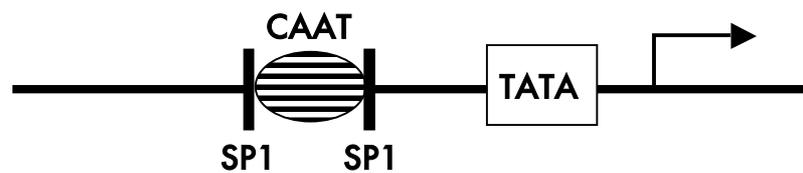
Transcription of viral genes begins once viral DNA enters the nucleus. The immediate early (IE) genes are the first set of genes transcribed in the nucleus. Transcription of IE genes is initiated by the recruitment of the cellular transcriptional machinery to IE gene promoters. Prior HSV protein synthesis is not required for the IE gene expression. The viral protein VP16 plays an important role in enhancing the expression of the IE proteins by forming a multi-protein complex with host cell proteins, Oct-1 and Host Cell Factor (HCF) (**Fig. 2A**) [24]. This complex then initiates transcription by binding to the TAATGARAT elements present in the IE promoter [25]. All viral mRNAs are synthesized by the host RNA polymerase II [26].

IE proteins such as regulatory proteins ICP4 and ICP27 initiate the transcription of the early (E) genes and their expression is essential for further viral gene expression. ICP4 is required for the transcription of all early and late genes [27]. In addition, it negatively regulates its own expression by binding to consensus sites within its promoter [28]. ICP27 is required for the lytic infection of HSV-1. This protein has been implicated in the switch from early to late gene expression during replication, and such functions as inhibition of mRNA splicing, RNA binding, and the transport of intronless viral mRNA out of the nucleus [29, 30]. E genes contain promoters with binding sites for the IE and a variety of cellular transcription factors (**Fig. 2B**). E proteins are involved in viral DNA replication. One viral protein, ICP8, is a ssDNA binding protein with a role in transcriptional regulation and is necessary for the transcription of late genes [31].

A. Immediate-Early Promoters



B. Early Promoters



C. Late Promoters

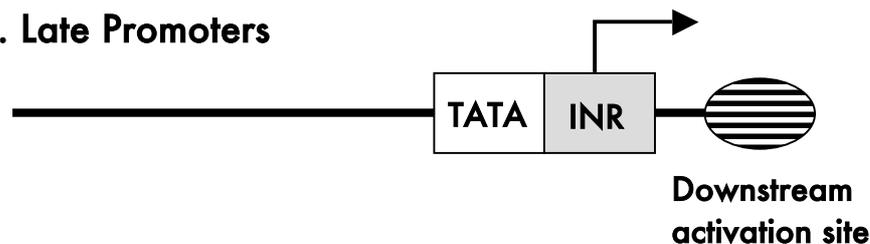


Figure 2. Schematic representation of the immediate-early, early, and late HSV-1 promoters. (A) IE promoters have the TAATGARAT and the TATA elements. This region provides the binding site for the multi-protein complex containing virion protein VP16, and two cell proteins Oct-1 and HCF. The binding of this complex mediates the transcriptional activation. (B) Expression of the early genes of HSV-1 requires a TATA element, two binding sites for the transcription factor SP1, and a CAAT element. (C) Late promoters have an initiator element (INR) in addition to the TATA element. Some late promoters also contain a downstream activation site.

The synthesis of viral DNA serves as a signal for a switch in the HSV gene expression. Transcription of late (L) genes begins to increase concurrently with DNA replication, while expression of early genes begins to wane. IE proteins can initiate transcription of γ_1 genes prior to DNA replication. Expression of true late genes γ_2 , however, occurs only after DNA replication (Fig. 2C). Majority of late genes encode structural proteins and are required for assembly of and egress.

1.6. VIRAL DNA REPLICATION

The HSV-1 linear, double-stranded DNA circularizes once it is released into the nucleus. HSV-1 DNA replication occurs in specialized compartments called replication compartments [32]. These structures are formed in the nucleus of the infected cells. In general, DNA replication proceeds in the following steps (Fig. 3):

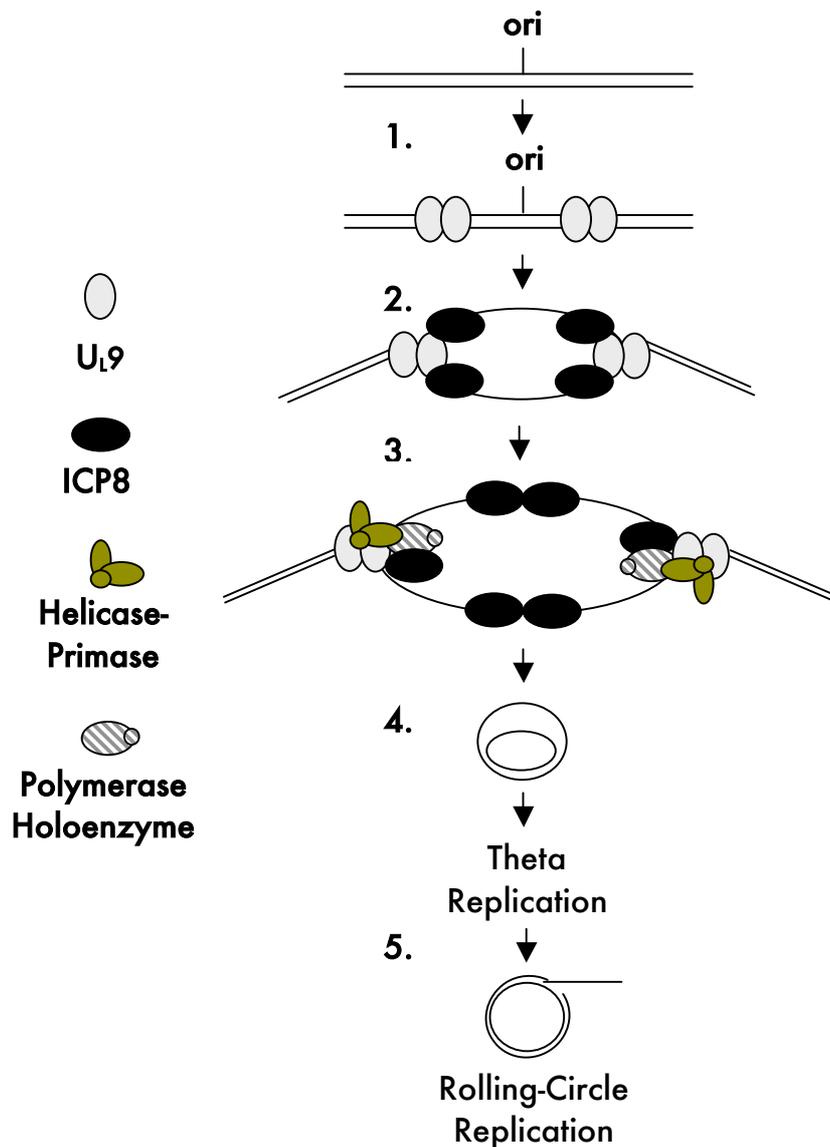


Figure 3. HSV-1 DNA replication. 1) U_L9, the origin binding protein, binds to specific sites at *ori*_L or *ori*_S, and starts to unwind the DNA. 2) The single-stranded DNA binding protein, ICP8, is recruited to the unwound DNA. 3) U_L9 and ICP8 recruit the Helicase-Primase complex (U_L5, U_L8, and U_L52) and the polymerase holoenzyme (U_L30 and U_L42) to the replication fork. 4) DNA synthesis initially proceeds via a theta replication mechanism, followed by a rolling-circle replication mechanism (5).

The origin binding protein, U_L9, contains ATP-binding and DNA helicase motifs, both of which are required for viral DNA replication [33]. U_L9 binds to specific sites at one of the origins, either *oriL* or *oriS*. The ssDNA and U_L9 then recruit ICP8 (single stranded DNA binding protein), which in turn stimulates the helicase activity of U_L9 to the unwind DNA. Together, U_L9 and ICP8 recruit five other replication proteins to the replication fork. These proteins consist of U_L5, U_L8 and U_L52 that make up the Helicase-Primase Complex [34, 35], and U_L30 and U_L42 that form the DNA Polymerase Holoenzyme [36]. ICP8 promotes the activity of the helicase-primase core subunit U_L5/U_L52 in the presence of U_L8, and modulates the polymerase activity of U_L30 [34-36]. DNA synthesis initially proceeds by the theta replication mechanism, and later switches to the rolling-circle replication mechanism [37].

1.7. VIRION ASSEMBLY, DNA ENCAPSIDATION AND EGRESS

Viral capsid assembly occurs in the nucleus and requires the synthesis of late proteins. Some capsid proteins lack nuclear localization signal (NLS) and must be transported from the cytoplasm to the nucleus by forming complexes with NLS-containing proteins. Cellular factors are not required for capsid assembly. A mature capsid is comprised of an outer shell containing capsid proteins VP5, VP19C, and VP23 [38, 39]. The major capsid protein VP5 forms pentons and hexons, and the minor proteins VP19C and VP23 form triplex structures. Accurate assembly of the icosahedral capsid is mediated by viral scaffolding protein VP22a. This protein is the major component of the inner core of the assembling nucleocapsid. VP5 and VP22a associate to form hexameric structural units within the core to which additional VP5 hexamers and VP19/VP23 triplexes are added. Once the nucleocapsid is assembled, the scaffolding protein VP22a must be removed from the structure so that the viral DNA can be accommodated. Therefore, VP22a is degraded by the viral protease VP24. Empty capsid shells are then loaded with viral DNA. Since the viral DNA is produced in concatemers, it needs to be cleaved so that only one genome-length is packaged into each capsid [38, 39].

After assembly, the nucleocapsid buds through the inner nuclear membrane into the space located between the inner and outer leaflets of the nuclear envelope [40]. During this process, it acquires tegument proteins and a primary lipid envelope studded with viral glycoproteins. The virus can then proceed by two proposed mechanisms: the re-envelopment model [41, 42] or the luminal pathway model [43]. In the re-envelopment model, the enveloped virus fuses with the outer nuclear membrane, releasing the free nucleocapsid into the cytoplasm. The virion is re-enveloped by budding into the golgi compartment. The re-enveloped virion is then secreted from the cell by a vesicular route. The luminal pathway model suggests that the enveloped virion is transported, in vesicles or within the lumen of ER, from the inner nuclear space to the golgi, and subsequently released from the cell by a secretory route. Recent evidence provided by electron microscopy and glycoproteins retrieved from the ER favor the re-envelopment model [44, 45].

1.8. LATENCY AND REACTIVATION

One of the major hallmarks of HSV-1 infections is its ability to establish life long latent infection of neurons. The major site of HSV-1 latent infection is the trigeminal ganglia of the sensory neurons. During latency, the HSV-1 genome remains in the nucleus of the neuron as circular, extra-chromosomal DNA [2]. No viral progeny is produced at this stage of infection and only a limited gene transcription occurs. The only abundant viral RNAs detected are those belonging to a family of transcripts referred to as the latency-associated transcripts or LATs [46, 47]. Proteins in this family have been implicated in silencing viral gene transcription so that the virus remains in a state of quiescence. The virus remains in this state until the proper signals reactivate the virus and new progeny are generated. The exact signals required for reactivation are subject to debate and will not be discussed here.

2. APOPTOSIS

2.1. GENERAL INTRODUCTION

Apoptosis, or programmed cell death, is a highly regulated process activated during normal development and in response to various stimuli that disturb cell metabolism and physiology [48, 49]. Apoptosis is generally described as a well ordered dismantling of the cell, and characterized by the following morphological features: cell shrinkage, loss of plasma membrane integrity, and cell membrane blebbing. The biochemical changes include phosphatidylserine exposure, mitochondrial release of cytochrome c (cyt c), chromatin condensation, and DNA fragmentation (reviewed in reference [50]). The final result of apoptosis is the breaking down of the cell into smaller apoptotic bodies, which are eventually cleared away by phagocytic cells. Apoptotic cell death differs from necrotic cell death in that little inflammation is produced since the cell contents are not shed into the extracellular fluid.

2.2. GENERAL APOPTOTIC PATHWAYS

Two principal pathways termed the extrinsic and the intrinsic pathways are known to initiate apoptosis. A family of death receptors belonging to the tumor necrosis factor (TNF) receptor superfamily mediate the extrinsic pathway. Members of the B cell lymphoma-2 (Bcl-2) family of proteins regulate the intrinsic pathway, which can also be termed 'Bcl-2-controlled' pathway (for reviews see references [51-53]). Although these pathways differ in their mode of initiation, they eventually converge at the point of activating members of the proteolytic enzymes, called caspases. These enzymes in turn deliver the final hit in destroying the cell.

2.2.1. CASPASES

CysteinyI aspartate specific proteases (caspases) are synthesized as inactive proteins called pro-caspases, containing a large and a small catalytic domain. Caspases are divided into initiators and executioners based on the architecture of their N-terminal pro-domain, and they differ in their mode of activation. The initiator caspases are characterized by having a long pro-domain which serves as

the protein-protein interaction domain. The executioner caspases instead possess a shorter pro-domain. The long pro-domain of some caspases contain specialized domains such as the N-terminal caspase-activating recruitment domain (CARD) found in caspase-9 or the N-terminal death effector domain (DED) found in caspase-8 (Fig. 4) (for in-depth reviews of caspases, see references [54-59]).

The initiator caspases are activated by one-step process, which involves their recruitment into specialized protein complexes [57, 60]. Activation of the executioner caspases, however, requires a two-step process. First, a proteolytic cleavage separates the large and the small subunits. This cleavage is usually carried out by an initiator caspase. Next, the pro-domain is removed auto-catalytically to fully activate the caspase [57, 61].

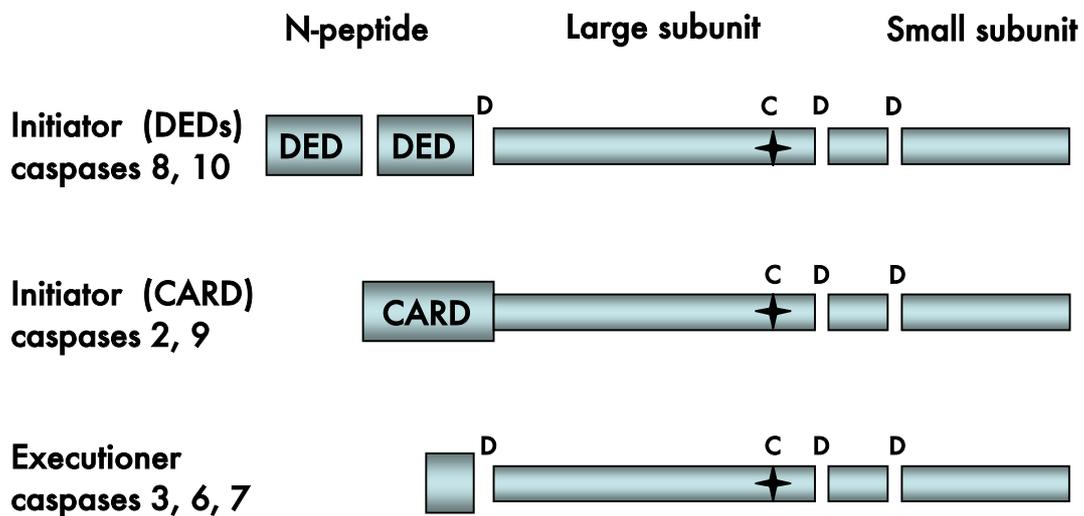


Figure 4. The domain structure of human caspases. The pro-domains of initiator caspases containing the DED and CARD domains are absent in the executioner caspases. Aspartyl residues at which proteolytic cleavage occurs and the active site cysteine are indicated.

2.2.2. THE EXTRINSIC APOPTOTIC PATHWAY

As mentioned above, the extrinsic pathway is activated by a variety of death receptors belonging to the TNF receptor family. Induction of apoptosis mediated by the Fas receptor represents an example for this pathway. Stimulation of Fas results from binding of the Fas ligand to the Fas receptor on the target cell surface (Fig. 5). This interaction leads to the intracellular binding of the adaptor-protein

Fas-associated death domain (FADD) to the Fas receptor through the interaction of their death domains (DD). FADD in turn serves to recruit pro-caspases-8 or -10 into this complex, through the interaction of their death effector domains (DED), resulting in the formation of the death-inducing signaling complex (DISC) [62]. The close proximity of these zymogens in the DISC leads to their catalytic activation presumably by an allosteric mechanism involving dimerization [62-65]. Proteolytic cleavage of the large and small subunits might serve to enhance the stability of the dimers formed.

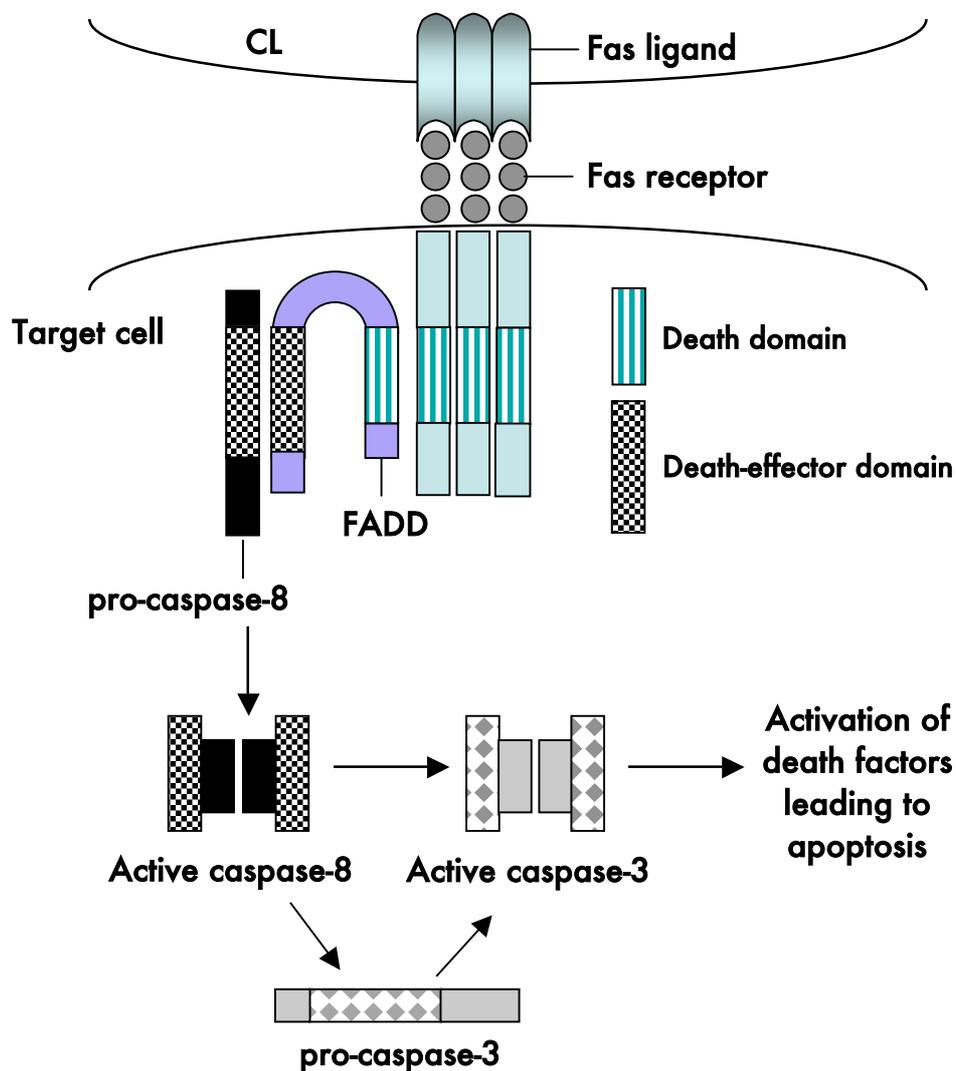


Figure 5. The cell extrinsic death pathway induced by ligation of the Fas ligand on the surface of cytolytic cell (CL) and the Fas receptor on the target cell surface. The cross-linking of Fas leads to the activation of caspases, which ultimately results in the organized dismantling of the target cell.

The activated caspases-8 or -10 then activate caspase-3 by cleavage [66]. The activated caspase-3 can then transmit the death signal by activating a variety of substrates such as the caspase-activatable DNase (CAD). CAD is held in an inactive state by forming a heterodimer with inhibitor of CAD (ICAD) in the nucleus. Upon induction of apoptosis, ICAD is cleaved by the activated caspase-3 facilitating the assembly of CAD into its active form. The activated CAD possesses endonuclease activity and cleaves DNA into 200 base pair fragments [67, 68].

2.2.3. THE INTRINSIC APOPTOTIC PATHWAY

The intrinsic apoptotic pathway is generally activated in response to intracellular stress signals such as cytokine deprivation, genotoxic damage, and detachment of adherent cells (anoikis) [52]. A breach in the mitochondrial integrity, which leads to the efflux of cytochrome c and other pro-apoptotic proteins, is the central key in the progression of apoptosis through this pathway via caspase-dependent and -independent mechanisms (**Fig. 6**) [52]. The intrinsic cell death pathway is also termed the 'Bcl-2-controlled' pathway since proteins belonging to the Bcl-2 family regulate the integrity of the mitochondrial membrane [53]. Members of this family play a crucial role, arbitrating the life-or-death decision, and will be discussed in detail in the next section.

Once cytochrome c is released from the mitochondria, it activates apoptotic protease activating factor-1 (Apaf-1) in the cytosol. Apaf-1 contains a large C-terminal WD40 repeat domain, which restrains its activity. Cytochrome c unleashes this inhibition and forms a multimeric complex termed apoptosome, consisting of ATP, activated Apaf-1 and pro-caspase-9 [69, 70]. Recruitment of pro-caspase-9 to this complex leads to its activation by an allosteric mechanism triggered by dimerization or oligomerization [63]. Activated caspase-9, like the DISC-activated caspases-8 and -10, triggers the activation of caspase-3 by executing the first catalytic cleavage in this caspase, which is followed by autocatalytic activation [63]. Activated caspase-3 subsequently activates downstream pro-apoptotic proteins leading to cell death (**Fig. 6**) [68, 71, 72].

It is important to mention that autocatalytic activation of caspase-3 can be inhibited by a set of cellular caspase-inhibitors, termed inhibitors of apoptosis

(IAP). IAPs were first described as viral proteins that blocked apoptosis. X-linked IAP (XIAP) is a human homologue capable of inhibiting caspase-3 activation by blocking the removal of the pro-domain [73]. The inhibitory activity of XIAP, however, can be antagonized by the release of mitochondrial proteins. Smac/DIABLO [second mitochondrial activator of caspases/direct inhibitor of apoptosis binding protein with low isoelectric point (PI)], like cytochrome *c*, resides in the mitochondrial inter-membrane space, and is released when the integrity of the mitochondrial membrane is breached [74, 75]. Once in the cytosol, Smac/DIABLO binds to XIAP and displaces it from caspase-3, and therefore, relieves the inhibitory effect of XIAP, allowing full activation of caspase-3. Thus, presence of active initiator caspases is not sufficient to induce apoptosis and removal of the IAP inhibition plays a critical role in the progression of cell death.

Other pro-apoptotic proteins released from the mitochondria serve as caspase-independent death effectors. Release of apoptosis-inducing factor (AIF) and endonuclease G (endo G) due to loss of mitochondrial membrane integrity, leads to their translocation to the nucleus. AIF induces chromatin condensation and formation of large DNA fragments ($\approx 50\text{kb}$) [76]. AIF is also able to induce loss of mitochondrial membrane potential and release of cytochrome *c*, indicating that it may have a dual function [76]. Endo G causes fragmentation of nuclear DNA into nucleosomal fragments and its function can be regulated by Bcl-X_L and presumably by Bcl-2 [77, 78].

2.2.4. THE BCL-2 FAMILY OF PROTEINS

The Bcl-2 proto-oncogene, the founding member of this family, was originally discovered at the t(14;18) (q32;q21) breakpoint, which is the cytogenetic hallmark of human follicular B cell lymphoma [79, 80]. The discovery of Bcl-2 presented a novel finding since it suggested a new role for proto-oncogenes. This protein had a unique functional role in blocking cell death independent of affecting cell proliferation [81, 82].

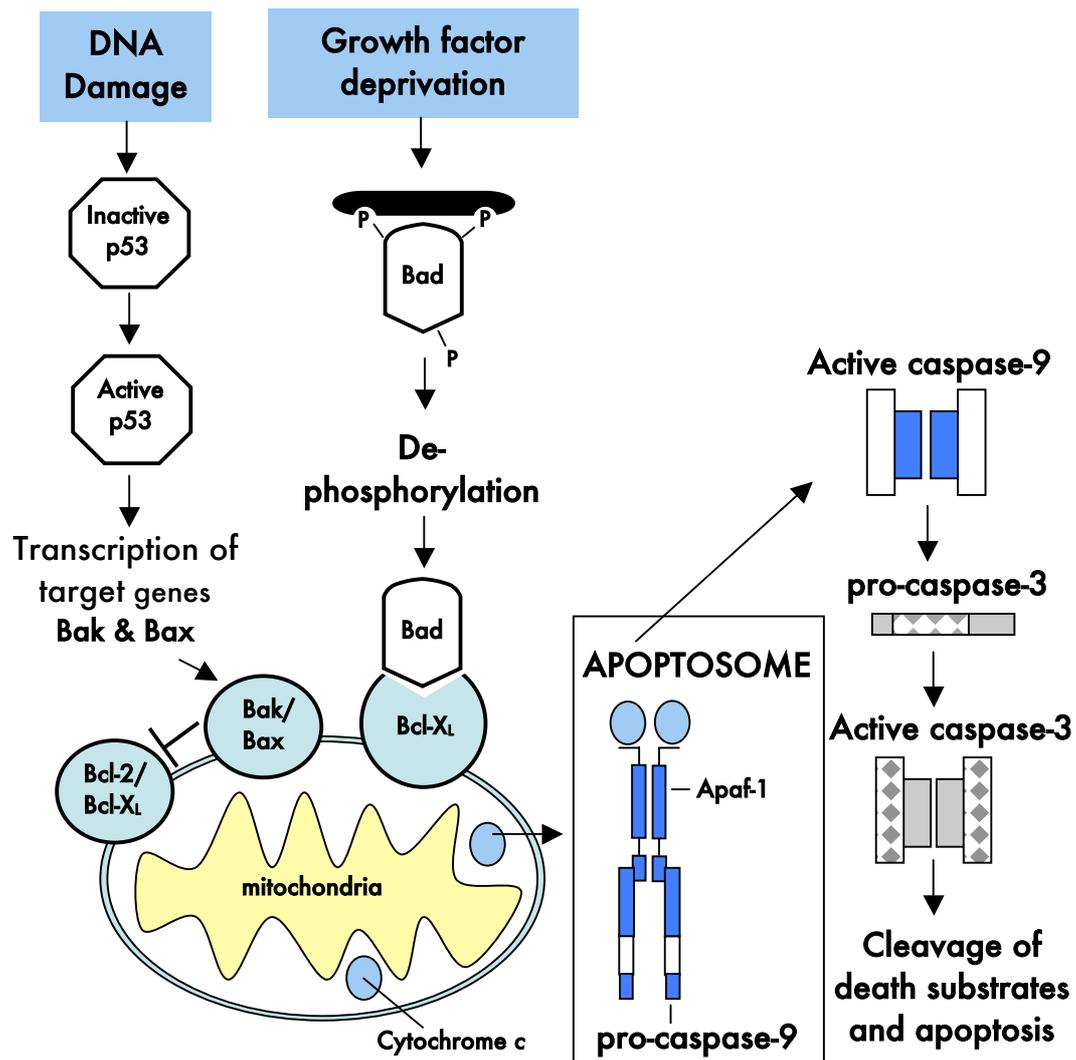


Figure 6. The cell intrinsic death pathway is activated by various stress signals. The pro-apoptotic proteins of the Bcl-2 family relay death signals to the mitochondria by antagonizing the function of the anti-apoptotic proteins Bcl-2 and Bcl-X_L. Permeabilization of the mitochondrial outer membrane leads to the release of apoptogenic factors such as cytochrome c, which forms a complex with Apaf-1 to activate caspase-9. Activated caspase-9 will, in return, active caspase-3 to initiate activation of other death substrates. Anti-apoptotic function of Bad and its activation/de-activation by cellular kinases will be discussed in sections 6.1 and 6.2. P53 is activated in response to DNA damages and induces transcription of pro-apoptotic proteins Bak and Bax, which in turn induce cell death.

The Bcl-2 family consists of pro-survival and pro-apoptotic proteins that are characterized by the presence of distinct conserved sequence motifs known as Bcl-2 homology (BH) domains designated BH1, BH2, BH3 and BH4 (Fig. 7) [83]. These proteins can be divided into three groups based on their domain architecture. The anti-apoptotic members include Bcl-2 and Bcl-X_L, and share all the

four BH domains (BH1-4). They also possess a C-terminal hydrophobic domain that is predicted to be responsible for membrane localization [83, 84]. Members of this group are known to become associated with the mitochondrial outer membrane and serve to maintain the mitochondrial integrity [53].

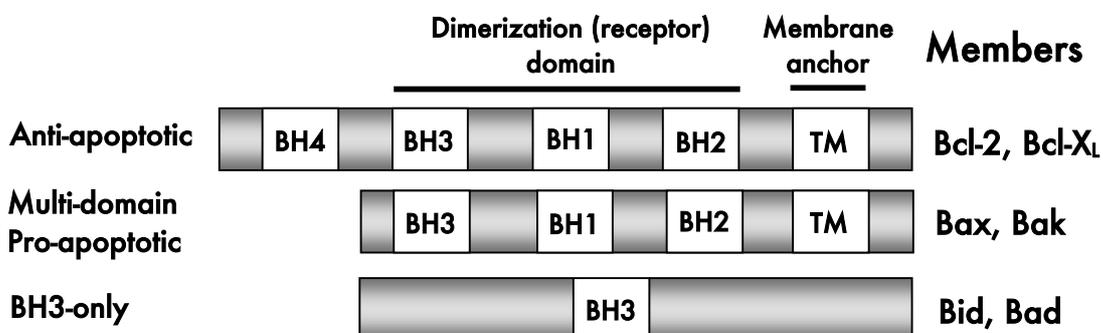


Figure 7. Members of the Bcl-2 family of proteins. The organization of the Bcl-2 homology domains among the family members is depicted.

The pro-apoptotic members of the Bcl-2 family are further divided into two groups. One group consists of the multidomain proteins Bax and Bak sharing the BH domains BH1-3. These proteins also contain the C-terminal hydrophobic domain. However, Bax will only associate with the mitochondrial membrane upon activation by apoptotic signals. Homo-dimerization of Bak and Bax, and hetero-dimerization with the anti-apoptotic members play an important role in their function. The other pro-apoptotic group comprises of the BH3-only proteins including Bid and Bad. Proteins in this family act as sentinels that detect distinct apoptotic stress signals and relay the death signal to the executioners Bak or Bax (for in-depth reviews of Bcl-2 proteins, see references [83, 85, 86]).

Structural studies on the Bcl-2 family members have provided detailed information on their molecular mechanism of action. Members of this family, show similar fold despite their overall divergence in amino acid sequence. Their three-dimensional structure has revealed the presence of two central hydrophobic α -helices, forming the core, surrounded by six or seven amphipathic α -helices. The two first α -helices are separated by a long, unstructured loop [85].

The solution structures of Bcl-X_L, Bcl-2, and Bcl-X_L complexed with Bak or Bad have provided more detailed insights into the significance of the BH domains

[87-90]. Several conclusions are deduced from these studies. First, the BH1-3 domains of Bcl-2 and Bcl-X_L define a prominent hydrophobic groove on the surface, which is somewhat wider in Bcl-2. The presence of this hydrophobic groove serves as a docking site for the BH3 domain of the pro-apoptotic proteins, and therefore, is the functional part of these proteins and is required for hetero-dimerization. Mutational analysis of the BH domains where mutations disrupted the formation of the hydrophobic groove ablates the anti-apoptotic function of Bcl-2 and Bcl-X_L [91].

Second, the wider elongated groove of Bcl-2 along with differences in topology and electrostatic character of this binding groove, affect the specificity for binding different pro-apoptotic proteins [89]. Third, the structural analysis of Bcl-X_L has also shown that it resembles the membrane insertion domains of diphtheria toxin and of colicins [87]. Based on this structural similarity, and experiments using synthetic membranes, it was postulated that the Bcl-2 family of proteins may be involved in pore formation in membranes [92-94].

And last, the structural data from Bax demonstrate that the putative C-terminal transmembrane tail occupies the hydrophobic groove, and masks the BH3 domain inside the hydrophobic core [95]. In this conformation, Bax is inactive and in its cytosolic form. Insertion of Bax into the mitochondrial membrane, and its interaction with Bcl-X_L requires a conformational change, which is brought about by apoptotic signals [96-100]. This conformational change would lead to the flipping out of the C-terminal tail essential for targeting the protein to the mitochondria, and the exposure of the BH3 domain required for hetero-dimerization [95, 97].

3. LYMPHOCYTE-MEDIATED APOPTOSIS

Cytolytic lymphocytes (CLs), which comprise of cytotoxic T cells (CTLs), natural killer cells (NK), and lymphokine activated killer cells (LAK), provide the most important line of defense against viral infections. Although these effector cells are initially activated by different cell surface receptors, they share common effector mechanisms for induction of apoptosis of the target cells. These cells have numerous ways to bring about the demise of the target cells. CLs trigger cell death by two principal pathways: the receptor-mediated pathway and the granule-

mediated pathway [101]. Both pathways rely on targeted proteolysis of cellular substrates for induction of death. These mechanisms and proteins involved will be discussed below.

3.1. RECEPTOR-MEDIATED APOPTOSIS

As discussed in previous chapters, the receptor-mediated pathway involves activation of the TNF family of receptors such as Fas. Engagement of the Fas receptor on the target cell surface by the Fas ligand on the surface of the CLs leads to induction of apoptosis through multiple protein-protein interactions involving caspases. The mechanistic details of receptor-mediated pathway follow those of the extrinsic apoptotic pathway, and will not be discussed here (see section 2.2.2 and Fig. 5).

3.2. GRANULE-MEDIATED APOPTOSIS

The granule-mediated pathway involves a mechanism in which upon target cell recognition, the effector cell cytoplasm is rearranged, and results in the exocytosis of pre-formed granules and the delivery of the key effector molecules. The contents of the lytic granules are released in the secretory synapse, the space formed between the effector and the target when contact has been made [102]. Granzyme B (GrB) and perforin (PFN) are two best-characterized proteins stored in lytic granules. GrB is the main component of the granules, which has been implicated in rapid DNA fragmentation, a hallmark feature of apoptosis.

3.2.1. PERFORIN

Perforin is an essential component of the lytic granules. PFN was initially regarded as a pore forming protein capable of inducing death by damaging target cell membrane due to the destruction of normal cellular gradients (Fig. 8) [103-105]. Experiments with perforin deficient mice have established the pivotal role for this protein. CLs from PFN knockout mice have abolished activity in their ability to lyse targets, and PFN-deficient mice are more susceptible to tumors and infections [106, 107]. PFN alone can induce lysis of cells, however, it does not induce the ordered breakdown of cells observed in apoptosis. This observation suggests that the

activity of perforin must be synergized with the activity of other granule components for the transduction of the apoptotic signal.

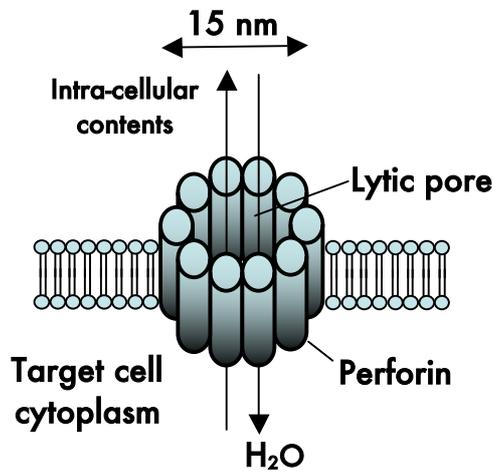


Figure 8. Formation of poly-perforin pore in the target cell membrane results in the loss of plasma membrane homeostasis, with excessive uptake of water and loss of intra-cellular contents, leading to the lysis of the cell. The size of the lytic pore is approximately 15 nm.

3.2.2. GRANZYME B

Granzyme B is a 32 kDa serine protease produced as a zymogen in cytotoxic lymphocytes. Activation of GrB requires cleavage by cathepsin C, which is localized to the cytotoxic granules [108]. GrB's potential to induce death within effector cells requires a tight regulation for its activity. Maturation of GrB within the granules protects CLs from inadvertent apoptosis. Moreover, GrB is optimally active at neutral pH, thus limiting its proteolytic activity in the acidic pH of granules. In addition, cytotoxic effectors contain a potent GrB inhibitor, known as proteinase inhibitor-9 (PI-9) [109]. PI-9 is a human serpin, which is also expressed in endothelial, epithelial, and mature dendritic cells [110, 111]. PI-9 is localized to the cytoplasm and the nucleus, acts as a pseudo-substrate for GrB, and forms tight, irreversible complex with GrB [112]. Therefore, PI-9 has an important role in protecting CLs against autolysis as well as the lysis of antigen-presenting and bystander cells since expression of PI-9 was shown to render cells resistant to GrB-induced death. Another level of self-protection is provided by surface expression of lysosomal protease cathepsin B, which can be detected on the surface of cytotoxic cells after degranulation [113]. Cathepsin B was proposed to degrade membrane-bound perforin on effector cells.

GrB is post-translationally modified by glycosylation in the Golgi with mannose-6-phosphate (M6P)-containing carbohydrate moieties [114]. GrB is also electrostatically bound to proteoglycan serglysin (SG) within lytic granules. SG is a proteoglycan distinguished by linkage of chondroitin sulfate or heparan sulfate glycosaminoglycans (GAGs) to serine-glycine repeats in the central portion of the core protein [115]. The GAG chains are negatively charged, and act as a scaffold for the cationic proteins GrB and PFN in the form of multimeric complexes within the granules. Association of GrB with serglysin might have a role in its uptake by target cells.

3.2.2.1. GRANZYME B UPTAKE AND ENTRY INTO CELLS

Several models have been proposed for the entry or uptake of GrB into the target cells. These models are all in agreement with the requirement of perforin for the full activity of granzyme B, despite its mode of entry into target cells. Granzyme B was originally thought to enter the cells by diffusing freely through pores made by perforin in the cytoplasmic membrane. This early view on perforin's activity is criticized since pores formed by poly-perforin do not appear large enough to allow diffusion of uncomplexed granzymes (**Fig. 9**).

Recent studies have elegantly demonstrated that target cells internalize GrB alone independent of PFN [116]. GrB is taken up either by receptor-mediated endocytosis or through macro-pinocytosis. Under these conditions, GrB remains confined to the endocytic vesicles, and cells remain healthy and viable [117]. Based on experiments with inhibitors of endosomal trafficking, it is thought that PFN is required for the disruption of endosomes and release of granzyme B into the cytosol [118]. Thus, enabling GrB to access key substrates and initiate the death signal. The fact that treatment of cells with PFN after internalization of GrB results in the morphological and biochemical features that are associated with apoptosis further supports this observation [118, 119].

More than one pathway has been suggested for the uptake of GrB into target cells. The cation-independent mannose-6-phosphate receptor (CI-MPR) has been identified as a putative receptor for GrB [120]. CI-MPR has been shown to bind and internalize monomeric GrB presumably by binding to mannose-6-

phosphate moieties on GrB. Moreover, CI-MPR shuttles the freshly synthesized GrB from ER to the granules, where it is sequestered with SG, during GrB biosynthesis [114]. SG-bound GrB may contain exposed mannose-6-phosphate groups and bind to CI-MPR on the surface where it is endocytosed in a clathrin-dependent process (Fig. 9). These observations are further supported by the findings, which demonstrate that only granule-purified GrB but not recombinant GrB is biologically

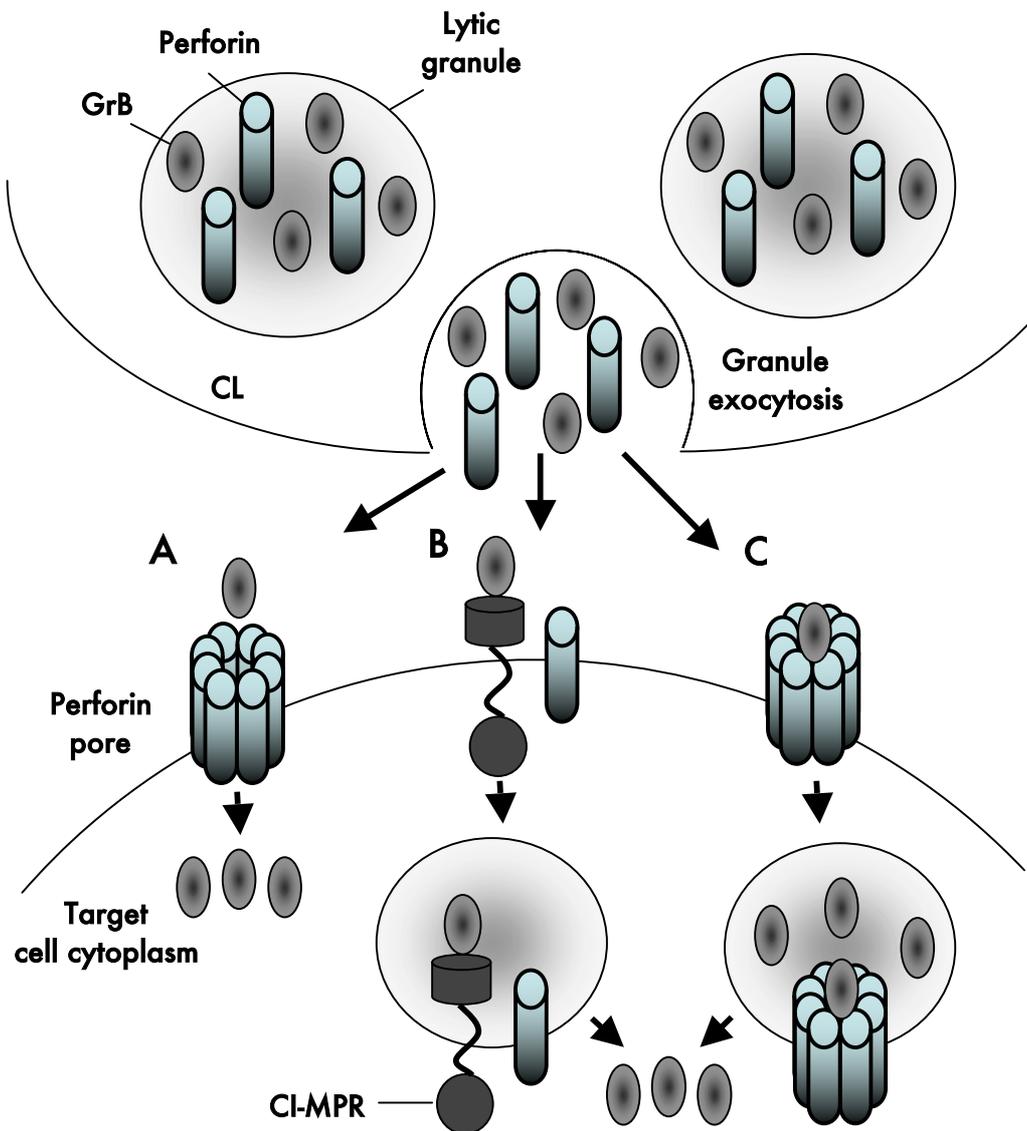


Figure 9. Entry of GrB into target cell cytoplasm. (A) GrB was thought to enter target cell via poly-perforin pores. (B) Recent evidence suggest that GrB is endocytosed by the target cell after binding its receptor, CI-MPR. (C) GrB may also be internalized in response to local membrane damages induced by perforin.

active [114]. Interestingly, de-phosphorylation of granule-purified GrB led to the loss of its biological activity, and binding to CI-MPR, thus implicating phosphorylation as an important post-translational event in the maturation and uptake of GrB [114].

GrB released in the secretory synapse can also be internalized in response to local membrane damages induced by perforin [121, 122]. In this model, both GrB and PFN are taken up as the target cell attempts to repair the lesion (Fig. 9). The balance between GrB entry pathways might reflect the amount of GrB and PFN delivered in the synapse, as well as the membrane properties of target cells.

3.2.2.2. CELL DEATH SIGNALING BY GRANZYME B

As C. Bleackley has named it, granzyme B is a natural-born killer. Once in the cytosol, GrB targets a variety of cellular proteins, and induces death both in a caspase-dependent and -independent manner. Initially, GrB was thought to act as an upstream caspase-like molecule since it has a preference for proteolytic cleavage after aspartate residues a feature shared by the caspase family members. Several pro-caspases are processed by GrB *in vitro* and *in vivo*, with the highest preference given to pro-caspases-3 and -8 [123-125]. Addition of mitochondria to GrB-activated extracts amplifies this apoptotic signal implicating GrB in activating the mitochondrial apoptotic pathway [126]. Moreover, over-expression of Bcl-2 localized to the mitochondria was shown to inhibit GrB-induced loss of mitochondrial membrane potential, release of cytochrome c, nuclear translocation of GrB, and DNA fragmentation, further supporting the involvement of mitochondria in GrB-induced cell death [127].

Recently, it was demonstrated that GrB targets pro-caspase-3 for cleavage upon entry to the target cell cytosol [128]. This cleavage, however, does not lead to the potent activation of caspase-3. Moreover, full activation of caspase-3 induced by GrB can be blocked by over-expression of Bcl-2 [129, 130]. These observations linked adequate activation of caspase-3 to the mitochondrial death pathway. The question remaining was how did granzyme B transduce the death signal. The missing link that brought the two pathways together was identified as the cytosolic and pro-apoptotic protein named Bid. Bid is a member of the Bcl-2

family of proteins with an apparent molecular weight of 22 kDa (p22). Bid is synthesized as an inactive protein, and kept inactive via an intra-molecular interaction between its C-terminus and the N-terminal located BH3 domain. Bid has been shown to be a substrate for cleavage by caspase-8 and GrB [131]. Caspase-8 cleaves Bid at D60, whereas granzyme B cleavage site is at D75. Bid is the most preferred substrate of GrB and is cleaved within minutes after treatment of targets with GrB. This cleavage generates the cleavage product p14, and leads to the exposure of the BH3 domain in the N-terminal region. The p14 Bid is then myristolated and rapidly migrates to the mitochondria where it interacts with Bak and Bax, and induces the release of apoptosis-inducing proteins from the inter-membrane space, such as cytochrome c and Smac/DIABLO [129]. GrB plays a dual role by doing so. It induces activation of caspase-9, which in turn activates more of the caspase-3 proteins. Also, it relieves the IAP-induced inhibition of caspase-3 by releasing Smac/DIABLO from the mitochondria, thus facilitating the massive amplification of the caspase cascade (**Fig. 10**).

Granzyme B also induces caspase-independent DNA fragmentation and cell death. The ability to induce rapid DNA fragmentation is a unique characteristic of GrB. Experiments with GrB-deficient CTLs have shown that deficient cells do not induce DNA fragmentation as early as the wild-type CTLs [132]. GrB targets the nucleus where it cleaves a variety of nuclear proteins, many of which are also targets for caspases, such as poly(ADP-ribose) polymerase (PARP) and ICAD [133, 134]. As mentioned before, PARP and CAD activities induce inhibition of DNA repair and fragmentation of DNA, respectively (**Fig. 10**). The type of cell death induced in the absence of activated caspases does not involve cytochrome c release or phosphatidylserine (PS) exposure. This observation is consistent with the characteristics of the type of cell death induced by direct cleavage of ICAD mediated by GrB. Granzyme B can also induce the release of AIF and endo G from the mitochondria, which would mediate caspase-independent DNA fragmentation and cell death [135-137].

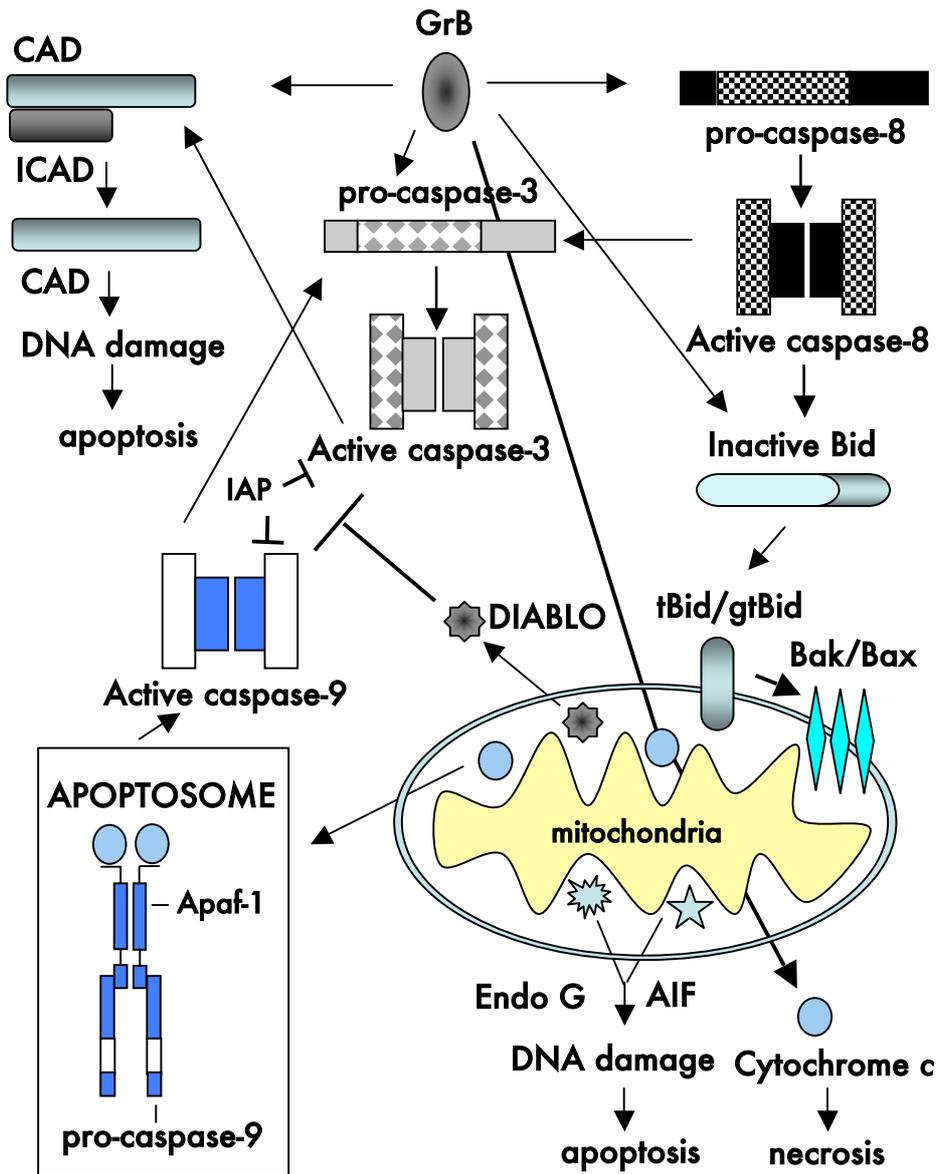


Figure 10. GrB-induced cell death. GrB can initiate apoptotic cell death through direct cleavage of pro-caspase-3, or indirectly, through cleavage of pro-caspase-8 and Bid. Full processing and activation of caspase-3 requires release of DIABLO from the mitochondria, which relieves IAP inhibition of this caspase. Processing of Bid results in the translocation of its truncated form to the mitochondria, and subsequent release of cyt c, which together with Apaf-1 activate caspase-9. Other factors such as Endo G and AIF are also released from the mitochondria and mediate caspase-independent DNA fragmentation. GrB can also directly execute cell cytotoxicity by inducing cyt c release from the mitochondria leading to necrosis. GrB is also able to cleave ICAD and activate CAD to induce DNA fragmentation directly.

4. MODULATION OF APOPTOSIS BY HSV-1 PROTEINS

Infection of human epithelial cells with herpes simplex virus has profound effects on its host cells with a lytic replication cycle, which leads to the destruction of cells. The cytopathic effect of infection with the wild-type HSV-1 observed commonly in adherent cells is described as the rounding up of the cells progressively with increasing times of infection. Other manifestations of an HSV-1 infection are listed as: (i) detachment due to the loss of matrix binding proteins on the cell surface; (ii) cytoskeletal destabilizations; (iii) nucleolar alterations; (iv) chromatin aggregation or damage, and (v) a decrease in cellular macromolecular synthesis [138-140]. This cytopathology is a consequence of the virus taking over the host cell in order to replicate its genome and produce progeny.

Induction of apoptosis is another consequence of HSV-1 infection. The morphological and biochemical changes that result from virus-induced apoptosis appear to be different than those induced by virus replication. These changes include cell shrinkage, membrane blebbing, nuclear condensation and fragmentation of chromosomal DNA into nucleosomal oligomers, and are all characteristics of cells dying from apoptosis. Infected cells, however, do not exhibit classical signs of apoptosis upon virus infection since the virus expresses proteins that block apoptosis induced by the virus infection. The virus has also evolved multiple proteins, which enable it to evade immune detection and block cell death induced by a variety of stimuli. Inhibition of apoptosis would allow sufficient time for the virus to replicate the viral DNA and produce progeny. This would ensure the survival of the virus and its spread to new cells and eventually to a new host, as well as establishment of latency.

Interaction of the virus with human epithelial cells prior to 1 h post infection (p.i.) results in the induction of apoptosis in the absence of de novo viral protein synthesis and translation of the HSV-1 IE mRNA [141]. Experiments where viral protein synthesis was blocked by temporal addition of cyclohexamide (CHX) have indicated that viral proteins synthesized between 3 and 6 h p.i. are essential for blocking virus-induced apoptosis [141, 142]. This time period has been termed the 'preventions window'. The 3 h p.i. time period corresponds to the onset of early

viral gene expression and the time period prior to 6 h p.i. corresponds to the transition phase from immediate early to early phase of viral gene expression [142]. This definition of the prevention window is consistent with the requirement for the immediate early protein expression for the prevention of apoptosis in infected human cells [142, 143]. The immediate early proteins have regulatory functions and cooperate to regulate the expression of all other viral genes. Inhibition of apoptosis by HSV-1 is therefore a multi-component phenomenon emphasizing on the fact that preventing cell death by the virus is crucial for its survival. Several proteins of HSV-1 involved in this process are discussed below. For a general scheme of modulation of apoptosis during HSV-1 infection, see **Figure 11**.

4.1. ICP27

Infected cell protein 27 (ICP27) is a multifunctional, immediate early protein of HSV-1. ICP27 has been implicated in having a prominent role in the inhibition of host mRNA biogenesis [144, 145]. ICP27 collaborates with *vhs* (virion host shutoff protein) to reduce the abundance of host mRNA during infection. *Vhs* contributes to this by increasing the rate of cellular mRNA degradation in the cytoplasm [146, 147]. This effect is further magnified by the function of ICP27 in reducing the pre-mRNA splicing efficiency of host mRNAs and repression of primary transcripts during infection [42, 148].

ICP27 also plays an important role in the inhibition of virus-induced apoptosis at an early time point of infection. Experiments with the ICP27 deletion mutant virus have shown that ICP27 rescues infected cells from dying by apoptosis. ICP27-deficient virus induces apoptosis by a mechanism, which involves activation of caspase-3 and the processing of death substrates, CAD and PARP [141]. The action of ICP27 is also dependent on the accumulation of β and γ_1 gene products U_s3 and *gD*, respectively, which points to the fact that ICP27 orchestrates the synthesis of multiple viral factors which block apoptosis in the HSV-1-infected cells [149]. ICP27, therefore, plays a central role in this prevention process.

4.2. ICP47

Infected cell protein 47 (ICP47) is an immediate early, cytosolic protein encoded by HSV-1. ICP47 has been shown to block presentation of the viral peptides to MHC class I-restricted CD8T cells in human fibroblasts [150]. ICP47 exerts its effect by interacting with the transporter associated with antigen processing (TAP), thereby, preventing the binding and translocation of antigenic peptides to the endoplasmic reticulum (ER), and their loading onto MHC class I molecules. Peptide loading is necessary for the stable association of β_2 -microglobulin with the heavy chain of MHC class I, and its subsequent transport from the ER to the cell surface. As a result of this interaction between ICP47 and TAP, MHC class I molecules are retained in the ER and the viral antigens are not presented on the cell surface. Down-regulation of MHC class I molecules plays an important role in masking virus-infected cells from recognition by the immune system especially by CD8T cells. Recognition of viral antigens would trigger signals by CD8T cells that would lead to the destruction of virus-infected cells.

4.3. U_s5

The HSV-1 U_s5 gene contains a small open reading frame, which encodes the membrane-associated glycoprotein J (gJ). Viruses deleted for U_s5 are phenotypically normal in cell culture, with normal plaque formation and cell-to-cell spread of the virus [151, 152]. U_s5 can localize to the cytoplasmic membrane of infected cells; however, no apparent function was initially assigned to this viral protein [153]. Recently, Jerome et al. ascribed an anti-apoptotic function to U_s5. They demonstrated that this viral protein strongly inhibits apoptosis induced by various stimuli [154, 155]. Cells expressing U_s5 were shown to inhibit caspases-3, -6, -8 and -9 activation after Fas ligation and UV irradiation. U_s5 was also implicated in the inhibition of GrB-induced apoptosis by a mechanism, which involves blocking the cleavage and activation of caspase-3 by granzyme B [155].

4.4. GLYCOPROTEIN D

Glycoprotein D (gD) is one of the major structural components of the HSV-1 virion. As mentioned in section 1.4, gD plays an important role in virus entry as well as

spread of HSV-1. Several observations have pointed to the importance of gD in protection of HSV-1-infected cells from apoptosis mediated by Fas. First, HSV-1 infection induces a persistent activation of the transcription factor NF- κ B [156]. This protein is present in the cytoplasm in an inactive form due to the inhibitory action of an associated protein, inhibitory κ B (I κ B) (for review, see references [157, 158]). In response to a variety of stimuli, I κ B is phosphorylated by the I κ B kinase, and degraded. Degradation of I κ B leads to the release and activation of NF- κ B. Activation of NF- κ B signaling was demonstrated to protect cells from Fas-mediated apoptosis by promoting the expression of anti-apoptotic proteins [159].

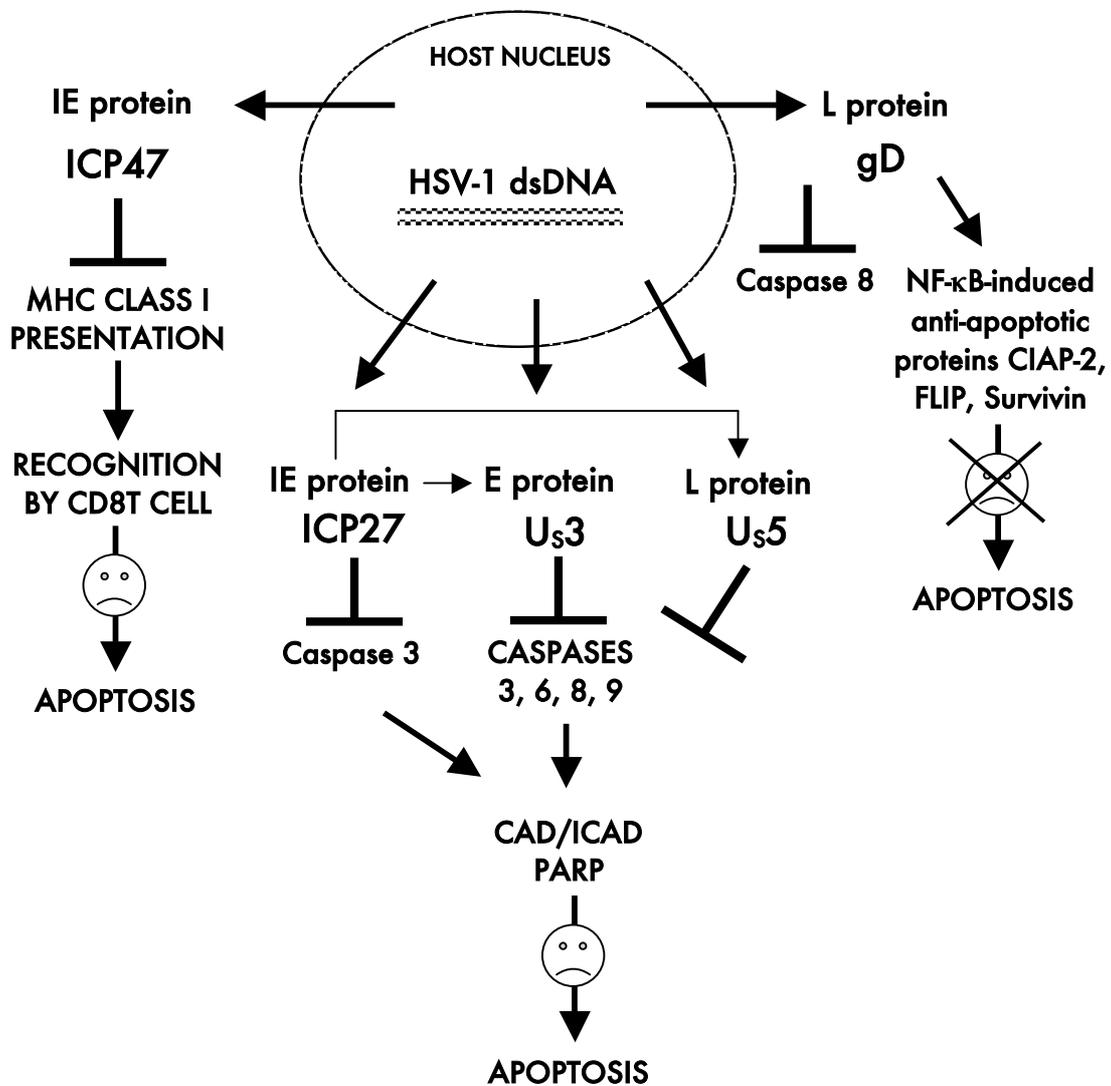


Figure 11. Modulation of apoptosis during HSV-1 infection. IE infected cell proteins produced during HSV-1 infection induce transcription of various viral genes to prevent apoptosis of infected cells. Alternatively, some IE proteins function directly in blocking cell death.

Second, the cell receptor for gD, HveA, belongs to the TNF family of receptors, which play important role in mediating signal transduction leading to receptor-induced apoptosis [160-162]. Over-expression of HveA in cells, or engagement of HveA with its natural ligand, LIGHT, has been shown to stimulate the activity of the transcription factor NF- κ B in different cellular systems [160, 163, 164]. Recent data demonstrate that gD delivered in *trans* can block apoptosis induced by infection with gD-deficient virus strains [165, 166]. These data suggest the possibility that engagement of gD with HveA could lead to the activation of NF- κ B. Finally, the mechanism of action of gD was suggested by Medici et al. where it was shown that gD by itself could activate NF- κ B in response to Fas ligation [167]. This activity correlated with a reduction in the level of caspase-8 activation, and an up-regulation of NF- κ B-induced anti-apoptotic proteins c-IAP2, FLIP and survivin.

4.5. U_s3

The U_s3 gene is encoded by an open reading frame that maps in the unique sequences of the S component (unique short) of HSV-1 DNA [168]. The gene codes for a protein kinase with an apparent molecular mass of 66 kDa. Sequence analysis of the amino acids encoding U_s3 provided strong evidence that it shared highly conserved motifs that are unique to the catalytic domain of other protein kinases [169]. Furthermore, the protein kinase activity of U_s3 was established by screening extracts from infected cells for activity against artificial substrates such as protamine. Using such technique, it was demonstrated that U_s3 can utilize ATP to phosphorylate substrates with serine or threonine residues on the C-terminal side of multiple arginine residues [170]. The consensus sequence for U_s3 phosphorylation was subsequently determined to be RRR-R/X-S/T-R/Y where Y is defined as any amino acid with the exception of acid residues and a preference for arginine, alanine, valine and serine [170, 171].

Although expression of U_s3 is not essential for the growth of virus in cell culture, its necessity in blocking apoptosis has been firmly established [172]. The anti-apoptotic activity of U_s3 was originally ascribed to the viral protein ICP4 [143]. Leopardi et al. reported that Vero cells infected with the HSV-1 mutant d120, which carries a deletion in both copies of the ICP4 gene, exhibited

morphological changes such as extensive condensation of chromatin, vacuolization and blebbing of the cytoplasm, attributed to induction of apoptosis. Further biochemical analysis of d120-infected cells demonstrated that these cells had undergone chromosomal DNA fragmentation, another characteristic of cells undergoing apoptosis [143]. These experiments together established that HSV-1 encodes a protein that is involved in blocking morphological and biochemical features characteristic of apoptosis induced by the virus infection.

More extensive analysis of the d120 mutant revealed that this strain had a secondary mutation mapping to the U_s3 region [173]. Using complementation assays with genetically engineered recombinant viruses containing or lacking the U_s3 gene, it was established that the absence of the protective activity of the d120 mutant was due to the mutation in the U_s3 gene and not that of the ICP4 gene. Moreover, infection of Vero cells with the recombinant HSV-1 mutant R7041, which does not express U_s3, produced similar morphological and biochemical changes as the d120-infected cells [173]. Furthermore, infection of cells with the recombinant HSV-1 strain R7306, in which the expression of U_s3 was reconstituted, blocked apoptosis induced by the virus infection [173]. These data collectively implicated U_s3 as the anti-apoptotic protein of HSV-1.

5. AIMS OF THIS THESIS

The aim of this research project was to identify mechanisms by which the U_s3 protein kinase of herpes simplex virus type 1 blocks apoptosis induced by various stimuli. Here we studied the effects of U_s3 on the induction of cell death by a variety of exogenous stimuli and by cytolytic cells.

The aims of this thesis are as follows:

- 1) Describe the function of U_s3 in protecting HSV-1-infected cells against the extrinsic apoptotic stimulus mediated by CD8T cells. A possible substrate for U_s3 was identified *in vitro*.
- 2) Compare cytotoxic activities of MHC class I-restricted and non-restricted cytolytic lymphocytes against HSV-1-infected cells.
- 3) Study the effect of U_s3 expression in cells undergoing apoptosis mediated by the intrinsic and the extrinsic apoptotic pathways by treatment with exogenous agents, sorbitol and anti-Fas antibody, respectively. A second possible substrate for U_s3 was identified *in vitro*.

6. RESULTS AND DISCUSSION

Programmed cell death, apoptosis, plays a major role in controlling virus infections. Inevitably, viruses have learned to intercept with pathways leading to cell death during co-evolution with their hosts. Viruses subvert the apoptotic machinery of their hosts for two major purposes; to increase viral production and to enhance viral spread. It would seem reasonable that most viruses would target the heart of the apoptotic machinery to prolong the life of the infected host. Targeting components of mitochondrial death machinery would therefore present an important key point in regulating cell survival. This reasoning is based on the fact that mitochondria have been implicated in integrating and unifying different apoptotic pathways; e.g. triggered by death receptors, granules exocytosis, and chemical agents, into one final pathway culminating in apoptotic death [174]. The Bcl-2 family is intimately linked to the mitochondria and can either negatively or positively regulate the process of cell death [83]. Therefore, modulation of the activity of this family of proteins by viruses would present the most suitable action.

Large DNA viruses such as those belonging to the herpes family of viruses have been afforded the luxury to encode many proteins involved in preventing premature death of their hosts. All known gamma herpesviruses, for example, encode homologues of the Bcl-2 family of proteins (for review see reference [175]). Kaposi's sarcoma-associated herpesvirus (KSHV) also known as human herpesvirus 8 (HHV8) is a human gamma2 herpesvirus that encodes a Bcl-2 homologue termed KSBcl-2 [175]. The 3-dimensional structure of KSBcl-2 is strikingly similar to its cellular homologues with recognizable BH1-2 sequence motifs. Interestingly, the BH3 domain which is thought to be important for inducing death, is poorly conserved in the viral homologue. KSBcl-2 also has a cleft similar to the Bcl-2 family members. This cleft was suggested to serve as a binding groove for the BH3 domains of its Bcl-2 partners. Recently, it was shown that a peptide corresponding to the BH3 domain of BAK binds to KSBcl-2 efficiently [176]. KSBcl-2 functions only during the lytic replication cycle, pointing to its importance in maintaining cells viable long enough for increasing viral load and enhancing virus transmission.

HSV-1, as previously discussed, encodes a variety of proteins that cooperatively block the destruction of infected cells long enough to ensure the survival of the virus [141, 149, 150, 154, 155, 165-167, 173]. However, no known homologue for Bcl-2 has been identified for HSV-1. We have shown that the U_s3 protein kinase is a component of this complex machinery and functions as an anti-apoptotic protein by targeting and inactivating proteins of the Bcl-2 family, and thus blocking the mitochondrial apoptotic pathway [177, 178]. By doing so, U_s3 manipulates both cell intrinsic and cell extrinsic pathways of death since these pathways converge at the level of mitochondria. Here, I will present a summary of our findings and will discuss the significance of our observations in the following chapters.

In **paper I**, we demonstrated that U_s3 protects HSV-1-infected cells from lysis by MHC class I-restricted CD8T cells [178]. This inhibitory effect was not due to a defect in antigen presentation since infected and uninfected cells expressed equal levels of MHC class I molecules. Thus ICP47, which was previously shown to tamper with assembly and therefore retention of MHC class I molecules in the ER, did not play a role at this time point of infection or in our experimental model. Expression of U_s3 had no effect on T cell activation since effector cells exposed to targets produced similar amounts of TNF α . These results suggested that U_s3 exerts its inhibitory function by acting downstream of antigen presentation. Further experiments led to several observations demonstrating that expression of U_s3 in targets which had been exposed to CD8T cells: 1) was associated with inhibition of caspase-3, -8 and -9 activation, and 2) resulted in a significant reduction in cleavage of the pro-apoptotic protein Bid. In addition, U_s3 targeted the processing of Bid since recombinant human GrB failed to cleave Bid in cytosolic extracts from U_s3 positive cells, and recombinant human Bid served as substrate for U_s3 phosphorylation *in vitro*. Our data provide the first evidence where an HSV-1 protein blocks the cell extrinsic death pathway by targeting a component of the mitochondrial apoptotic machinery. This function serves as a strategy for viral escape from T cell lysis (**Fig. 12**).

In **paper II**, we demonstrated the ability of the host immune system to overcome the anti-apoptotic function exerted by U_s3. We extended our analysis to

the role of U_s3 in infected cells against cytotoxicity mediated by NK and LAK cells since these effector cells share common mechanisms in inducing death of virus-infected cells. We showed that, in contrast to their lower sensitivity to CD8T cell lysis, HSV-1-infected cells were lysed by NK or LAK cells as efficiently as the uninfected controls. Both MHC-restricted and non-restricted cytolytic effects were significantly dependent of the activity of GrB and were efficiently blocked by treatment with a GrB inhibitor. However, in contrast to a significant induction of Bid cleavage mediated by CD8T cells, Bid was not cleaved when targets were co-cultured with LAK or NK cells suggesting that these effectors induce lysis by a mechanism that does not involve cleavage of Bid. These differences in sensitivity to lysis by different effectors are likely to be important for the respective role of MHC restricted and non-restricted effectors in the control of HSV-1 infection.

In **paper III**, we identified a second target for U_s3, which is also a component of the mitochondrial death machinery [177]. We used a set of stable U_s3 transfected cell lines with constitutive and inducible expression of the protein to show that U_s3 alone is sufficient in inhibiting apoptosis induced by virus infection, sorbitol or Fas antibody treatment. Inhibition of apoptosis in these cell lines correlated with the inhibition of the activation of caspases-3 and -9. Moreover, Bad was rapidly phosphorylated in cells expressing high levels of U_s3 following virus infection or induction from a tetracycline-regulated plasmid, and Bad also served as substrate for U_s3 phosphorylation *in vitro*. Thus, U_s3 appears to inhibit both the cell intrinsic and the cell extrinsic apoptotic pathways by mimicking or hijacking a cellular anti-apoptotic response (**Fig. 12**).

6.1. U_s3 MAY INDUCE STRUCTURAL MODIFICATIONS IN BID & BAD

The activity of the BH3-only pro-apoptotic proteins of the Bcl-2 family, Bad and Bid, constitutes a critical decisional point in apoptotic pathways, upstream to the irreversible damage to cellular constituents. Bid and Bad possess amphipathic domains required for protein-protein interactions but lack the hydrophobic C-terminal membrane-anchoring domains [179-181]. These characteristics of the BH3-only proteins allow their translocation between the cytosol and the mitochondria, where they interact with their membrane-bound partners anchored in the

mitochondrial outer membrane. This suggests that Bid and Bad may serve as death ligands, and act as sensors that receive death signals in the cytosol, and relay those signals to the mitochondria by binding to their partners, serving as receptors [181]. Thus, the activity of these proteins must be kept in check by post-translational mechanisms to prevent induction of inappropriate cell death in healthy cells. Post-translational modifications, therefore, serve as a key instrument in regulating the pro-apoptotic activities of Bid and Bad.

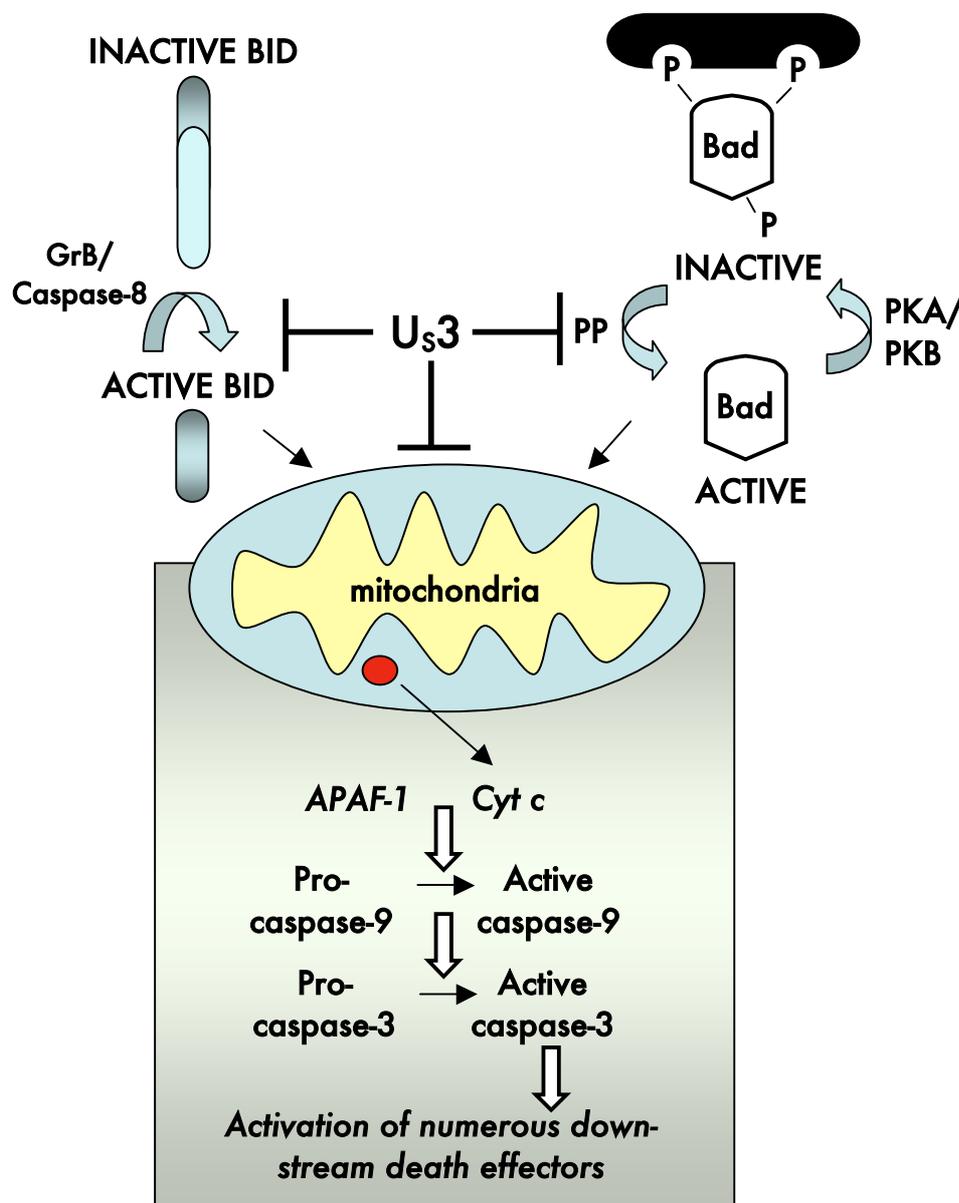


Figure 12. Inactivation of pro-apoptotic proteins Bid and Bad by the U_S3 protein kinase of HSV-1. U_S3-mediated post-translational modifications of Bid and Bad inhibits activation of the mitochondrial death pathway induced by different apoptotic stimuli. PP= protein phosphatase.

Phosphorylation of these proteins by cellular kinases has been previously reported, and numerous studies have firmly established the requirement for phosphorylation in rendering both proteins inactive in inducing death [182-186]. We propose that U_s3-induced phosphorylation events may play a crucial role in modulating the apoptotic activities of Bid and Bad, in a similar manner as under normal cellular conditions where cellular kinases render these proteins inactive. Further evidence in support of this hypothesis is provided by structural analyses, which demonstrate the requirement for post-translational modifications in regulating pro-apoptotic functions of Bid and Bad [85, 89, 187, 188]. These analyses would also provide reasoning as to why HSV-1 may choose to target and inactivate apoptosis-inducing proteins by phosphorylation. In agreement with these studies, we have identified Bid and Bad as substrates for U_s3 phosphorylation *in vitro* in **papers I and III**, respectively [177, 178].

Two criteria must be met for the pro-apoptotic functions of Bid and Bad. The first requirement is one of cellular localization. Both Bid and Bad must be targeted to the mitochondria for their pro-apoptotic activities. The second criterion is exposure of the BH3 death domain [182, 183, 189, 190]. Based on sequence and secondary structure alignments, members of the Bcl-2 family can be grouped into two structural categories [187]. The members of group I, including pro-apoptotic molecules Bid, Bax and Bak, have their BH3 domains buried, and rely on conformational changes to release these domains from their structural constraints and to induce their pro-apoptotic activity. The group II members are either the truncated form of group I (e.g. tBid), or have an unrelated tertiary structure (e.g. Bad). The members of this group have their BH3 surface exposed and as a result would be constitutively active.

Under normal cellular conditions, Bid is phosphorylated and present in its inactive form [191]. The BH3 domain of Bid is a part of the hydrophobic groove, formed by BH1-3 motifs, a surface that mediates both dimerization and apoptotic events. The hydrophobic surface of the amphipathic BH3 helices were shown to mediate dimerization with Bcl-2 [88, 183]. This hydrophobic face of the BH3 domain is buried and not accessible for avid interaction with its binding partners when Bid is in its inactive conformation. As a result, Bid does not form homodimers, and does

not serve as a BH3 motif receptor or donor and therefore can not interact with other Bcl-2 members [187]. Under apoptotic conditions, proteases such as caspase-8 or GrB, mediate cleavage and removal of the N-terminus of Bid to produce the truncated form, tBid and gtBid, respectively [131]. For simplicity, the truncated form of Bid will be referred to as tBid throughout the text. This cleavage results in marked changes in the character of Bid surface due to the exposure of the BH3 domain and a significant change in the surface charge and hydrophobicity in tBid [187]. These changes are likely to contribute to the rapid change in cellular distribution of Bid molecule upon cleavage. Moreover, inactive form of Bid has a large negative charge sufficient to prevent close association with the mitochondrial outer membrane, which itself, is among the most negatively charged biological membranes. This negativity is strongly reduced in tBid, and would explain why tBid is able to integrate in the mitochondrial membrane (**Fig. 13A, B**) [187].

It should be noted that post-translational modifications by means of de-phosphorylation may serve as the 'on switch' to provide a rapid mechanism for structural and/or conformational activation. Recently, using murine Bid, Desagher et al. have provided the evidence linking de-phosphorylation to cleavage-induced activation of Bid [191]. They demonstrated that Bid is constitutively phosphorylated by Casein Kinases (casein kinases I and II) under normal cellular conditions at several serine (S) residues in the vicinity of the caspase-8 cleavage site. Phosphorylation of serine residues at positions 61 and 64 of the murine Bid was shown to be the most important event in blocking cleavage by recombinant active caspase-8. These serine residues proceed immediately after the caspase-8 consensus cleavage sequence LQTD. Interestingly, phosphorylation of these sites correlated only with the inhibition of caspase-8-induced Bid cleavage in response to Fas triggering and had no effect on GrB-mediated cleavage of Bid [191].

The U_s3 protein kinase may impose a similar mechanism of inactivation on Bid cleavage mediated by caspase-8 and GrB. It is quite possible that U_s3 may induce a similar conformational restraint by phosphorylating residues near caspase-8 cleavage site or other available residue(s) possibly around GrB cleavage site (**Fig. 13C**). Interestingly, two serine residues at positions 76 and 78 are present at close proximity of GrB proteolytic cleavage sequence IEPD. These

serine residues are conserved in human and in murine Bid. Moreover, this site contains arginine residues at positions 68, 71, 84, and 88 in the human Bid. These arginine residues create a basic environment which is compatible with the extremely basic consensus sequence proposed for the U_s3 phosphorylation site. Phosphorylation of S76 and/or S78 by U_s3 may counteract the activity of phosphatases that are activated by apoptosis-inducing signals such as exposure to CD8T cells. This in return would render Bid resistant to cleavage and activation. Such phosphorylation event may be responsible for our observed phenomenon where CD8T cell- and GrB-induced cleavage of Bid is inhibited in U_s3 expressing cells.

An equivalent structural and/or conformational change could be envisaged for Bad, where phosphorylation of Bad by U_s3 may prevent exposure of the BH3 domain. As it has been previously shown, the pro-apoptotic function of Bad is tightly regulated by serine phosphorylation at multiple residues [182-186]. In healthy cells or in the presence of survival signals such as growth factors, Bad is highly phosphorylated at several residues, and preferentially binds to and is sequestered by 14-3-3 scaffold protein. Protein kinases A (PKA) and B (PKB) are among cellular kinases, that phosphorylate Bad at serine residues S155, S112, and S136, respectively [184, 185, 192]. A death stimulus, such as withdrawal of growth factors, results in rapid de-phosphorylation of Bad, and thus its activation. Activated Bad can then interact with either Bcl-2 or Bcl-X_L to neutralize their anti-apoptotic function, and this neutralization is believed to account for the pro-apoptotic function of Bad [182, 183].

As previously noted, Bad contains an exposed BH3 domain and therefore is constitutively active in its native, non-phosphorylated form [126]. In the case of Bad, phosphorylation may also result in structural changes that would hinder the interaction of the BH3 motif with its binding partners, thus render Bad inactive. Datta et al. have recently shown that phosphorylation of Bad at S155 induced by survival factors disrupts its binding to Bcl-2 and promotes cell survival [193]. Interestingly S155 is located in the BH3 domain of Bad. This finding would further support our hypothesis where a U_s3-induced phosphorylation may serve as a means to block the activation of Bad in response to apoptotic stimuli.

Taken together, the findings here highlight the significance of phosphorylation-induced modifications in modulating the pro-apoptotic functions of the Bcl-2 family of proteins, namely that of Bid and Bad.

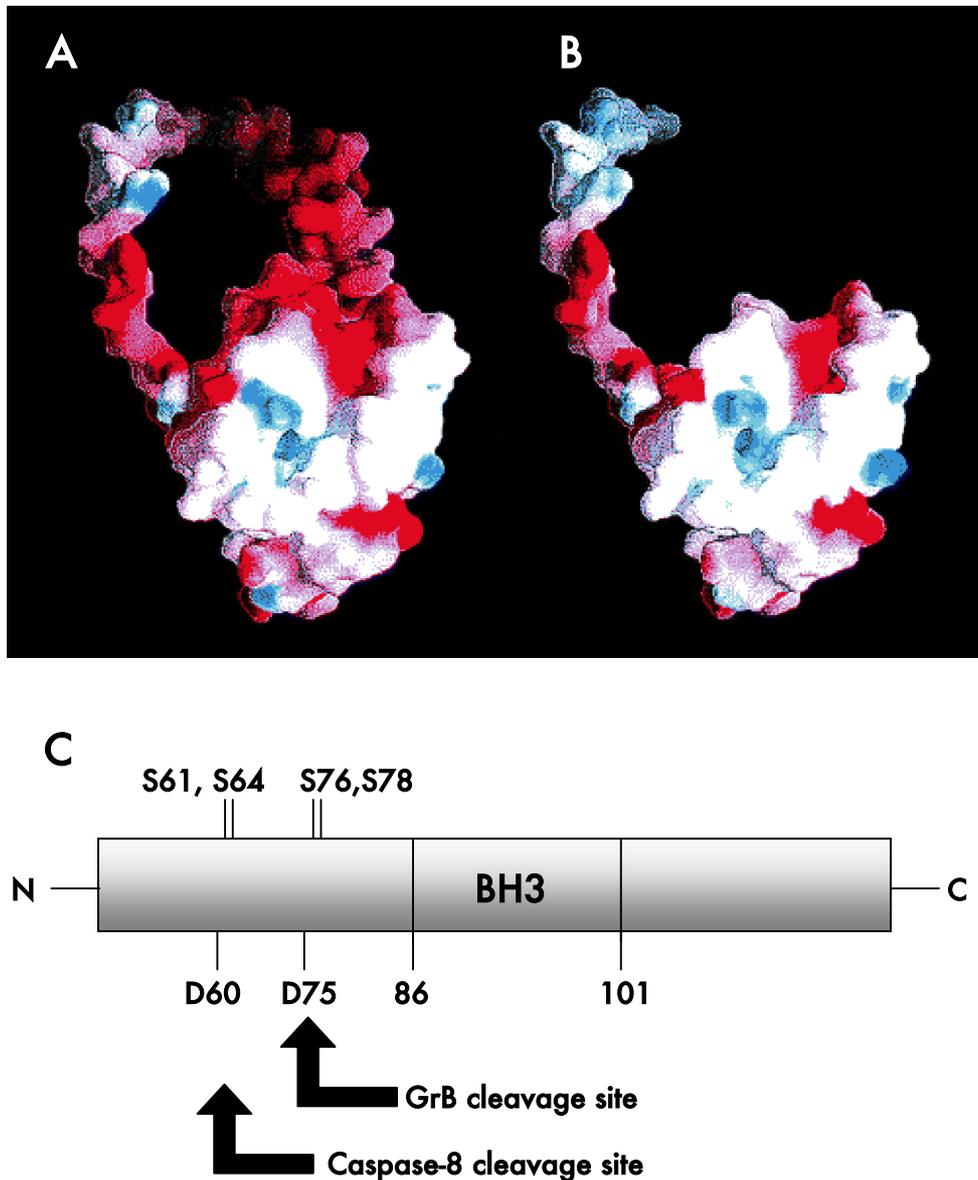


Figure 13. Three-dimensional structures of full-length (A) and cleaved Bid (B). Striking structural differences can be observed between the two forms of Bid. Note that the BH3 domain and the binding groove are exposed in cleaved Bid. **Schematic diagram of Bid structure (C).** Cleavage of Bid at D60 and D75 by caspase-8 and granzyme B, respectively, leads to the exposure of the BH3 domain and activation and its translocation to the mitochondria. Serine residues S61 and S64 are targets for phosphorylation by Casein kinases. S76 and S78 are the putative phosphorylation sites for U_s3.

6.2. U_s3 MAY TARGET SUBSTRATES OF CELLULAR SURVIVAL KINASES

Many cell types utilize the PKA and PKB signaling pathways to transform environmental signals into biochemical or cellular signals essential for survival (for in-depth reviews on PKA and PKB see references [194-197]). PKA is activated by cyclic adenosine mono-phosphate (cAMP) generated after ligation of a G-protein coupled surface receptor. Studies have shown that activation of PKA in response to cAMP inhibits apoptosis induced by a variety of stimuli including γ -irradiation, and treatment with the DNA damaging agent etoposide. PKB is activated by the action of phosphoinositol 3-kinase (PI-3-K) which itself is activated by tyrosine kinases coupled to growth factor and G-protein coupled receptors. Activation of PKB also has an anti-apoptotic effect since it has been shown to block cell death induced by different stimuli such as growth factor removal. The anti-apoptotic activities of PKA and PKB are tightly associated with phosphorylation of Bad, and this function is especially important for neuronal cell survival [184, 185, 192].

It is worth mentioning that many viruses require up-regulation of the PKB signaling pathway to sustain long-term infections [198]. The hepatitis B X protein (HBx) of hepatitis B virus (HBV) is one of the viral proteins produced after random integration of viral DNA into the host during chronic HBV infection and HBV-associated hepatocellular carcinoma (HCC). This viral protein functions as a transcriptional transactivator of a variety of cellular and viral promoter and enhancer elements [199]. HBx was shown to block transforming growth factor (TGF)- β -induced apoptosis in hepatoma cells by inducing PI-3-K activity and phosphorylation of both Bad and PKB [200]. This phosphorylation event in turn correlated with the inhibition of caspase-3 activation in a p53-dependent manner, thus blocking cell death [201].

Several observations implicate U_s3 in mimicking the functions of PKA and PKB. The first argument in support of this hypothesis is based on the optimal consensus sequence for U_s3 (see section 4.5), which closely resembles the target sequences of PKA and PKB. Therefore, it seems plausible that U_s3 would target substrates of PKA and PKB that are essential for cell survival. As previously mentioned, phosphorylation of Bad by PKA and PKB is the key event not only for their signaling pathways but also in linking apoptotic signals to the mitochondrial

death pathway [184]. Phosphorylation of Bad by U_s3 might, therefore, be of great significance in neuronal cells where induction of apoptosis would be highly unfavorable. Significance of Bad in cell survival was recently demonstrated in transgenic mice containing *BAD*^{3SA/3SA} mutation, in which the three serine residues 112, 136 and 155 of Bad, were changed to alanines [202]. Data from these mice demonstrated that neuronal cell populations were dependent on survival factor-mediated phosphorylation and inactivation of Bad. These mice were also hypersensitive to Fas-mediated cell death, and exposure to γ -irradiation. Furthermore, the phenotype of mice with *BAD*^{3SA/3SA} and *PKB*^{-/-} mutations are very similar, confirming that PKB could be an important regulator of Bad activity *in vivo* [202].

U_s3 may also target nuclear substrates of PKB such as Forkhead family of transcription factors. Phosphorylation of Forkhead proteins by PKB blocks apoptosis through regulation of cell death genes, including Fas ligand [203, 204]. In the absence of survival factors and PKB activity, Forkhead transcription factors translocate to the nucleus and initiate gene transcription [205]. Expression and secretion of the Fas ligand, which acts in an autocrine or paracrine manner, activates the cell surface Fas receptor and the caspase cascade leading to apoptosis [206]. In the presence of growth factor signals, PKB is activated and phosphorylates the Forkhead family members, resulting in their sequestration by 14-3-3 and inactivation, in a similar manner as Bad [205]. Interestingly, Fas ligand was shown to be potently upregulated by Forkhead family members in neurons upon survival factor withdrawal [207]. This finding indeed would have a great significance in terms of why would the function of U_s3 parallel that of PKB especially in neurons. Blocking induction of Fas ligand by U_s3 due to virus-induced stress signals would prevent death of the reservoir cells for HSV-1.

Further evidence in support of the notion that the function of U_s3 parallels that of PKB is provided by their similar anti-apoptotic activities. PKB has been shown to: 1) inhibit UV light-mediated cell death by blocking caspase-3 activation and preventing the release of cytochrome c, and 2) inhibit apoptosis induced by over-expression of the pro-apoptotic Bcl-2 family members Bak, Bax, and Bad [208]. U_s3 was also shown to: 1) block UV light-induced caspase-3 activation [154] and cytochrome c release [209-211], and 2) block apoptosis induced by

over-expression of Bid, Bad, and Bax [212]. Both kinases are therefore capable of inhibiting apoptosis induced by the release of cytochrome *c*, and by both the BH3 only and the Bax subfamily of the pro-apoptotic Bcl-2 family of proteins.

Another possible function for U_s3 would be targeting PKA or PKB for phosphorylation directly, thus bypassing their need for activation by receptor signaling. This idea is supported by a recent publication where it was demonstrated that: 1) activation of PKA by forskolin (a common agent used to activate PKA) blocked apoptosis induced by a U_s3 mutant virus strain; 2) U_s3 phosphorylated several peptides known to contain PKA phosphorylation sites, and one of these peptides contained the auto-phosphorylation site for the regulatory subunit of PKA, RII α , and 3) PKA RII α subunit was shown to be phosphorylated in U_s3-dependent manner *in vitro* [213].

The presented data here point to the fact that U_s3 might functionally overlap PKA and PKB pathways by modulating the activity of these kinases either by targeting them directly or by targeting their substrates for phosphorylation. Modulation of these signaling pathways by U_s3 provides the virus with an important alternative to block the mitochondrial death pathway, and serves as a function that would substitute for the lack of expression of any known HSV-1 Bcl-2 homologue.

6.3. U_s3 MAY DIFFERENTIALLY MODULATE CYTOTOXIC FUNCTIONS OF CYTOLYTIC LYMPHOCYTES

Cytolytic lymphocytes (CTL, LAK and NK) are the major immunological effectors involved in clearing HSV-1 infections. NK cells provide the first line of innate immune defenses against HSV-1 followed by that of the acquired antigen-specific CTL responses [214, 215]. CLs share common pathways in inducing cell death despite their different modes of activation. Unlike CD8T and LAK cells, NK cells are spontaneously cytotoxic and do not require prior immunization for their lytic activity. Moreover, while CD8T cell triggering and activation is MHC class I-dependent, NK cell functions are regulated by a set of inhibitory and activating receptors that act in MHC class I-dependent and -independent manners, respectively [216-219].

Several lines of evidence demonstrate that CD8T lymphocytes play an important role in controlling HSV-1 infections in experimental mouse models. First, primary sensory neurons were shown to up-regulate expression of MHC class I molecules during the acute phase of infection and several days after virus clearance [220]. Up-regulation of MHC class I in neurons opposes the conventional view that lack of neuronal MHC class I expression protects this vital cell type against the cytotoxic attack mediated by CD8T lymphocytes [221]. Second, expression of MHC class I genes was shown to profoundly influence the severity of acute infection in the sensory nerve ganglia, in that severity of disease was two to three orders of magnitude higher in H-2^K than in H-2^D animals [222]. This would suggest that CD8T cells play an important protective role against HSV-1 infection since the function of MHC class I is to present viral antigens to CD8T cells and induce their activation in response to the presented foreign peptides [223].

Third, quantitative histological and histochemical approaches show that the host immune response mediated by CD8T cells does not destroy infected neurons [224]. The number of viral antigen positive neurons in spinal ganglia of immunocompetent hosts at the peak of HSV-1 infection is significantly greater than neuronal loss. However, a strikingly high proportion of ganglionic neurons are destroyed in mice depleted of CD8T cells. Thus, termination of productive ganglionic infection is not dependent on destruction of infected neurons. Furthermore, mice treated with anti-CD8 antibody fail to clear the virus from the nervous system [224]. Taken together, these data demonstrate that neuronal survival depends on adequate recognition of infection by CD8T cells.

The function of NK cells might be more crucial during early phases of infection, to limit the extent of viral spread prior to elicitation of specific immune responses. Rager-Zisman et al. elaborately demonstrated a direct role for early involvement of NK cells in protection of mice against HSV-1 infection [225]. In their study, they pretreated mice with cyclophosphamide (CY), an agent which is known to depress NK activity with a maximal effect between days 1 and 4 after treatment, prior to infection with HSV-1. They showed that treatment of these mice, that are naturally resistant to HSV-1 infection, with CY rendered them susceptible to infection and led to a mortality rate of 100% between days 5 and 8 of infection.

Pathogenesis of HSV-1 in CY treated mice correlated with a significant high titer of the virus in brain and liver, which are known to be target organs for herpes simplex virus in the mouse [226]. Passive transfer of spleen cells from normal donors restored resistance of these mice to HSV-1 infection. This protective ability was attributed to cells that were of NK1.1 and GM₁⁺ phenotype, both of which are markers characteristic of NK cells [227]. Additional evidence in support of the critical role of NK cells in controlling HSV-1 infection is provided by observations indicating that: 1) NK cells prevent HSV-1 production in cell culture; 2) cells infected with HSV-1 are more sensitive to NK lysis due to expression of viral proteins [228-231]; 3) NK cells protect mice from mortality due to HSV-1 encephalitis and death after intranasal and ocular infections, respectively [232, 233], and 4) NK cells prevent direct anterior-to-posterior spread of HSV-1 in the eye [234].

In papers I and II, we have identified U_s3 as an important viral protein in modulating the sensitivity of target cells to lysis by CD8T cells but with virtually no effect on lysis mediated by LAK or NK cells [178]. The absence of the inhibitory activity of U_s3 in response to NK cells, whose function may be crucial in early time points of infection and against epithelial cells, may reflect the lack of importance in protecting cells of mucosal surfaces from apoptosis. The presence of this anti-apoptotic activity, on the other hand, would be required by the virus to protect neurons from late CD8T cell cytotoxic responses. In line with this hypothesis, the U_s3 protein kinase was shown to have a protective effect in primary afferent neurons infected with HSV-2 [235, 236]. Consistent with this finding and the above assumption is the fact that the anti-apoptotic activity of U_s3 against apoptosis induced by treatment with numerous agents including thermal shock, sorbitol, TNF α , anti-Fas antibody, and ceramide is cell type dependent [209]. Galvan et al. demonstrated that infection of the SK-N-SH cell line, which is a human neuroblastoma cell line, with HSV-1 protected this cell line from apoptosis induced by the agents above. Infection of HeLa cells with HSV-1; However, showed no protective effect against treatment with the same agents [209]. This protective function of HSV-1 was attributed to the anti-apoptotic activity of U_s3.

The differential role of U_s3 in modulating cytotoxic responses of CLs to HSV-1-infected cells may represent an interplay between host cell type being infected and the type of immune cells present at the time of infection. This interaction will allow a short window of opportunity for HSV-1 to infect cells of the nervous system before being cleared from the host entirely. The survival of the virus is thus ensured once it has infected neurons. This interaction will also ensure protection of neuronal cells from death whose destruction might not only have deleterious consequences for the host but also devastating effects for the virus itself.

7. CONCLUDING REMARKS

The findings of this thesis implicate U_s3 as a key component of the anti-apoptotic machinery evolved in HSV-1. U_s3 appears to be an exceptionally powerful protein since it can sufficiently target and inactivate multiple components of various apoptotic mechanisms. The function of U_s3 is further complimented by the action of other viral proteins. Together, these proteins cooperate and ensure the survival of the virus under harsh conditions set to eliminate this foreign pathogen from its host.

FIGURE ACKNOWLEDGMENTS

Figures 1 and 3 were adapted from **T. J. Taylor et al.** (2002) Herpes simplex virus. *Frontiers in Bioscience* **7**, 752-764.

Figure 2 was adapted from **J. P. Weir et al.** (2001) Regulation of herpes simplex virus gene expression. *Gene* **271**, 117-130.

Figure 4 was adapted from **J. C. Reed et al.** (2004) The domains of Apoptosis: A Genomics Perspective. *Sci. STKE* **239**, 1-29:re9.

Figure 13 (A and B) was adapted from **J. M. McDonnell et al.** (1999) Solution Structure of the Proapoptotic Molecule Bid: A structural Basis for Apoptotic Agonists and Antagonists. *Cell* **96**, 625-634.

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