Chapter 2
Modulation of Apoptotic Pathways by Herpes Simplex Viruses

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1. Molecular Mechanisms Implicated in Virus-Induced Apoptosis

Viral infections perturb many strictly monitored biochemical processes and thus inhibit cellular protein synthesis, disrupt membrane integrity, modify metabolism, elicit cytokine production, modulate the activity of signaling pathways, alter cellular gene expression, and affect cell-cycle progression. Such perturbations, in turn, frequently trigger apoptosis either of the infected or bystander cells (Razvi and Welsh, 1995; Roulston et al., 1999; Ameisen et al., 2003; Koyama and Adachi, 2003).

Apoptotic cell death is a type of cell deletion characterized by stereotypic cytomorphological changes, such as nuclear compaction, DNA fragmentation to nucleosome-sized fragments, cytoplasmic condensation, membrane blebbing, and cell shrinkage, resulting in cellular breakdown into membrane-bound apoptotic bodies phagocytosed without evoking an inflammatory response (Wyllie et al., 1980). Depending on the origin of the death-inducing signal, the biochemical steps of apoptosis can be mediated by intrinsic and extrinsic pathways. The apoptotic response can be divided into three phases: (i) initiation, (ii) effector phase, and (iii) degradation (see Fig. 2.1).

2. The Intrinsic Apoptotic Pathway

2.1. Inductive Phase

The intrinsic apoptotic response is triggered by stimuli that are generated inside the cells. Developmental death signals, viral infections, lack of survival factors, hypoxia, oncogene activation, contradictory signals for cell-cycle progression, telomere shortening, and DNA damage caused by irradiation, cytotoxic drugs, or other harmful stimuli all may act as inducers of apoptotic cell demise (Razvi and Welsh, 1995; Roulston et al., 1999; Ameisen et al., 2003). The molecular events constituting the induction phase of the intrinsic pathway are extremely

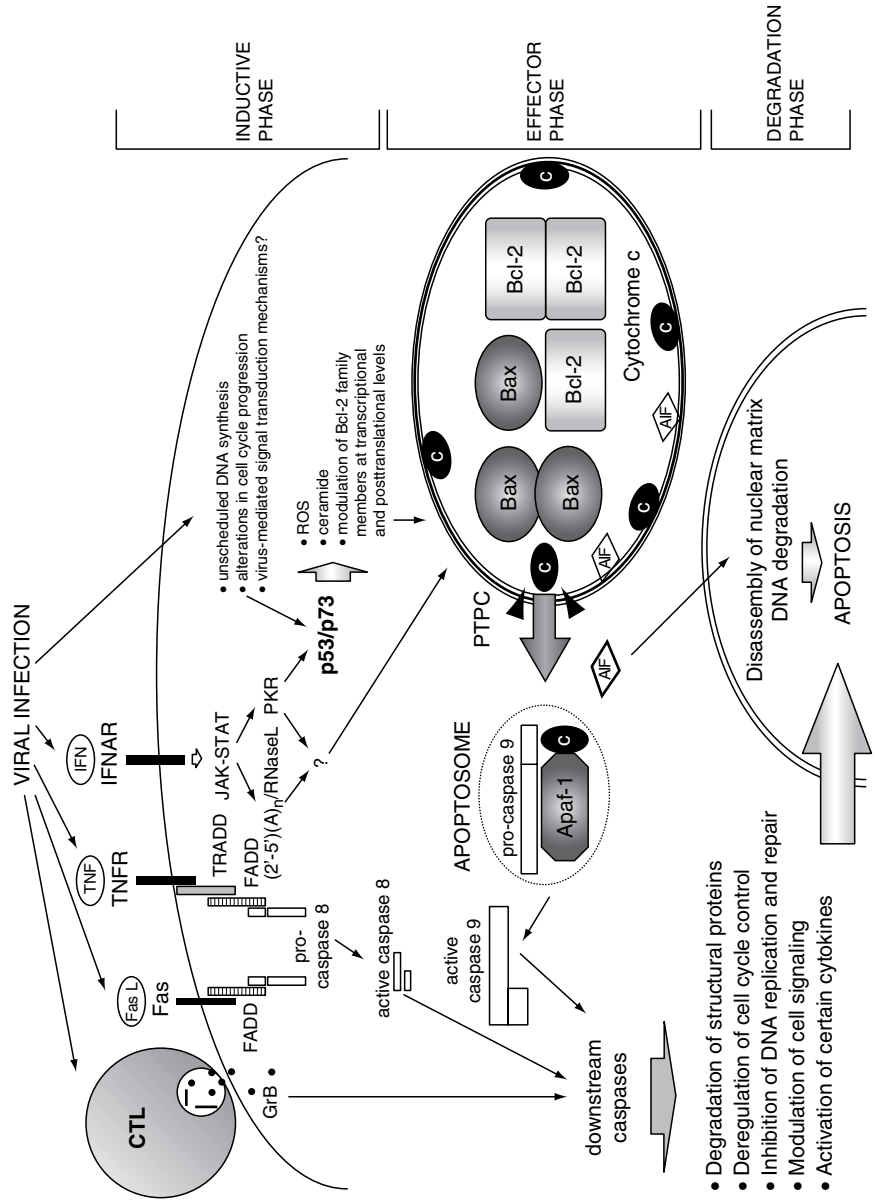

FIGURE 2.1. Apoptotic processes triggered by viral infections.

heterogenous and can be mediated by p53-dependent and some p53-independent mechanisms (Razvi and Welsh, 1995; Roulston et al., 1999; Ameisen et al., 2003).

2.2. *Effector Phase*

The proapoptotic and antiapoptotic pathways, which have been activated by viral infections, are integrated and amplified in the mitochondria. Several proapoptotic components of the apoptotic machinery perturb mitochondrial membrane integrity and disrupt transmembrane potential ($\Delta\Psi_m$) leading to the release of intermembrane proteins, such as cytochrome *c* into the cytoplasm (Kroemer et al., 1995, 1997; Kluck et al., 1997; Susin et al., Li et al., 1997a; Zou et al., 1999). Cytochrome *c* binds to the cytosolic apoptotic protease-activating factor-1 (Apaf-1); the complexes formed are stabilized by Apaf-1–mediated hydrolysis of ATP/dATP (Li et al.,1997a). Apaf-1 recruits and activates pro-caspase-9 and thereby facilitates the assembly of cytochrome *c*/Apaf-1/pro-caspase-9 complexes, termed apoptosomes (Zou et al.,1999). The formation of these complexes represents the commitment step in the mitochondria-initiated apoptotic pathway. In response to apoptotic stimuli, other proteins, including apoptosis-inducing factor (AIF), endonuclease G (Endo G), HtrA2/Omi, and second mitochondrial activator of caspases/direct inhibitor of apoptosis binding protein with low isoelectric point (Smac/Diablo), are also released from mitochondria (Susin et al., 1999; Chai et al., 2000; Du et al., 2000; Li et al., 2001; Candé et al., 2002; Hegde et al., 2002; Verhagen et al., 2002; van Gurp et al., 2003). AIF transmits death signal from the mitochondria to the nucleus and causes chromatin condensation and degradation of double-stranded DNA to fragments of 50 kb (Susin et al.,1999; Candé et al., 2002). Endo G can attack dsDNA, single-stranded DNA or RNA and thereby facilitate DNA and also RNA degradation during apoptosis (Li et al., 2001). The serine protease HtrA2/Omi and Smac/Diablo are negative regulators of the inhibitor of apoptosis proteins (IAPs), which control the activity of caspase-3, -7, and -9 (Chai et al., 2000; Du et al., 2000; Hegde et al., 2002; Verhagen et al., 2002; van Gurp et al., 2003). The HtrA2/Omi-mediated proteolytic degradation of IAPs abrogates their inhibitory effect on caspases leading to the activation of caspase-dependent apoptotic processes (Hegde et al., 2002; Verhagen et al., 2002; van Gurp et al., 2003). HtrA2/Omi can also trigger caspase-independent apoptosis through its own serine protease activity. Binding of Smac/Diablo to IAPs triggers apoptosis by promoting the release and activation of caspases (Chai et al., 2000; Du et al., 2000; van Gurp et al., 2003). Proteins belonging in the Bcl-2 family play a pivotal role in the control of the mitochondria-initiated caspase activation pathway (Kroemer, 1997; Borner, 2003).

2.3. *Degradation Phase*

The caspase family of cysteine proteinases consisting of 14 members (caspase-1 to caspase-14) can be characterized by its specificity for cleavage after aspartic acid residues (Thornberry and Lazebnik, 1998; Riedl and Shi, 2004). Caspases are synthesized as inactive proenzymes consisting of a prodomain, a large

(~20 kDa) and small (~10 kDa) subunit connected by a linker region (Thornberry and Lazebnik, 1998; Earnshaw et al., 1999; Garrido and Kroemer, 2004; Philchenkov, 2004; Riedl and Shi, 2004). Activated upstream (initiating) caspases, such as caspase-2 (also called ICH-1, Nedd-2), -8 (also called FLICE, MACH, Mch5), -9 (also called Mch6, ICE-LAP6), -10 (also called FLICE2), and -11 (also called mICH-3, mCASP-11) cleave and activate downstream (effector) caspases including caspase-3 (also called CPP-32, Yama, Apopain), -6 (also called Mch2), and -7 (also called Mch3, ICE-LAP3, CMH-1) responsible for the execution of apoptosis (Thornberry and Lazebnik, 1998; Earnshaw et al., 1999; Garrido and Kroemer, 2004; Philchenkov, 2004; Riedl and Shi, 2004). Effector caspases trigger the proteolysis of specific subsets of cellular proteins (Thornberry and Lazebnik, 1998; Earnshaw et al., 1999; Garrido and Kroemer, 2004; Philchenkov, 2004; Riedl and Shi, 2004). These caspase substrates include proteins involved in cell structure, signaling, cell cycle, DNA repair or function as cytokines (Thornberry and Lazebnik, 1998; Earnshaw et al., 1999; Garrido and Kroemer, 2004; Philchenkov, 2004; Riedl and Shi, 2004). Caspase-mediated cleavage may lead to the degradation and inactivation of several proteins, such as nuclear lamins, actin, STAT1, Raf1, Akt1, NF-κB, pRb, poly(ADP-ribose) polymerase (PARP), DNA-dependent protein kinase (DNA-PK), and the inhibitor of caspase-activated DNase/DNA fragmentation factor-45 (ICAD/DFF-45). Many other proteins, including mitogen-activated protein kinase/ERK kinase kinase1 (MEKK1), cytosolic phospholipase A_2 (cPLA2), protein kinase C (PKCδ and PKCθ), p21-activated kinase 2 (PAK2), IL-1β, IL-16, and IL-18, may become constitutively activated by caspases. Activated signal transducers act to turn on death-promoting pathways and to turn off survival pathways. The proteolytic degradation of structural proteins leads to cytomorphological changes characteristic of apoptosis, including mitochondrial damage, DNA fragmentation, and the formation of apoptotic bodies. Inflammatory cytokines processed on caspase cleavage attract phagocytes and facilitate the removal of apoptotic cells.

3. Cellular Regulators of Apoptosis

The cellular regulators of apoptosis include Bcl-2 family member proteins, the p53 thanscription factor family, and the interferon system. Their interactions are shown on Figure 2.1, but they are not discussed here (for details, see the reviews by Gross et al., 1999; Levrero et al., 2000; Irwin et al., 2001; Borner, 2003; Chawla-Sarkar et al., 2003; Table 2.1).

4. The Extrinsic Apoptotic Pathway

The extrinsic apoptotic response is triggered by several members of the tumor necrosis factor (TNF) cytokine family through engagement of “death receptors” belonging in the TNF receptor (TNFR) family (Almasan and Ashkenazi, 2003; Dempsey et al., 2003; Ware, 2003, 2005).

TABLE 2.1. IFN-α/β–inducible proteins and their characteristics.

Protein	Characteristics	Principal activity
Proteins involved in the antiviral and/or proapoptotic responses		
(2′-5′)$(A)_n$ synthetase/RNase L system	Multienzyme system	Synthesis of 2′,5′-oligoadenylates and cleavage of ssRNA antiviral and proapoptotic effect
PKR kinase	Serine-threonin kinase	Inhibition of translation of viral mRNA antiviral and proapoptotic effect
Mx proteins	GTPases of the dynamin family	Inhibition of virus replication
STAT1	Transcription factor (STAT family)	Signal transduction of IFN-α/β
IRF-1, IRF-2, IRF-3, IRF-7, ISGF3γ/IRF-9	Transcription factors (IRF family)	Modulation of IFN-α/β gene expression, antiproliferative, antiviral, and proapoptotic effect
Fas	Cell-surface receptor, TNFR family	Distinct roles in the biochemical events of apoptosis
TRAIL	Member of the TNF family	
Caspase-8	Cysteine protease	
XAF-1	XIAP-interacting protein	
RID-2	Inositol hexakisphosphate kinase 2	
PML	Component of nuclear bodies	
DAP/ZIP-kinases	Serine-threonin kinases	
PLSCR1	Membrane protein affecting movement of plasma membrane phospholipid	
Proteins involved in the immune response		
MHC class I	Transmembrane protein of the immunoglobulin supergene family	Antigen processing and immune recognition
MHC class II	Transmembrane protein of the immunoglobulin supergene family	Antigen processing and immune recognition
β_2 microglobulin	Light chain of MHC class I	Antigen processing and immune recognition
Proteins with poorly characterized roles as mediators of biological effects of IFN-α/β	GBP-1, Ifi 17/15-kDa protein, ISG15, ISG54, ISG56, ISG58, metallothionein II, IP10, 200 family, 9-27, 6-16, 1-8, C56, 561, dsRNA adenosine deaminase, RAP46, hypoxia-inducible factor-1, NF-IL6-β	

Death ligands (DL), including TNF-α, FasL, lymphotoxins (LT), LT-related inducible ligand that competes for glycoprotein D binding to herpesvirus entry mediator on T cells (LIGHT), TRAIL, and ectodysplasin-A2 (EDA-A2), are type II transmembrane glycoproteins forming trimeric structure that is essential for their biological activity. The TNF family ligands occur in membrane-bound and secreted forms and interact with one or more specific cell-surface receptors (Almasan and Ashkenazi, 2003; Dempsey et al., 2003; Ware, 2003, 2005).

TNFR family members can be subdivided into two subfamilies, which may either contain death domain (DD) or have a recognition motif for TNFR-associated factors (TRAFs), respectively. The DD containing receptors transmit apoptotic signals, while TNFRs recruiting TRAFs activate signal transduction pathways implicated in cell survival. Recently, a novel TNFR family member, the X-linked ectodermal dysplasia receptor (XEDAR), has been identified, which in spite of the absence of a discernible DD is capable of inducing apoptosis. Thus, XEDAR may represent the third subgroup of the TNFR family (Almasan and Ashkenazi, 2003; Dempsey et al., 2003; Ware, 2003, 2005).

Death receptors (DR), including TNFR1, Fas/CD95, TRAIL-R1 and -2, DR3 and DR6, as well as ectodermal dysplasia receptor (EDAR) activate apoptosis after ligand binding through recruitment of adaptor proteins and upstream pro-caspases to form a complex termed death-inducing signaling complex (DISC) (Almasan and Ashkenazi, 2003; Dempsey et al., 2003; Ware, 2003, 2005). A characteristic structural feature of adaptor proteins, such as TNFR-associated DD-containing protein (TRADD) and Fas-associated DD-containing protein (FADD), is that they contain a DD and also a death effector domain (DED). DD and DED of the adaptors enable them to bind the DD of the activated receptor and to interact with pro-caspases-8 and/or -10, respectively (Almasan and Ashkenazi, 2003; Dempsey et al., 2003; Ware, 2003, 2005). Upon formation of the DISC, by autocatalysis, pro-caspases-8 and -10 become activated and released from the cellular membrane to process downstream caspases leading to a series of proteolytic events that contribute to apoptosis (Almasan and Ashkenazi, 2003; Dempsey et al., 2003; Ware, 2003, 2005). The cells fully armed to execute this extrinsic, caspase 8/10-dependent apoptotic program are termed type I cells. In type II cells, the signal generated by death receptors has to be amplified in the mitochondria to trigger apoptosis. It has been shown that the Bcl-2 family member Bid can provide a link between the death receptor–initiated (extrinsic) and mitochondria-dependent (intrinsic) apoptotic pathways (Trapani et al., 1999; Korsmeyer et al., 2000; Degli Espoti et al., 2003; Martin and Vuori, 2004; Sprick and Walczak, 2004).

The death receptor–mediated apoptosis is controlled by the cellular FLICE-inhibitory protein (cFLIP) family (Krueger et al., 2001). These proteins, by binding to the DISC, can inhibit pro-caspase-8 (also called FLICE) recruitment and can also prevent proteolytic processing of caspase-8. Thus, cFLIPs can inhibit apoptosis signaling originated from the death receptors (Krueger et al., 2001).

5. The Effects of Herpes Simplex Virus 1 (HSV-1) and Herpes Simplex Virus 2 (HSV-2) on Cell Fate

HSVs invade the body through the skin and mucous membranes and cause a lytic infection in epithelial cells. Virus particles then pass through the sensory nerve endings and are transported to sensory ganglia by retrograde axonal flow. HSVs establish latent infection in neurons, characterized by the lack of virus replication

and by the presence of viral RNA transcripts, termed latency associated transcripts (LATs). The viral genome is maintained in an episomal state in the neurons, and from this cellular reservoir reactivation may occur (Whitley, 1996).

HSVs are capable of infecting different histological types of cells, including epithelial cells, fibroblasts, neurons of sensory ganglia and central nervous system, lymphocytes, monocytes, and dendritic cells. Lytic infection leads to cell death, while the latently infected neurons survive. Both necrotic and apoptotic mechanisms are implicated in the cytopathogenicity evoked by HSV-1 and HSV-2. Recent observations demonstrate that the development of apoptosis after HSV infections largely depends on the histological type of infected cells. A great body of experimental evidence also indicates that HSVs evolved complex strategies to counteract the process of apoptotic death triggered by the infection (Whitley, 1996; Roizman and Knipe, 2001).

6. Induction of Apoptosis by HSV-1 and HSV-2

Interesting studies have shown that the infection of the epithelial HEp-2 cell line with wild-type (wt) HSV does not lead to apoptosis (Galvan and Roizman, 1998; Aubert et al., 1999). HSV could induce apoptosis only in the absence of *de novo* protein synthesis in this cell line (Aubert et al., 1999). Inhibition of protein synthesis by using cycloheximide possibly abrogates the synthesis of proteins required for the inhibition of cell death. It has also been demonstrated that deletion mutant HSV strains lacking the genes that encode infected cell protein (ICP) 4, ICP22, or ICP27 are able to trigger apoptotic cell death (Leopardi and Roizman, 1996; Galvan and Roizman, 1998; Aubert and Blaho, 1999; Aubert et al., 1999, 2001; Galvan et al., 2000; Zhou and Roizman, 2000; Zachos et al., 2001; Hagglund et al., 2002; Sanfilippo et al., 2004). Apoptosis induced by these deletion mutant viruses in HEp-2 cells was shown to involve the activation of several caspases, decreased expression of Bcl-2, mitochondrial release of cytochrom *c,* and PARP cleavage (Leopardi and Roizman, 1996; Galvan and Roizman, 1998; Aubert and Blaho, 1999; Aubert et al., 1999, 2001; Galvan et al., 2000; Zhou and Roizman, 2000; Zachos et al., 2001; Hagglund et al., 2002; Sanfilippo et al., 2004). Collectively, these data indicate that HSVs trigger both the intrinsic and the extrinsic apoptotic pathways, however ICP4, ICP22, and ICP27, as well as several other viral proteins block the development of apoptosis in epithelial cells. Studies focusing on the proapoptotic effect of these viruses have also shown that virion binding, membrane fusion, and uncoating are not sufficient and *de novo* viral protein synthesis is not required for the induction of apoptosis in response to HSV infection (Sanfilippo et al., 2004). To identify the viral gene products that display apoptogenic capabilities, a mutant, designated to HSV-1 *d*109, possessing deletions of all five immediate early (IE) genes, has been constructed. Infection with the *d*109 mutant virus does not allow viral gene expression and protein synthesis and does not trigger apoptosis (Sanfilippo et al., 2004). These data indicate that the transcription of (IE) genes is the necessary

trigger for the HSV-mediated induction of apoptosis (Goodkin et al., 2004; Sanfilippo et al., 2004). It has been suggested that the presence of IE mRNAs in the infected cells, or the splicing, export, and translation of these viral mRNAs, by perturbing important cellular functions may initiate the cellular suicide program (Goodkin et al., 2004; Sanfilippo et al., 2004).

In contrast with the epithelial HEp-2 and Vero cell lines, laboratory signs of apoptosis, such as the annexin V-labeling or internucleosomal DNA fragmentation, were revealed in HSV-infected dendritic cells, activated T-lymphocytes, and monocytoid cell lines, such as HL-60 and U937 (Mastino et al., 1997; Fleck et al., 1999; Jones et al., 2003; Muller et al., 2004; Bosnjak et al., 2005). It has also been reported that both HSV-1 and HSV-2 elicit apoptosis in mouse pituitary gland, and corneal epithelial cells (Wilson et al., 1997; Gautier et al., 2003; Miles et al., 2003, 2004; Aita and Shiga, 2004). Together, these data demonstrate that the development of HSV-induced apoptosis is cell type specific (Mastino et al., 1997; Wilson et al., 1997; Fleck et al., 1999; Gautier et al., 2003; Jones et al., 2003; Miles et al., 2003, 2004; Aita and Shiga, 2004; Muller et al., 2004; Bosnjak et al., 2005). Elevated p53 protein level was detected in HSV-1–infected dendritic cells, indicating that this virus, by stabilizing this transcription factor, may possess the potential to activate the p53-dependent intrinsic pathway of apoptosis (Muller et al., 2004). Interestingly, in the same study, p53 was found to be sequestered in the cytoplasm of the infected cells (Muller et al., 2004). Although the role of viral macromolecules in the cytoplasmic sequestration of p53 has not been investigated, this novel finding clearly demonstrates that translocation of p53 into the nucleus is inhibited in HSV-1–infected cells. Moreover, no alterations were revealed after HSV-1 infection in the expressions of bcl-2 and bax genes, two known transcriptional targets of p53 (Muller et al., 2004). Thus, the transcriptional effect of p53 does not seem to play a major role in the proapoptotic effect of HSV-1 in dendritic cells. However, it cannot be excluded that the transcription-independent activity of p53 may be implicated in HSV-1–induced apoptosis, because this transcription factor can inhibit the antiapoptotic function of Bcl-2 and Bcl-x_L through direct physical interaction at the mitochondrial membrane. Experiments using the monocytoid HL-60 cell line revealed that HSV-2 decreases the level of Bcl-2 protein (Mastino et al., 1997). Thus, the effect of HSV on the expression of Bcl-2 family members may be cell type specific. Several data demonstrated that HSVs are capable of inducing the production of IFNs and therefore have the potential to activate the IFN-mediated apoptotic response. HSV infection was also shown to elicit the synthesis of some death ligands, such as TNF-α and TRAIL and to activate caspase-8 in immature dendritic cells (Muller et al., 2004). In HSV-1–infected immature dendritic cells, decreased levels of the long variant of cFLIP (cFLIP$_L$) protein and increased levels of cFLIP mRNA were detected, indicating that HSV-1 affects cFLIP$_L$ expression at the protein level (Muller et al., 2004). Because cFLIP$_L$ is a powerful cellular inhibitor of caspase-8, decreased cFLIP$_L$ expression may lead to caspase-8 activation and induction of apoptosis in HSV-infected cells (Muller et al., 2004). In contrast, HSV-1 exerted no effect on the expression of cFLIP$_L$ in mature dendritic cells

(Muller et al., 2004). These data demonstrate that HSV activates the extrinsic pathway of apoptosis in immature dendritic.

Together, these data indicate that both the intrinsic and the extrinsic apoptotic pathways are triggered by HSVs (Leopardi and Roizman, 1996; Koyama and Adachi, 1997; Mastino et al., 1997; Wilson et al., 1997; Galvan and Roizman, 1998; Fleck et al., 1999; Aubert et al., 1999, 2001; Galvan et al., 1999, 2000; Aubert and Blaho, 1999, 2001; Zhou and Roizman, 2000; Zachos et al., 2001; Hagglund et al., 2002; Gautier et al., 2003; Jones et al., 2003; Miles et al., 2003, 2004; Aita and Shiga, 2004; Blaho, 2004; Goodkin et al., 2004; Muller et al., 2004; Sanfilippo et al., 2004; Bosnjak et al., 2005). These viruses affect several components of the apoptotic machinery; HSV infection increases the level of p53, downregulates the expression of Bcl-2, activates caspases, and decreases the level of $cFLIP_L$ protein in certain experimental systems. A characteristic feature of the cytopathogenicity evoked by these viruses is that it highly depends on the histological type of the infected cells (Leopardi and Roizman, 1996; Koyama and Adachi, 1997; Mastino et al., 1997; Wilson et al., 1997; Galvan and Roizman, 1998; Fleck et al., 1999; Aubert et al., 1999, 2001; Galvan et al., 1999, 2000; Aubert and Blaho, 1999, 2001; Zhou and Roizman, 2000; Zachos et al., 2001; Hagglund et al., 2002; Gautier et al., 2003; Jones et al., 2003; Miles et al., 2003, 2004; Aita and Shiga, 2004; Blaho, 2004; Goodkin et al., 2004; Muller et al., 2004; Sanfilippo et al., 2004; Bosnjak et al., 2005). It has already been suggested that the HSV-evoked cellular response can be classified into three subgroups (Mastino et al., 1997). Inhibition of apoptosis dominates in persistently infected tissues of the central nervous system and maintains cell survival (Table 2.2). Cells in which HSV causes lytic infection die primarily by way of necrosis, while apoptosis is efficiently blocked in epithelial cells of the mucocutaneous tissues by the synthesis of protein products coded by the IE and several other viral genes. Finally, the virus elicits apoptotic demise in myeloid and lymphoid tissues (Table 2.2).

It has also been proposed that apoptosis induction in immature dendritic cells may represent an immune escape strategy for HSV, because death and functional impairment of this professional antigen-presenting cell type may disturb the development of the adaptive immune response required to control the infection (Mastino et al., 1997; Fleck et al., 1999; Jones et al., 2003; Bosnjak et al., 2005). The enhanced expression of death ligands, such as TRAIL, by the infected cells may also represent another tactic serving immune evasion, as the membrane-bound form of TRAIL can trigger apoptosis in the infiltrating lymphocytes (Muller et al., 2004). However, apoptosis of the HSV-infected myeloid and

TABLE 2.2. The effects of HSV on cell fate.

	Cell type		
	Neurons	Epithelial cells	Hematopoietic cells
Virus replication	–	+	+
Type of infection	Latent	Lytic	Lytic
Dominant effect	Antiapoptotic	Antiapoptotic	Proapoptotic
Biological consequence	Survival	Necrosis	Apoptosis

lymphoid cells may cross-prime those antigen-presenting cells, which phagocytose the apoptotic macrophages and dendritic cells and thereby promotes the initial phase of the immune response. Apoptosis may also contribute to the development of organ-specific dysfunctions observed during HSV infections, but the precise role these viruses play in the pathogenic mechanism and in the immune response remains to be elucidated (Perkins et al., 2003a; Pretet et al., 2003; Sanfilippo and Blaho, 2003; Irie et al., 2004; Aurelian, 2005). As HSVs are under evaluation as virotherapy vectors in the treatment of malignant tumors and some other diseases (Meignier and Roizman, 1989; Andreansky et al., 1996, 1997; Roizman, 1996; Advani et al., 1999, 2002; Markert et al., 2000; Markovitz and Roizman, 2000; Spear et al., 2000; Skelly et al., 2001; Yamada et al., 2001; Chung et al., 2002; Perkins, 2002; Aubert and Blaho, 2003; Curi et al., 2003; Kamiyama et al., 2004; Stanziale et al., 2004; Currier et al., 2005; Kim et al., 2005), further studies allowing more insight into the proapoptotic features of these viruses are of great medical importance.

7. Inhibition of Apoptosis by HSV-1 and HSV-2

HSVs encode several products, including ICP4, ICP22, ICP27, US3, US5/gJ, U_L14, glycoprotein D (gD), ICP34.5, and LAT, which possess powerful antiapoptotic activity.

7.1. α4/ICP4, U_S1/α22/ICP22, and U_L54/α27/ICP27

ICP4 is a phosphoprotein that binds DNA in a sequence-specific manner and transactivates most β and γ genes, but it also acts as a repressor. It is an IE protein of HSV and possesses an essential function in virus replication. Three forms designated as ICP4a, 4b, and 4c have been described, having apparent molecular masses of 160, 163, and 170 kDa, respectively. ICP4a was shown to be present in the cytoplasm of infected cells, while ICP4b and 4c accumulated in the nucleus (Blaho and Roizman, 1991; Roizman and Knipe, 2001).

ICP22 is a phosphorylated and nucleotidylylated regulatory protein. It is an IE protein, which is required for the optimal expression of ICP0 and of a subset of γ proteins, but it is dispensable for virus replication (Roizman and Knipe, 2001).

ICP27 is a multifunctional, regulatory phosphoprotein with an apparent molecular mass of 63 kDa that affects viral RNA processing and export. ICP27 is an IE protein possessing an essential function in virus replication (Roizman and Knipe, 2001). Experiments demonstrating that cells infected with replication-incompetent deletion mutant viruses lacking the genes that encode ICP4 or ICP27 die by way of apoptosis provided clear evidence for the antiapoptotic function of these proteins (Leopardi and Roizman, 1996; Galvan and Roizman, 1998; Aubert and Blaho, 1999; Aubert et al., 1999, 2001; Galvan et al., 2000; Zhou and Roizman, 2000; Zachos et al., 2001; Hagglund et al., 2002; Sanfilippo et al., 2004). It has also been revealed that ICPs are synthesized between the time period

(3 and 6 h postinfection) that corresponds with the so-called apoptosis prevention window (Sanfilippo et al., 2004). This finding indicates that the accumulation of ICP4 and ICP27 correlates with the inhibition of apoptosis during HSV replication (Aubert et al., 2001; Sanfilippo et al., 2004). As ICP4 and ICP27 are essential for the expression of β and γ genes, it is possible that at least in part they exert antiapoptotic effect by stimulating the synthesis of some other early and late viral genes (Leopardi and Roizman, 1996; Galvan and Roizman, 1998; Aubert and Blaho, 1999; Aubert et al., 1999, 2001; Galvan et al., 2000; Zhou and Roizman, 2000; Roizman and Knipe, 2001; Zachos et al., 2001; Hagglund et al., 2002; Goodkin et al., 2004; Sanfilippo et al., 2004). Additionally, ICP4 and ICP27 may also affect cellular apoptotic processes directly (Leopardi and Roizman, 1996; Galvan and Roizman, 1998; Aubert and Blaho, 1999; Aubert et al., 1999, 2001; Galvan et al., 2000; Zhou and Roizman, 2000; Roizman and Knipe, 2001; Zachos et al., 2001; Hagglund et al., 2002; Goodkin et al., 2004; Sanfilippo et al., 2004). However, the contribution of direct and indirect mechanisms to the antiapoptotic effect of these viral proteins at present is unknown. Other experiments have shown that during the course of apoptosis triggered by ICP4-deficient HSV, ICP22 was cleaved by cellular caspases (Munger et al., 2003). The observation demonstrating that ICP22-deletion mutant HSV exhibits decreased protective effect as compared with wt virus further supports the antiapoptotic role of ICP22 (Aubert et al., 1999).

7.2. U_S3 and U_S5/gJ

U_S3 is a serine/threonin kinase with an apparent molecular mass of 66 kDa (Roizman and Knipe, 2001). It phosphorylates several virus-encoded proteins, such as the U_L34 membrane protein and U_S9 tegument protein (Roizman and Knipe, 2001). U_S3 may also target cellular proteins and modulate their activity by phosphorylation. The optimal consensus sequence for U_S3 phosphorylation was determined to be $(R)_nX(S/T)YY$, where $n = 3$, X can be Arg, Ala, Val, Pro, or Ser, and Y can be any amino acid with the exception of acidic residues. The consensus sequence recognized by U_S3 is similar to the consensus recognized by PKA and Akt/PKB. (Purves et al., 1987; Roizman and Knipe, 2001; Benetti and Roizman, 2004).

Several studies have demonstrated that U_S3 inhibits apoptosis triggered by replication-incompetent deletion mutants or other apoptogenic stimuli (Leopardi et al., 1997; Hata et al., 1999; Jerome et al., 1999; Asano et al., 2000; Munger and Roizman, 2001; Munger et al., 2001; Benetti et al., 2003; Cartier et al., 2003a, 2003b; Mori et al., 2003; Sloan et al., 2003; Ogg et al., 2004). The molecular mechanism of U_S3-mediated inhibition of apoptosis has been investigated in detail. It has been revealed that the enzymatic activity of U_S3 is required for the anti-apoptotic activity, since a catalytically inactive form of U_S3 was unable to inhibit apoptosis. It has also been shown that U_S3 exerts its anti-apoptotic effect by blocking the activation of some pro-apoptotic BH3-only members of the Bcl-2 family (Leopardi et al., 1997; Hata et al., 1999; Jerome et al., 1999; Asano et al.,

2000; Munger and Roizman, 2001; Munger et al., 2001; Benetti et al., 2003; Cartier et al., 2003a, 2003b; Mori et al., 2003; Sloan et al., 2003; Ogg et al., 2004).

The activity of Bad is known to be negatively regulated by phosphorylation. In the absence of apoptogenic stimuli, phosphorylation events, mediated by several cellular kinases, including PKA and Akt/PKB, maintain Bad in latent, inactive conformation and thereby preclude the activation of the cellular apoptotic machinery. Interesting studies have shown that the U_S3 protein kinase can phosphorylate peptides containing PKA phosphorylation sites, intact proteins being natural substrates of PKA and even PKA, itself (Munger and Roizman, 2001; Benetti et al., 2003; Ogg et al., 2004). Further experiments have demonstrated that U_S3 inhibits apoptosis by activating PKA and by phosphorylating proteins targeted by PKA (Munger and Roizman, 2001; Benetti et al., 2003; Ogg et al., 2004). It has also been revealed that Bad, an important substrate of PKA, can be phosphorylated by U_S3. The U_S3-mediated phosphorylation of Bad thus may compensate the lack of survival signals by inhibiting the proapoptotic effect of this BH3-only member protein (Munger and Roizman, 2001; Benetti et al., 2003; Ogg et al., 2004).

Recent studies have shown that the phosphorylation status of Bid regulates its susceptibility to caspase-8–mediated or granzyme B–mediated cleavage. Phosphorylation of Bid by cellular kinases, including casein kinases, is thought to reduce its spontaneous degradation and to ensure its specific susceptibility to proteolysis by caspase-8. Interesting experiments have shown that Bid may also serve as substrate for U_S3-mediated phosphorylation (Cartier et al., 2003a; Ogg et al., 2004). The post-translational modification of Bid by U_S3 blocked its cleavage by granzyme B. U_S3 phosphorylation possibly caused conformational change in the structure of Bid rendering this protein resistant to proteolytic processing (Benetti et al., 2003; Ogg et al., 2004). Moreover, reduced cleavage of Bid by granzyme B was shown to play a role in the resistance of cells expressing U_S3 to lysis mediated by CTLs (Cartier et al., 2003a). It has been revealed that several other proteins contain known or putative U_S3 phosphorylation sites. These proteins may play important roles in the apoptotic response and include members of the forkhead transcription factor family (AFX, FKHR, and FKHRL1), members of the Bcl-2 family (Bcl-x_L and Bcl-2, in addition to Bad and Bid), Akt/PKB, caspases-9, IκB, and some others. These data raise the possibility that U_S3 kinase may operate as a broad-spectrum inhibitor of apoptosis that possesses the capability to modulate numerous signal transduction pathways and several components of the cellular death machinery (Leopardi et al., 1997; Hata et al., 1999; Jerome et al., 1999; Asano et al., 2000; Munger and Roizman, 2001; Munger et al., 2001; Benetti et al., 2003; Cartier et al., 2003a, 2003b; Mori et al., 2003; Sloan et al., 2003; Ogg et al., 2004).

The U_S5 gene encodes a 10-kDa protein termed gJ (Roizman and Knipe, 2001). gJ has a single N-glycosylation site. The glycosylated form of this protein migrates near 17 kDa. It has been shown that U_S5/gJ deletion mutant virus has impaired antiapoptotic activity (Jerome et al., 2001). Transfected U_S5 was shown to confer protection against Fas- or UV light-induced apoptosis, and cells

expressing gJ were resistant to the apoptotic action of CTL cells or granzyme B (Jerome et al., 2001).

Although HSVs do not encode known homologues of Bcl-2, their U_S3 kinase is able to complement this lack by modulating the activity of some Bcl-2 family members. This HSV-encoded enzyme, by mimicking the functions of cellular signal transduction pathways involved in survival, affects the apoptotic processes in a pleiotropic and cell-type-specific way (Leopardi et al., 1997; Hata et al., 1999; Jerome et al., 1999; Asano et al., 2000; Munger and Roizman, 2001; Munger et al., 2001; Benetti et al., 2003; Cartier et al., 2003a, 2003b; Mori et al., 2003; Sloan et al., 2003; Ogg et al., 2004). The U_S3-mediated inhibition of target cell lysis by CTLs represents a novel and important escape strategy by which HSVs may continue their replication even in the face of a vigorous antigen-specific adaptive immune response (Cartier et al., 2003a; Sloan et al., 2003). Moreover, U_S5/gJ can serve as another tool for the inhibition of immune effector mechanisms (Jerome et al., 2001).

7.3. U_S6/gD

gD is an envelope glycoprotein required for attachment and penetration of HSV. gD was shown to bind to several different cell-surface receptors, including nectin 1, which is a member of the nectin family of intercellular adhesion molecules, herpesvirus entry mediator A (HVEM), which is a member of the TNFR family, and cation-independent mannose-6 phosphate receptor (Zhou et al., 2000a, 2003; Roizman and Knipe, 2001; Zhou and Roizman, 2002a, 2002b). Three functional domains have been identified in the structure of gD: one domain is required for the antiapoptotic effect of gD, and two domains are involved in membrane fusion (Zhou et al., 2000a, 2003; Zhou and Roizman, 2002a, 2002b). Remarkable studies by using the SK-N-SH cell line have shown that gD delivered in *trans* inhibits apoptosis triggered by gD deletion mutants viruses (Zhou et al., 2000a). Other interesting experiments have demonstrated that cocultivation of U937 cells with gD stable transfectants or exposure to the soluble form of gD blocks Fas-induced apoptosis (Medici et al., 2003). Moreover, further data provided clear evidence on the important role of NF-κB in the protection by gD against Fas-mediated apoptosis.

7.4. U_L14

U_L14 protein is a tegument protein with an apparent molecular mass of 25 kDa. This protein plays some role in the egress of virions from the infected cells during the replication cycle of HSV (Roizman and Knipe, 2001). U_L14 protein was shown to be endowed with HSP-like properties. HSPs are molecular chaperons that limit stress-induced cellular damage at least in part by inhibiting apoptosis. An interesting study has recently shown that transfected U_L14 confers protection against apoptosis triggered by osmotic shock and certain drugs, including etoposide, camptothecin, and staurosporine (Yamauchi et al., 2003). Moreover, U_L14

deletion mutant virus exhibited weaker inhibition of apoptosis than the rescued virus in HEp-2 cells (Yamauchi et al., 2003). It has been proposed that the U_L14-mediated inhibition of apoptosis, by maintaining nuclear integrity in the late phase of infection, may facilitate egress and cell to cell spread of HSV (Yamauchi et al., 2003).

7.5. $R_L2/\gamma_1 34.5$/ICP34.5

ICP34.5 is encoded in the inverted repeats of the unique long (U_L) sequence of HSV (Roizman and Knipe, 2001). There are two copies of the ICP34.5 gene in the genome (Roizman and Knipe, 2001). The N-terminal portion of the ICP 34.5 contains a sting of Arg. The middle region of the ICP34.5 gene carries variable numbers of repeats encoding proline-alanine-threonine (PAT repeat) (Chou and Roizman, 1990; Bower et al., 1999). The numbers of PAT repeats may range from 3 to 22 in different HSV strains and determine whether the protein is restricted to the cytoplasm or can also be present in the nucleus. Viruses with various PAT repeats were shown to differ in their plaque morphology and neurovirulence (Chou and Roizman, 1990; Bower et al., 1999). The C-terminal portion exhibits homology to the corresponding domains of the growth arrest and DNA damage 34 (GADD34) and myeloid differentiation 116 (MyD116) proteins and binds to protein phosphatase 1α (PP1α), as well as to proliferating cell nuclear antigen (PCNA) (Brown et al., 1997).

It has been revealed that ICP34.5 forms complexes with PP1α and counteracts the effect of PKR (Chou et al., 1995; He et al., 1997a, 1997b, 1998). ICP34.5 redirects PP1α to dephosphorylate eIF2α and thereby relieves the PKR-mediated block in protein synthesis (Chou et al., 1995; He et al., 1997a, 1997b, 1998). Thus, in the presence of ICP34.5, protein synthesis may continue even in the presence of activated PKR (Chou et al., 1995; He et al., 1997a, 1997b, 1998). These data are consistent with those results demonstrating that infection with ICP34.5 deletion mutant HSV (ICP34.5$^-$) activates PKR, which in turn phosphorylates and thereby inactivates eIF2α leading to protein synthesis shutoff. It has also been revealed that serial passages of the ICP34.5$^-$ virus in cell cultures resulted in further alterations in the HSV-1 genome (Cassady et al., 1998b). These secondary mutations have been precisely analyzed, and ICP34.5$^-$ virus, carrying an insertion containing the native U_S10 and U_S11 genes driven by the α47 promoter, was able to complement ICP34.5 and could preclude the block in protein synthesis (Cassady et al., 1998b). In the ICP34.5$^-$ compensatory mutant, U_S10 and U_S11 genes were converted to early genes (Cassady et al., 1998b). Together, these data show that, in addition to ICP34.5, HSV-1 has elaborated a second, cryptic mechanism to counteract the action of PKR (Cassady et al., 1998b). It has been demonstrated that the ICP34.5$^-$ induces apoptotic cell death (Chou and Roizman, 1992; Coukos and Roizman, 1992; Lan et al., 2003). Thus, the HSV ICP34.5 protein inhibits the IFN-mediated intrinsic apoptotic pathway by blocking the effects of PKR.

7.6. R_L3/LAT

LATs are encoded by inverted repeat sequences flanking U_L sequences of the HSV genome (Roizman and Knipe, 2001). This region is termed latency-associated transcriptional unit (LATU) (Roizman and Knipe, 2001). The primary LAT transcript is 8.3 kb. Splicing of the 8.3-kb transcript yields a stable 2-kb LAT intron and an unstable 6.3-kb LAT. The 2-kb LAT can also be further spliced in infected neurons to yield a 1.5-kb transcript. The majority of the 2-kb LAT is not polyadenylated and is not capped. Within the LATU, a gene that is antisense and partially overlaps the LAT gene was recently identified and termed antisense to LAT (Meignier et al., 1988; Perng et al., 2000b; Roizman and Knipe, 2001; Kang et al., 2003; Kent et al., 2003; Bloom, 2004).

The functions of LATs were unknown for a long time, however remarkable data proved that these viral transcripts promote establishment and reactivation from latency. It has also been revealed that LAT exhibits antiapoptotic effect and thereby promotes the survival of the infected cells. Moreover, novel findings strongly support that the role of LATs in latency and reactivation is linked to their antiapoptotic effect (Perng et al., 2000a; Inman et al., 2001; Thompson and Sawtell, 2001; Roizman and Knipe, 2001; Ahmed et al., 2002; Henderson et al., 2002; Perng et al., 2002; Hunsperger and Wilcox, 2003; Jin et al., 2003, 2004; Kent et al., 2003; Peng et al., 2003, 2004b, 2005; Bloom, 2004; Branco and Fraser, 2005).

Experiments using a series of viruses containing deletions in various regions of the LAT gene, as well as plasmids expressing various portions of LAT, have shown that the expression of just the first 1.5 kb of the primary LAT transcript is sufficient to decrease the ratio of apoptotic cells and caspase-9 activation. Because this region was also shown to be sufficient to confer high spontaneous reactivation phenotype for HSV-1 in animal model system, LAT-mediated apoptosis inhibition and reactivation appear to be linked. These data have also provided strong evidence that LAT has two antiapoptosis regions, which map from LAT nucleotides 76 to 447 and from 1500 to 2850 (Inman et al., 2001; Ahmed et al., 2002; Jin et al., 2003; Kang et al., 2003; Kent et al., 2003; Bloom, 2004; Peng et al., 2004b).

The effect of LATs on apoptotic processes involves complex mechanisms. LAT was shown to inhibit caspase-8– and caspase-9–induced apoptosis, indicating that LAT affects both the intrinsic and the extrinsic pathways of apoptosis. It has already been revealed that LAT modulates the expression of certain Bcl-2 family members (Peng et al., 2003). An increase in the mRNA level of the proapoptotic Bcl-x_S was detected in Neuro-2A cells infected with the McKrae LAT null mutant (LAT^-) compared with cells inoculated with wt HSV-1 (Peng et al., 2003). Consistant with this result, the transfected LAT inhibited the accumulation of Bcl-x_S mRNA (Peng et al., 2003). Moreover, this finding suggested that LAT promotes splicing of Bcl-x to Bcl-x_L rather than to Bcl-x_S or affects the stability of the two splice variants of Bcl-x (Peng et al., 2003). Triggering the accumulation of Bcl-x_L in neurons, by leading to an antiapoptotic shift in the stoichiometric

ratio of Bcl-2 family members, may promote survival and enhance the latency-reactivation cycle of HSV.

Interesting recent studies have revealed that LAT$^-$ HSV-1 elicits higher IFN-α and IFN-β production, proceeding with faster kinetics, than that induced by wt HSV-1 in cultures of SK-N-SH and Neuro-2A cells, as well as in trigeminal ganglia of infected mice (Peng et al., 2005). Furthermore, higher proportions of IFN-α-5, -6, or -10 and lower proportions of IFN-α-1, -7, -14, -17, and -21 were detected in the LAT$^-$ HSV-1–infected human neuroblastoma cells than in cultures infected with wt virus, respectively. The fact that LAT possesses the ability to modulate differentially the expression of several IFN-α subtypes is of great importance because the various IFN-α subtypes elicit different biological effects (Peng et al., 2005). Together, these careful studies demonstrate that LAT interferes with and delays IFN expression and suggest that LAT-mediated inhibition of the IFN-system may promote survival of HSV-infected neurons during latency (Peng et al., 2005).

7.7. ICP10

Both HSV-1 and HSV-2 encode a ribonucleotide reductase enzyme (RR) composed of large (RR1) and small (RR2) subunits with apparent molecular masses of 140 kDa and 38 kDa, respectively. RR is not necessary for virus replication in dividing cells, whereas it is essential in neural cells. RR1 is an IE gene, whereas RR2 follows the expression kinetics of early genes. HSV-1 RR1 is termed ICP6, and HSV-2 RR1 is designated ICP10. RR contains a carboxy-terminal region responsible for RR activity, a transmembrane segment, and an amino-terminal domain possessing intrinsic serine/threonin kinase activity. The homology in the serine/threonin kinase domain of HSV-2 ICP10 and HSV-1 ICP6 is only 38%. HSV-2 ICP10 is able to exert both auto- and transphosphorylating effect, whereas HSV-1 ICP6 possesses only autophosphorylating activity.

It has recently been demonstrated that HSV-2 ICP10, but not HSV-1 ICP6, exerts antiapoptotic effect that involves the activation of the Ras/Raf/MEK/MAPK signal transduction pathway. ICP10 binds and phosphorylates the GTPase-activating protein Ras-GAP (Langelier et al., 2002; Perkins et al., 2002, 2003b; Chabaud et al., 2003; Gober et al., 2005a). Ras-GAP acts as a negative regulator of Ras activity by promoting the intrinsic GTPase activity of Ras. Inactivation of Ras-GAP by ICP10 results in an increase in the level of activated Ras leading to the activation of the downstream Raf/ MEK/MAPK kinase cascade and induction of the Bcl-2–associated athanogene (Bag-1) (Townsend et al., 2005). Bag-1 exists in three isoforms and interacts with a wide array of cellular proteins, including Raf, Bcl-2, HSPs, components of the proteasome, and several other proteins involved in apoptosis, cell signaling, growth, and division (Townsend et al., 2005). Bag-1 exerts antiapoptotic and cytoprotective effects, and thereby it may play an important role in the molecular mechanism of ICP10-mediated inhibition of apoptosis (Langelier et al., 2002; Perkins et al., 2002, 2003b; Chabaud et al., 2003; Gober et al., 2005a).

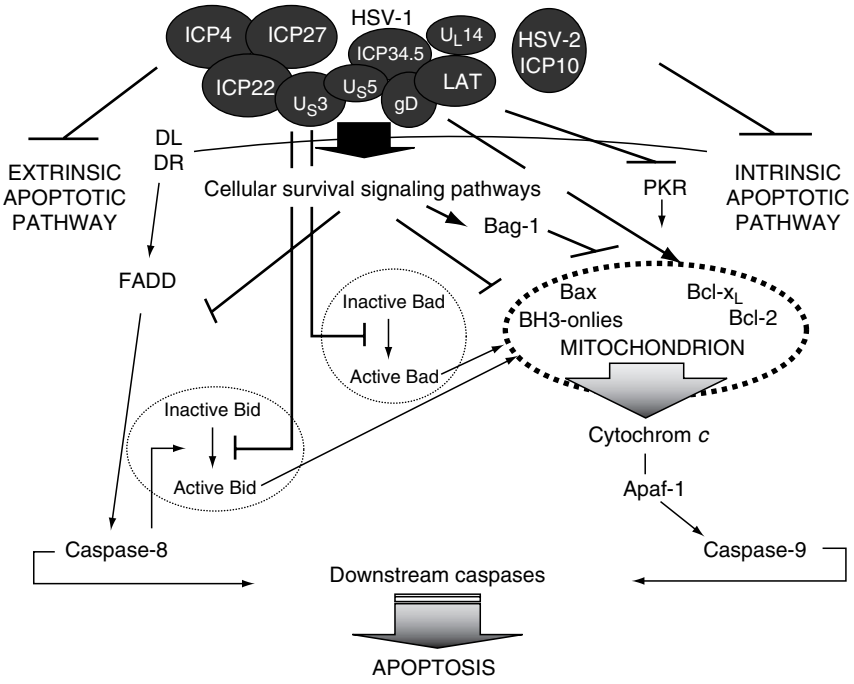

FIGURE 2.2. Antiapoptotic mechanisms activated by HSVs. HSVs activate survival kinases, counteract the action of PKR, and modulate the activity of several Bcl-2 family members, such as Bid, Bad, Bcl-2, and Bcl-x, leading to the inhibition of both the intrinsic and extrinsic pathways of apoptosis.

Together, these interesting data demonstrate that HSV elaborated several strategies to inhibit both the intrinsic and the extrinsic pathways of apoptosis (Fig, 2.2; Table 2.3). This virus is able to block the transcriptional activity of p53, to activate NF-κB (Goodkin et al., 2003; Taddeo et al., 2003, 2004; Gregory et al., 2004), to interfere with the IFN-system (Chee and Roizman, 2004), to modulate the activity of several Bcl-2 family members, to impede death receptor signaling and at the same time to stimulate survival kinases. HSV seems to promote cell survival from the very moment of attachment till the time of egress in a way that serves the needs of optimal virus replication. Apoptosis inhibition in HSV infections increases virus yields, helps spread, facilitates reactivation, and serves as an immune evasion mechanism. Table 2.3 shows the principal activites of the proapoptotic and the antiapoptotic gene products of HSV.

TABLE 2.3. Activities of the proapoptotic and the antiapoptotic gene products of HSV.

Virus	Effect of gene products	Mechanism of action
	Proapoptotic	
HSV-1 and HSV-2	IE mRNA	The presence, splicing, export, and translation of IE mRNAs may trigger apoptosis by perturbing important cellular functions.
	Antiapoptotic	
	ICP4, ICP22, and ICP27	Inhibit the cellular apoptotic process directly (e.g., by stabilizing Bcl-2).
		Inhibit the cellular apoptotic process indirectly by stimulating the expression of other HSV-encoded antiapoptotic genes.
	U_S3	Phosphorylates and thereby inactivates some proapoptotic BH3-only members of the Bcl-2 family, such as Bad and Bid, and possibly modulates several other components of the apoptotic death machinery.
	U_S5/gJ	Cooperates with U_S3 and inhibits Fas-induced apoptosis.
	U_S6/gD	Binds to cellular receptors, such as nectin 1, herpes virus entry mediator A, and cation-independent mannose-6 phosphate receptor, and inhibits Fas-induced apoptosis.
	U_L14	Exhibits HSP-like properties.
	ICP34.5	Forms complexes with protein phosphatase 1 α and counteracts the effect of PKR.
	LAT	Inhibits the activation of caspase-8 and -9.
		Stimulates the accumulation of the antiapoptotic Bcl-X_L.
		Inhibits and delays IFN expression.
HSV-2	ICP10	Phosphorylates and inactivates the GTPase-activating protein Ras-GAP leading to the activation of the Ras/Raf/MEK/MAPK kinase cascade and induction of the antiapoptotic Bag-1.

For further details, please refer to the text and the references cited therein.