[Frontiers in Bioscience 10, 2788-2803, September 1, 2005]

HERPES SIMPLEX VIRUS TYPE 2 ENCODES A HEAT SHOCK PROTEIN HOMOLOGUE WITH APOPTOSIS REGULATORY FUNCTIONS

Michael D. Gober, Samantha Q. Wales, and Laure Aurelian

Department of Pharmacology and Experimental Therapeutics, University of Maryland School of Medicine, Baltimore MD. 21201

TABLE OF CONTENTS

1. ABSTRACT

The decision to undergo apoptosis lies in the balance between pro- and anti-apoptotic proteins. Since virus replication relies on the cellular machinery, viruses have evolved various strategies to alter this balance. They target the Bcl-2 and signaling protein kinase (PK) apoptosis modulatory families by encoding homologues or altering the expression of the cellular proteins. The heat shock proteins (Hsp) are emerging as a new family of apoptosis modulatory proteins and are also a target of virus modification. Hsp function in protein folding and activation, often assisted by co-chaperones. They complex with nascent or damaged proteins and chaperone them for refolding and resumption of function, or for proteosomal degradation. Until recently, Hsp were considered strictly anti-apoptotic, possibly by virtue of their contribution to the removal of damaged and undesirable client proteins. However, recent studies have also begun to associate the Hsp with pro-apoptotic functions (1). Herpes simplex virus type 2 (HSV-2) encodes two proteins homologous to Hsp family members. One of these, known as ICP10PK, is a homologue to a newly cloned Hsp (H11) and modulates virus-induced apoptosis. ICP10PK is unique among the viral proteins that regulate apoptosis in that it targets all the families of apoptosis modulatory proteins. It activates the ERK signaling pathway, stabilizes Bcl-2 and upregulates Hsp70 and Hsp27 as well as the Hsp70 co-chaperone Bag-1. Its ability to commandeer these families of apoptosis regulators is required for HSV-2 replication and latency establishment/ reactivation.

2. INTRODUCTION

Unlike eukaryotic and prokaryotic organisms, viruses are unable to replicate independently. They evolved various strategies to hijack the cells, forcing them to become virus producing factories. A basic mechanism used by the cells to escape virus control is programmed cell death, also known as apoptosis. Apoptosis limits virus replication and prevents/reduces the infection of neighboring uninfected cells. It is an irreversible process

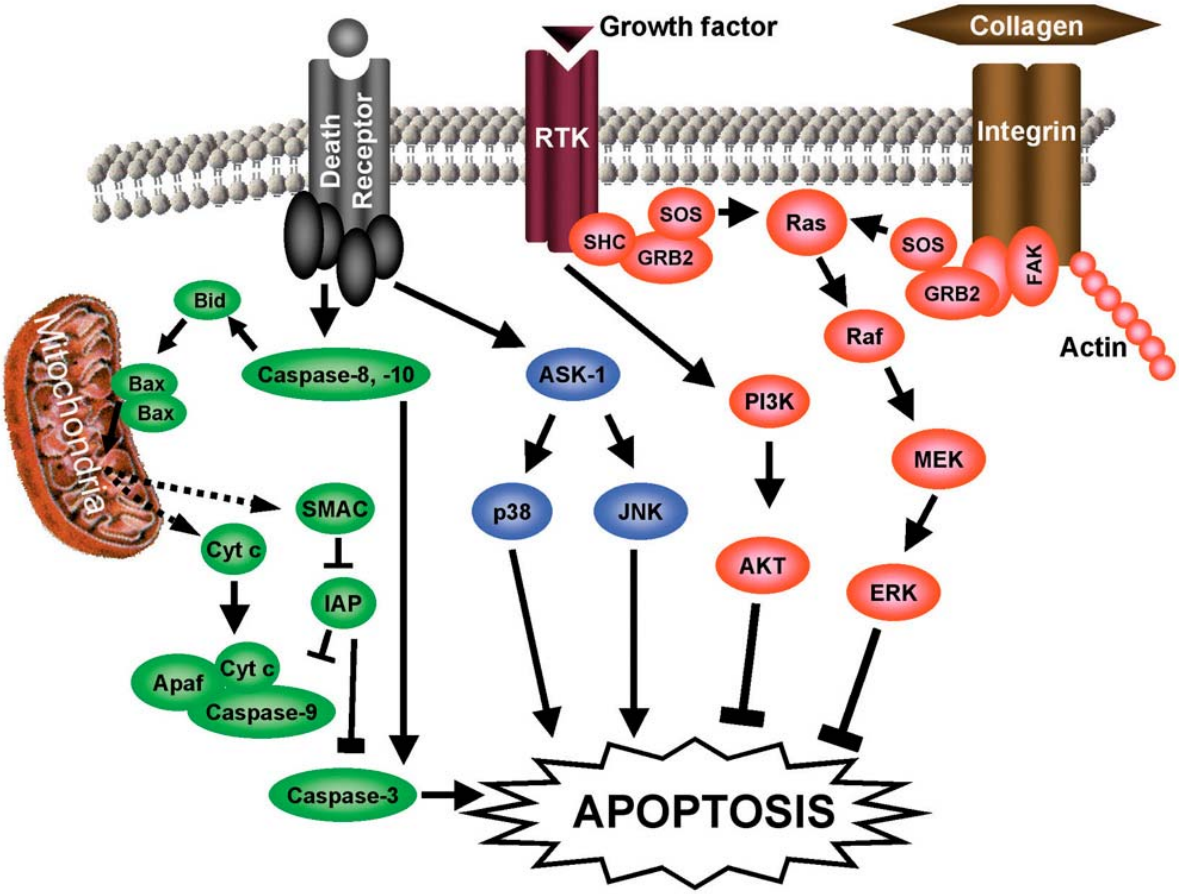

Figure 1. Protein kinase signaling pathways and apoptotic cascade. Schematic representation of protein kinase signaling pathways involved in apoptosis regulation. The intrinsic apoptosis cascade involves release (dashed arrows) of pro-apoptotic mediators such as cytochrome c (Cyt c) and Smac/DIABLO (Smac) resulting in caspase-9 and -3 activation and apoptosis. The extrinsic pathway involves "death" receptor activation such as interaction of Fas-ligand with Fas or tumor necrosis factor alpha (TNF-alpha) with the TNF receptor (TNFR) leading to capase-8 or -10 activation and resulting in activation of caspase-3 directly or by activating the intrinsic pathway. Activation of the death receptors can also lead to apoptosis through activation of c-Jun N-terminal kinase (JNK) or p38MAPK (p38) through activation of apoptosis signaling kinase 1 (ASK-1). Activation of a receptor tyrosine kinase (RTK) on the cell surface can inhibit apoptosis by activating the Ras/Raf/MEK/ERK signaling pathway or the phosphatidylinositol 3-kinase (PI3K)/ AKT signaling pathway. The Ras signaling pathway can also be activated by the interaction of integrins with the extracellular matrix such as collagen. Solid arrows indicate activation while solid lines resembling the letter T signify inhibition.

regulated by gene families that consist of pro- and anti-apoptotic members and the balance of which determines the cell's fate. Viruses have evolved various strategies to utilize the apoptosis modulatory genes in order to insure their survival. Here, we briefly review some of these mechanisms focusing on Hsp and an HSV-2 protein (ICP10PK), that is an Hsp homologue (2,3).

3. APOPTOSIS MODULATORY GENE FAMILIES

Apoptosis is a tightly regulated, irreversible process that results in cell death in the absence of inflammation. Execution of the apoptotic death program is an energy dependent process. It requires expression and activation of proteins which ultimately lead to nuclear and cytoplasmic condensation, intranucleosomal DNA cleavage, and blebbing of the cell into membrane-bound apoptotic bodies. Classically, apoptosis is mediated by cysteine proteases with aspartate specificity known as caspases. Signaling cascades involved in apoptosis are schematically represented in Figure 1.

Functionally distinct protein families regulate apoptosis. Two of these, the Bcl-2 and PK signaling proteins, are well recognized. The Bcl-2 family consists of over 20 proteins all of which contain at least one Bcl-2 homology domain (BH1- BH4). They regulate apoptosis by affecting mitochondrial permeability (4). The group consists of pro- and anti-apoptotic members that form homo- and heterodimers, regulating each others' function. Pro-apoptotic members, such as Bax and Bak, homodimerize leading to the permeablization of the mitochondrial outer membrane and the release of cytochrome c and apoptosis inducing factor (AIF). Anti-

apoptotic members, such as Bcl-2 or Bcl-X_L, form heterodimers with Bax and Bak, thereby preventing mitochondrial permeablization.

A diverse group of PKs that trigger pro- or anti-apoptotic signaling pathways (Figure 1) are also known to regulate apoptosis. Pro-apoptotic signaling pathways, such as those induced by environmental stress stimuli or the oligomerization of death receptors result in the recruitment of apoptosis-regulating kinase 1 (ASK1) and lead to activation of the pro-apoptotic c-Jun N-terminal kinase (JNK) and p38MAPK protein cascades (5). Anti-apoptotic signaling pathways are triggered by survival stimuli. One of these pathways involves a kinase cascade that initiates with c-Raf-1 and involves phosphorylation (activation) of MAP kinase kinase (MEK) and extracellular signal-regulated kinase (ERK, also known as MAPK). Another of the survival anti-apoptotic pathways involves Akt whose targets, along with ERK, include genes required for cell cycle progression and the altered balance between pro- and anti-apoptotic Bcl-2 proteins (6,7). Activation of the ERK or Akt survival pathways can override apoptotic cascades triggered by various stimuli (8,9).

Proteins in the inhibitor of apoptosis protein (IAP) family also regulate apoptosis (Figure 1). IAPs are characterized by the presence of one to three zinc binding regions of ~70 amino acids in length known as baculovirus IAP repeat (BIR) domains (10). Family members inhibit apoptosis by direct interaction with caspase-3, -7, or -9 (10). Pro-apoptotic members have not yet been identified. To inhibit IAP activity, mitochondria release Smac/Diablo which binds IAP, thereby allowing caspase activity (11) (Figure 1).

4. HEAT SHOCK PROTEINS ARE AN APOPTOSIS REGULATORY FAMILY

Hsp are a newly emerging family of apoptosis regulators. They are highly conserved proteins which are upregulated by various stress conditions and function as molecular chaperones in regulating homeostasis. Virtually all family members are associated with thermotolerance and cytoprotection (12), attributed at least in part, to apoptosis inhibition (13). However, the exact mechanism of Hsp anti-apoptotic activity is still unclear, and Hsp overload after extreme stress can also contribute to apoptosis (14). Increased Hsp expression can lead to their translocation to the cell surface, thereby increasing immune mediated cell death (15), and they can enhance other apoptotic signals (16, 17). A recent report that an Hsp family member (H11) has its own pro-apoptotic activity (1) establishes the Hsp as a *bona fide* family of apoptosis regulators that consist of both pro- and anti-apoptotic members.

Hsp are known to function in protein folding and activation, assisted by co-chaperones, such as Bag-1. Hsp70 complexes with nascent or damaged proteins and chaperones them for refolding and function resumption, or for degradation by the proteosome complex. Bag-1 is an Hsp70 co-chaperone that interacts with the ATP binding site of Hsp70. Through this interaction, (i.e. by competing for binding to the Hsp70 ATPase binding domain), Bag-1 downregulates the refolding chaperone properties of Hsp70 with client proteins being targeted instead for proteosomal degradation (18). Bag-1 associates with the proteosome in an ATP-dependent manner and promotes binding of Hsp70 to the proteolytic complex (19). Hsp90 has also been implicated in proteosomal degradation of client proteins (20). Removal of client proteins by proteosomal degradation is a potential mechanism of Hsp anti-apoptotic activity, leading to cell survival and proliferation. By the same token, however, removal of proteins that are required for cell survival could be deleterious and result in apoptosis. An interesting question is whether Hsp modulate apoptosis strictly as a passive or active chaperone, or whether they can also function by a chaperone-independent protein-protein interaction. Recent data indicate that Hsp70 inhibits JNK and AIF independent of ATPase (chaperone) activity (21), suggesting that protein-protein interactions are also involved in the apoptosis regulatory activity of the Hsp.

4.1. Hsp bridge cell signaling/apoptotic pathways and the immune response

Because Hsp interact with the proteasome, they can function in antigen presentation and stimulation of the immune response. Recent data indicate that Hsp are components of the putative presentasome, an organized cellular region in antigen presenting cells (APC) in which proteins are degraded and peptides are loaded onto the major histocompatibility complex (MHC) class I and/or II for presentation to T cells (adaptive immunity) (Figure 2). Hsp family members bind to antigenic peptides and facilitate presentation (22, 23). In addition Hsp can be released into the extracellular compartment and Hsp-peptide complexes are taken up by APC through the CD91 receptor for presentation on MHC class I and class II molecules (cross presentation) (22) (Figure 2). In addition Hsp also function as cytokines capable of stimulating antigen-independent (innate) immunity. Hsp70 induces APC to release inflammatory cytokines such as TNF-alpha, interleukin (IL)-1beta, IL-6 and RANTES through its interaction with the CD14/toll-like receptor (TLR) complex and promotes dendritic cell maturation (24, 25). Hsp70 ATPase activity does not appear to be required for induction of inflammatory cytokines or dendritic cell maturation, because the Hsp70 peptide binding fragment (amino acids 359-610) elicits a similar response (25). Thus, Hsp function both as intracellular chaperones/apoptosis modulators and stimulators of adaptive and innate immunity. By virtue of their ability to influence cell life and death decisions intracellularly and in the context of the immune response, Hsp are a logical target for virus modulation, for example within the context of immune evasion.

4.2. Hsp70

Hsp70 family members are the most conserved of all Hsp (26). Members can be either constitutively expressed (viz. Hsc70) or induced by a variety of stresses including hyperthermia, oxidation, or cytotoxic drugs (viz. Hsp70). Once expressed, Hsp70 influences protein folding,

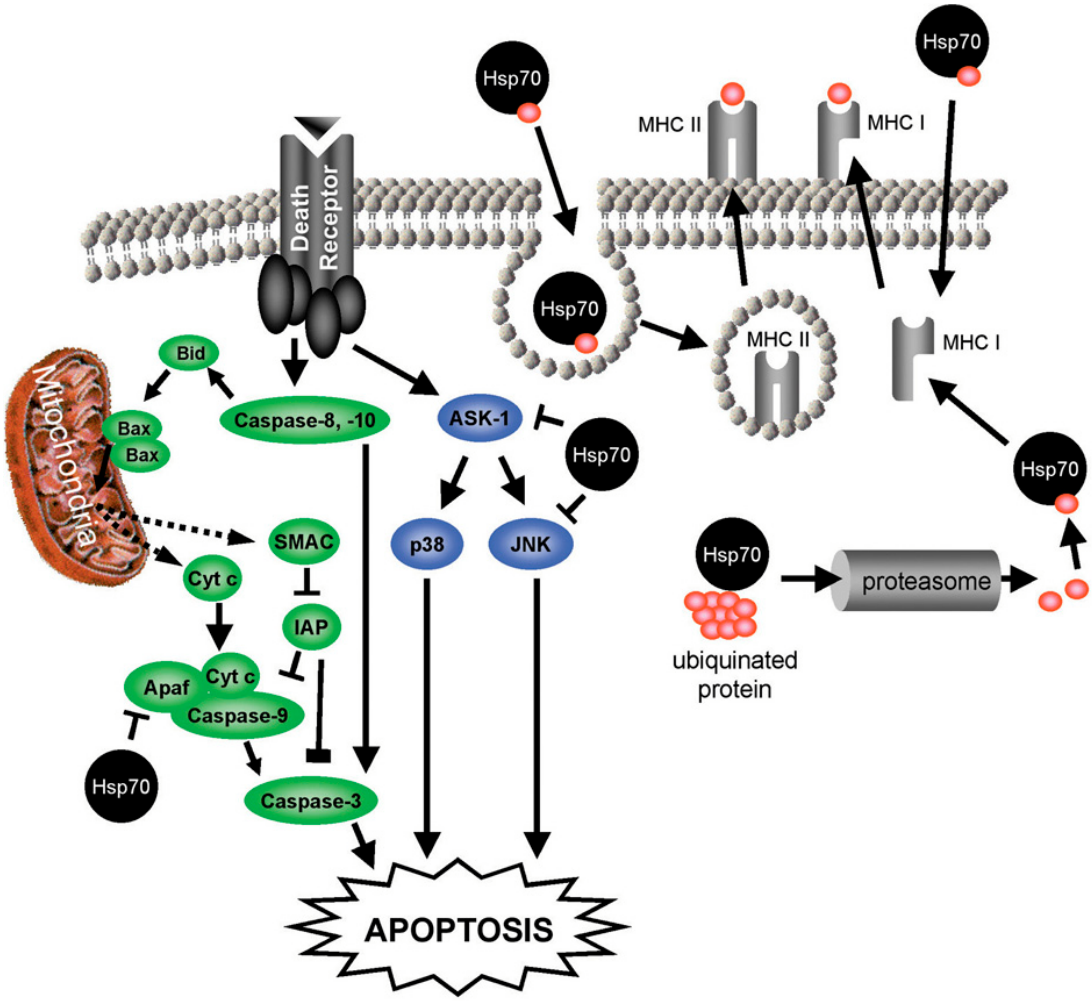

Figure 2. Hsp70 inhibits apoptosis and is involved in immune regulation by modulating protein degradation and antigen presentation. Hsp70 inhibits apoptosis by interfering with the formation of the apoptosome by binding to Apaf thereby preventing caspase-9 and caspase-3 activation. It also inhibits apoptosis by directly interacting with either ASK-1 or JNK. Hsp70 can aid in the degradation of ubiquinated proteins by chaperoning them to the proteasome. Hsp70 also binds the remaining peptides from the degraded protein and facilitates their presentation on the cell surface with the major histocompatibility complex class I (MHC I). The Hsp70-peptide complex, extruded from neighboring non-immune cells or nearby antigen presenting cells (APC), is endocytosed by the APC and the peptide is presented on the surface with MHC II. The Hsp70-peptide complex also transverses the plasma membrane and enters the intracellular antigen presentation pathway resulting the peptide presentation on the surface with MHC I.

prevents the formation of undesired protein aggregates, and when necessary, refolds misfolded proteins. Hsp70 facilitates the removal of damaged proteins by ubiquitin-mediated proteosomal degradation. It drives a multiprotein degradation complex that includes Hsp90, p60Hop, and the C-termini of the Hsc70-interacting proteins, CHIP and CAIR-1. The demonstration of a shift in the client-chaperone protein binding from Hsp90 to Hsp70 under distinct conditions, supports the central role of Hsp70 in this complex (18). These Hsp70 properties are attributed to the molecular chaperone activity of Hsp70, which is accelerated by catalyzing ATP hydrolysis to ADP and by associating with co-chaperones (viz. Hip, Hop, and Bag-1) in order to regulate ATPase activity (12). Significantly, however, Bag-1 also binds (and activates) Raf-1 kinase, a member of the PK signaling proteins. During periods of stress, the increased expression of Hsp70 competes for Bag-1, leading to downregulation of Raf-1 kinase activity and the arrest of DNA synthesis (27). Bag-1 also binds Bcl-2 increasing its stability and, thereby, its activity (28), providing a direct link for cross-talk between the two families.

In addition to maintaining the proper tertiary and quaternary state of various cellular proteins, Hsp70 has anti-apoptotic activity. Heat-induced expression of Hsp70 has been shown to reduce Fas-associated apoptosis in U937 macrophages (29), and A549 cells stably transfected with Hsp70 were protected from hyperoxia. Other apoptotic stimuli against which Hsp70 confers protection include ceramide (30), UV irradiation (31), and nitric oxide (32). The mechanism of Hsp70 mediated anti-apoptotic activity

has been studied in some detail. Increased expression of Hsp70 inhibited caspase-3 mediated PARP cleavage by inhibiting the processing of procaspase-3, rather than caspase-3 activity (33). A number of studies indicate that Hsp70 can also modulate apoptosis through direct protein-protein interaction. Hsp70 binds to Apaf-1 through its carboxy-terminal EEVD motif and prevents the recruitment and processing of procaspase-9 to the apoptosome (34). It can also regulate apoptosis by caspase independent mechanisms, for example by directly binding Ask-1 to prevent H_20_2-induced apoptosis (35) (Figure 2). Hsp70 also inhibits JNK activation by direct binding (36) or by increasing the activity of a JNK phosphatase (37). Finally, Hsp70 binds AIF and inhibits its translocation, thereby protecting from AIF-mediated apoptosis (21).

Significantly, Hsp70-mediated JNK and AIF inhibition is independent of ATPase (chaperone) activity, and involves direct protein binding (21, 36). However, chaperone activity is required for Hsp70-mediated caspase inhibition, as evidenced by the finding that Hsp70 inhibition of caspase-3 activation depends on ATPase activity. It seems, therefore, that the Hsp70 anti-apoptotic activity is both chaperone-dependent and independent, a choice that appears to be cell type specific and may be related to the duration and intensity of Hsp70 expression and the nature of the apoptotic stimulus. In the PEER T-lymphocyte cell line, transient Hsp70 expression blocked JNK activation, while activation was unaffected in cells that constitutively expressed Hsp70. Conversely, caspase-3 activation was inhibited by constitutively expressed Hsp70, while transiently induced Hsp70 was unable to inhibit caspase activation and PARP cleavage (33). Caspase-3 activity was not inhibited in stably transfected WEHI-S fibrosarcoma cells that overexpress Hsp70, but the cells were protected from apoptosis, suggesting that: (i) Hsp70 can also protect downstream of caspase-3, and (ii) the mechanism of action is cell-type specific (38). Supporting the conclusion that the role of Hsp70 in apoptosis regulation may be stimulus-specific, Hsp70 protected Jurkat cells from hyperthermia-induced apoptosis, but it enhanced Fas-mediated apoptosis on the same cells (16).

4.3. Hsp90

Hsp90 is constitutively expressed in most cells making up a large proportion of all cellular proteins (1-2%). Hsp90 has chaperone activity dependent on ATP hydrolysis. It interacts with specific proteins which include transcription factors, signaling PK, and nuclear hormone receptors (39). It binds Raf-1 kinase and maintains activation of the Ras/Raf/MEK/ERK survival pathway by preventing Raf-1 degradation (40). It also binds PI3-K and Akt, thereby maintaining the integrity of this survival pathway (41). Hsp90 also interacts with other proteins to modulate apoptosis. It binds to Apaf-1 preventing formation of the apoptosome and subsequent cleavage of caspase-9 (42). In HeLa cells, Hsp90 binds and stabilizes receptor interacting protein (RIP) which protects cells from tumor necrosis factor-alpha (TNF-alpha) induced apoptosis by deflecting the apoptotic signal towards NF-kB activation (43). However, in U937 cells, increased expression of Hsp90 has been associated with increased susceptibility to apoptosis induced by TNF-alpha and cycloheximide, indicating that under specific conditions, Hsp90 could enhance pro-apoptotic signals (44).

4.4. Hsp27

The small Hsp subfamily consists of nine different proteins grouped together based on their relatively small size (15-30 kDa) and the presence of a conserved alpha-crystallin motif. One of the best studied members of this group is Hsp27, which does not have ATPase activity. Hsp27 has energy-independent chaperone activity, the main function of which is to protect from protein aggregation (45). Increased Hsp27 expression inhibits apoptosis induced by Fas, the kinase inhibitor staurosporin, and anticancer agents such as actinomycin-D or etoposide (46,47). Various pathways are responsible for this anti-apoptotic activity. Hsp27 binds procaspase-3, inhibiting its processing (48). It also binds to cytosolic cytochrome c, thereby preventing apoptosome formation (49, 50). Hsp27 can also translocate to the mitochondria, where it prevents the release of pro-apoptotic signaling mediators such as cytochrome c and Smac/DIABLO (51, 52). Significantly, Hsp27 phosphorylation by MAPKAP kinase-2 causes its dimerization and subsequent interaction with Daxx. By binding and sequestering Daxx, Hsp27 prevents Fas/Ask1/JNK induced apoptosis (53). Like Hsp90, Hsp27 also interacts with Akt ensuring the maintenance of its kinase activity, which is important for survival of hyperthermia-stressed PC12 cells (54). To protect from oxidative stress, Hsp27 restores depleted glutathione levels by promoting the activation of glucose-6-phosphate dehydrogenase (55) and it helps retain the overall structural integrity of the cell by binding and stabilizing F-actin microfilaments (56). In certain cell types, Hsp27 overexpression enhances proteosomal degradation of ubiquinated proteins in response to stress stimuli such as etoposide or TNF-alpha. Hsp27 binds to polyubiquitin chains as well as to the 26S proteosome and is involved in the degradation of the main inhibitor of NF-kB, I-kBalpha (57). However, unlike Hsp70 and Hsp90, Hsp27 was not shown to enhance pro-apoptotic stimuli.

4.5. H11, a novel Hsp with pro-apoptotic activity

H11 (also known as Hsp22 or HspB8), is a member of the small Hsp family which includes Hsp27. It was initially identified during the search for a homologue to the PK domain of the HSV-2 large subunit of ribonucleotide reductase (ICP10) (2). H11 contains a degenerate alpha-crystallin motif and its expression is upregulated by heat stress (1, 58). However, it differs from canonical Hsp in that it is associated with the cell surface (2), it does not translocate to the nucleus upon heat shock (Figure 3A), and it has intrinsic auto- and trans-phosphorylating kinase activity (1,2,59,60). Using sequence analysis described after compilation of nuclear export sequences (NES) (61), putative leucine-rich NES motifs were identified in the H11 N- (residues 21-31) and C- (residues 157-166) termini (Figure 3B), presumably accounting for its cytosolic localization.

Like other Hsp, H11 has been associated with cell proliferation. Its overexpression in cardiac muscle

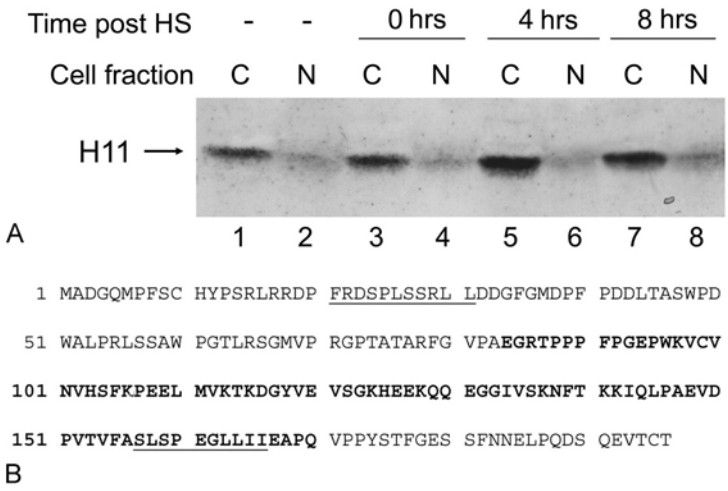

Figure 3. H11 remains in the cytoplasm after heat shock. (A) HEK293 cells were heat shocked (HS; 42.5 ºC for 1 hr) (lanes 3-8) or not (lanes 1-2). They were harvested immediately thereafter (0hr; lanes 3-4) or allowed to recover at 37ºC for 4 hrs (lanes 5-6) or 8 hrs (lanes 7-8) before harvest. To prepare cytoplasmic (C) and nuclear (N) fractions, cell pellets were resuspended in lysis buffer (50 mM Tris pH 8, 50 mM NaCl, 1% NP-40, 1mM DTT and protease inhibitors) and centrifuged at 7000g for 1 min. The pellet was separated from the supernatant (cytoplasmic extract), resuspended in lysis buffer that contained 450 mM NaCl, and sonicated for 30 seconds (nuclear extract). The cytoplasmic and nuclear extracts were immunoblotted with antibody specific for H11. (B) Complete amino acid sequence of H11. Two putative nuclear export signal (NES) sequences are underlined and the alpha-crystallin motif is bolded

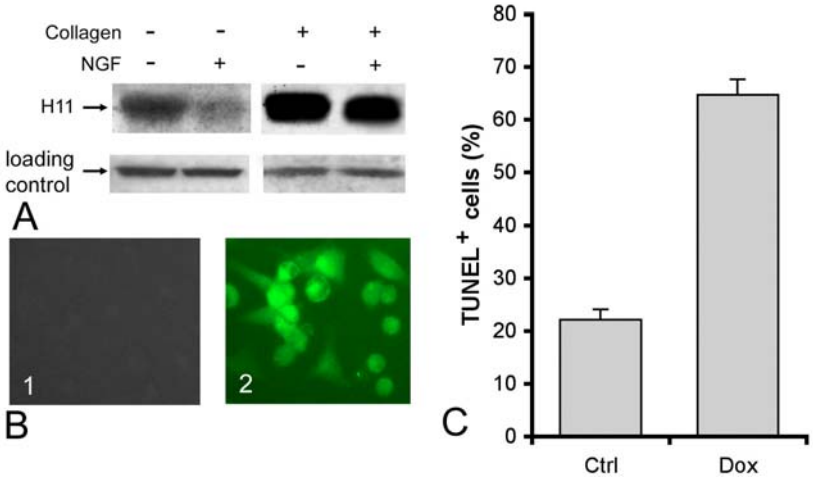

Figure 4. H11 induces apoptosis in differentiated PC12 cells. (A) Extracts of PC12 cells grown in the presence or absence of collagen and differentiated with NGF (100ng/ml; 5 days) were immunoblotted with H11 antibody as previously described (1). H11 expression was inhibited in NGF-differentiated cells grown in the absence, but not presence, of collagen, indicating that it is not involved in differentiation. (B) PC12 cells were stably transfected with H11 fused to EGFP (H11-EGFP) under the control of tetracycline responsive promoter. They were cultured with NGF (100 ng/ml; 5 days) followed by 2 days of culture without NGF in order to further reduce expression of the endogenous H11 (panel 1). Doxycycline (Dox; a tetracycline analogue) (2μg/ml) was added during the last 2 days of differentiation and the 2 days of NGF withdrawal to induce H11-EGFP. Dox treated (panel 2), but not untreated (panel 1) cells exhibited green fluorescence. (C) Duplicates of the cultures in (B) were assayed for apoptosis by TUNEL. Results are expressed as the % $TUNEL^+$ cells +/- SEM.

leads to hypertrophy and cytoprotection from ischemia, mediated by Akt activation that does not require H11 kinase activity (59,60). It is overexpressed in many stomach tumors (1) and in proliferating human keratinocytes (3) and rat pheochromocytoma (PC12) cells. Interestingly, expression is significantly reduced/inhibited when keratinocytes (3) and PC12 cells (Figure 4) are induced to differentiate [by increased Ca^{2+} ions or nerve growth factor (NGF), respectively]. In PC12 cells, H11 expression was significantly reduced by NGF differentiation when the cells were grown on plastic, but not when they were grown on collagen (Figure 4A).

Because collagen binds the NGF-responsive alpha1beta1 integrin receptor on PC12 cells (62), the data implicate H11 in the alpha1beta1 integrin signaling pathway that functions in cell cycle arrest (63,64). However, H11 is not required for differentiation, because the cells were differentiated when grown under both conditions, as determined by neurite outgrowth.

Why is H11 expression inhibited under some conditions? To answer this question we asked what happens to PC12 cells that are forced to express H11 through stable transfection with an expression vector for H11 fused to enhanced green fluorescent protein (H11-EGFP) under the control of a tetracycline-responsive promoter. The transfected cells were differentiated by culture with NGF and H11 expression was induced with Doxycycline (Dox; a tetracycline analogue). Virtually all the Dox treated cells evidenced green fluorescence indicative of H11 expression (Figure 4B) and this was associated with increased apoptosis, as determined by TUNEL (22 +/- 2% and 65 +/- 3%, TUNEL+ cells for Dox and no Dox, respectively) (Figure 4C). Consistent with the conclusion that H11 has pro-apoptotic activity, its expression was markedly decreased in certain tumors, notably melanoma (1). In these tumor cells, H11 expression was induced by treatment with the methylation inhibitor 5-aza-2' deoxycytidine (Aza-C) and induction was associated with apoptosis. Apoptosis involved the independent activation of the p38MAPK and caspase-3 pathways (1). Because p38MAPK activation is directly regulated by Lys 63 (K63)-linked polyubiquination (65), H11 may favor ubiquination, for example through chaperone activity. Kinase activity may also be involved in the H11 pro-apoptotic activity (1,60). In this context it may be important to point out that in cardiac myocytes, H11 pro-apoptotic acitivity appears to involve inhibition of casein kinase 2 (CK-2) activity (60).

Significantly, in some tumor cells (viz. HeLa and G361), H11 had a single amino acid substitution at residue 51 (H11-W51C) (1). This mutation results in 7 additional beta-turns in the predicted secondary structure and a significant increase in autokinase activity. Unlike the wild type, which triggers caspase-3 and p38MAPK dependent apoptosis, H11-W51C activates the Raf/MEK/ERK survival pathway and causes cell transformation. H11-W51C has dominant anti-apoptotic activity, as evidenced by the finding that stably transfected cells are protected from apoptosis induced by staurosporine or by transfection with the wild type H11 (1). Collectively, the data indicate that in certain tumor cells H11 is silenced by aberrant promoter hypermethylation while in others it is mutated to an anti-apoptotic and transforming phenotype. Such regulation was not previously described for other Hsp, but it is likely related to the pro-apoptotic activity of H11, which is undesirable from the standpoint of tumor development. The data also suggest that H11 is a promising target for cancer chemotherapy, since its forced expression triggers cell-type specific apoptosis.

It is tempting to point out that these properties resemble those previously described for the tumor suppressor protein p53. Classically, p53 induces apoptosis by a mechanism involving upregulation of pro-apoptotic genes, such as Fas and Bax (66, 67), and its expression can be inhibited in tumor cells by promoter hypermethylation (68). However, like the H11 cytoprotective activity in cardiac cells (59), p53 protects lung cancer cells from UV-induced apoptosis by binding and inhibiting JNK, and by blocking caspase 3 activation (69). Like H11, p53 expression is also decreased by differentiation in various cell types (70-72). Moreover, a single site mutation (replacement of Arginine^{175} with Histidine) reverses its activity from pro- to anti-apoptotic (73). Further studies are needed in order to identify the spectrum of H11 mutations, quantify the extent of H11 gene silencing by promoter hypermethylation, and determine their relationship to tumor development. Notwithstanding, the similarity of H11 to p53 underscores the commonality of strategies used by various genes to regulate apoptosis and indicates that Hsp play pivotal roles in cell life and death decisions. As such Hsp are desirable targets for hijacking/modulation by virus infection.

5. VIRAL REGULATION OF APOPTOTIC SIGNALING

5.1. Viruses encode homologues to the anti-apoptotic cellular proteins, notably Bcl-2.

In the context of virus infection, the balance between cell survival and apoptosis often determines the relative success of virus replication. One mechanism of ensuring successful virus replication is to tip the balance away from apoptosis by inhibiting death receptor pathways, such as TNF. Viruses achieve this goal by encoding homologues of the cellular anti-apoptotic proteins which interfere with the extracellular (death receptor) apoptotic cascade (Figure 1). For example, poxviruses encode several genes homologous to the TNF receptor. When secreted, these proteins effectively compete with the cellular receptor for TNF, thereby blocking activation of the extracellular apoptotic cascade (74). Human herpes virus type 8 (HHV-8) encodes a FLIP homologue which interacts with FADD and procaspase-8, blocking the latter's activation and subsequent apoptosis (75). Viruses also target downstream components of the apoptotic cascade. The 19 kDa protein encoded by the adenovirus E1B gene (E1B-19K) is a Bcl-2 homologue (76). It can directly bind to Bak and Bax, inhibiting their ability to oligomerize and act as mitochondrial membrane pores (77). HHV-8 and Herpesvirus saimiri also encode Bcl-2 homologues (78, 79). Despite their ability to bind and inhibit pro-apoptotic Bcl-2 family members, the viral Bcl-2 proteins evidence limited amino acid conservation (80). The EBV Bcl-2 homologue, BHRF1, shares only 25% overall sequence identity with cellular Bcl-2, and the HHV-8 Bcl-2 homologue, HHV8-Bcl-2, has only 15% overall identity with its cellular counterpart (78, 81).

Within the BH1 domain, BHRF1 and HHV8-Bcl-2 have 30% and 45% sequence identity with the BH1 domain of Bcl-2, respectively (78). This similarity is far lower than the 90% sequence identity shared by the BH1 domains of cellular anti-apoptotic Bcl-2 family members.

However, comparison of solution structure for the HHV8 and cellular Bcl-2 proteins revealed similar structural properties within the BH domains (82). Furthermore, both BHRF1 and HHV8-Bcl-2, have the same number of alpha-helices and the same overall folding pattern as their cellular counterpart (82,83). A major structural difference between the viral and cellular Bcl-2 proteins is a significant shortening of the loop region connecting the alpha1 and alpha2 helices (82,83). This region contains a caspase-3 cleavage site that, when acted upon by caspase-3, leads to ablation of the Bcl-2 anti-apoptotic activity (84). It also contains a phosphorylation site that inhibits Bcl-2 activity (85). By lacking this loop region, the viral Bcl-2 proteins are resistant to caspase-3 cleavage and phosphorylation, thus maintaining their anti-apoptotic activity (86). Relatively low sequence identity to their cellular counterparts is actually advantageous for the viral Bcl-2 homologues, because it removes key regulatory sites while maintaining a structure and function similar to those of the cellular Bcl-2 protein. Viruses also inhibit apoptosis by encoding homologues of the caspase inhibitor IAP, such as the baculovirus p35 protein (87).

5.2. Viruses alter expression/activation of PK signaling cascades

Another strategy used by viruses to regulate apoptosis is to alter the expression and activation of cellular apoptosis modulatory proteins. PKs involved in intracellular signaling pathways are a major target of viral modulation. The hepatitis B virus HBVx protein and the EBV LMP1 protein upregulate expression of the epidermal growth factor receptor (EGFR) leading to activation of the ERK pathway (88, 89). The bovine papilloma virus (BPV) E5 protein binds directly to the EGFR cytoplasmic domain, enhancing its kinase activity (90) and the human papilloma virus type 16 (HPV-16) E5 protein inhibits EGFR downregulation, presumably by direct binding (91). Human cytomegalovirus activates ERK by inhibiting a phosphatase that dephosphorylates it (92), and the HSV-2 protein ICP10PK activates Ras by binding the Grb2-Sos complex and by blocking the activity of the Ras inhibitory protein RasGAP (93).

Viruses also modulate the PI3-K/Akt survival pathway. The EBV latency protein LMP1 binds to the non-catalytic p85 subunit of PI3-K leading to the activation of its catalytic domain (94). A second EBV latency protein, LMP2A, interacts with Src, a tyrosine PK that activates the PI3-K/Akt pathway (95). Pathway activation is believed to promote latency maintenance by inhibiting apoptosis. However, the EBV protein BRLF1 is expressed during latency reactivation, and it also activates PI3-K signaling, suggesting that PI3-K/Akt is involved in latency reactivation (96). This is likely required in order to inhibit apoptosis long enough to allow for virus replication. Other viral genes that activate the PI3-K pathway by direct binding include HBVx (97), the CMV envelope glycoproteins gB and gH (98) and the human immunodeficiency virus type 1 (HIV-1) Tat protein (99). Viruses can also inhibit pro-apoptotic kinases, as is the case for the HIV-1 protein Nef, which binds and inhibits Ask-1 (100).

5.3. Viruses encode Hsp homologues

Very few viruses have been shown to encode homologues of Hsp family members. One of these, Closterovirus, is a plant RNA virus that encodes an Hsp70 homologue known as p65 (also known as Hsp70h) (101). p65 is required for efficient virion assembly, and is involved in cell-to-cell virus movement, presumably through its association with microtubules (102). It resembles Hsp70 in the ATPase domain (greater than 30% homology), and has functional ATPase activity (103). However, p65 does not resemble the protein binding domain of Hsp70 family members, and it does not bind to denatured proteins (103). ATPase-dependent chaperone activity was not described. Therefore, despite its similarity to Hsp70, p65 probably does not retain apoptosis modulatory activity.

To the extent of our knowledge, HSV-2 is the only human viral pathogen that encodes Hsp homologues. One of these is UL14 that has 27% sequence identity with the protein binding domain of Hsp70, but lacks ATPase activity. Like Hsp70, UL14 undergoes nuclear translocation after heat shock, it appears to aid in protein folding (104), and it has anti-apoptotic activity (105). The other HSV-2 gene that is an Hsp homologue is ICP10PK. ICP10PK is located at the amino-terminus of the viral large subunit of ribonucleotide reductase (R1; ICP10). It is unique to HSV as no other R1 protein has such a PK domain. This finding, originally interpreted to indicate that ICP10PK was co-opted from a cellular gene (106), is supported by the subsequent finding that it is homologous to H11 (2,3). ICP10PK has a degenerate crystallin motif similar to that in small Hsp family members (107), and direct alignment with anchored PK motifs revealed 32% identity (23/71 identical residues) and 59% homology (42/71 identical and functionally homologous residues) between the H11 and ICP10 PK catalytic cores (Figure 5A). This level of sequence homology is similar to that seen for viral Bcl-2 homologues and their cellular counterparts. Presumably, in the process of its co-option, the cellular gene fell under the control of the viral R1 promoter, losing regulatory constraints while retaining ATPase-independent chaperone activity (107).

5.4. Viruses regulate Hsp expression

Viruses also regulate the expression of cellular Hsp. The adenovirus E1a protein upregulates Hsp70 by directly activating its promoter (108) and HSV1/2 increase Hsp70 expression, a function that is presumably mediated by the viral immediate early genes (109). Other viruses, such as SV40, are also known to induce Hsp expression (110). However, it is still unclear whether the purpose of Hsp upregulation is to control apoptosis and/or aid in protein folding.

In addition to being homologous to an Hsp, ICP10PK upregulates the expression of Hsp family members. Immunoblotting with antibodies that recognize Hsp27 or both the constitutively expressed Hsc70 and inducible Hsp70, indicated that primary cortical cultures do not express Hsp27 and only minimally express Hsc70 (Figure 5, lane 1). However, Hsp70 was induced by HSV-2 as early as 30

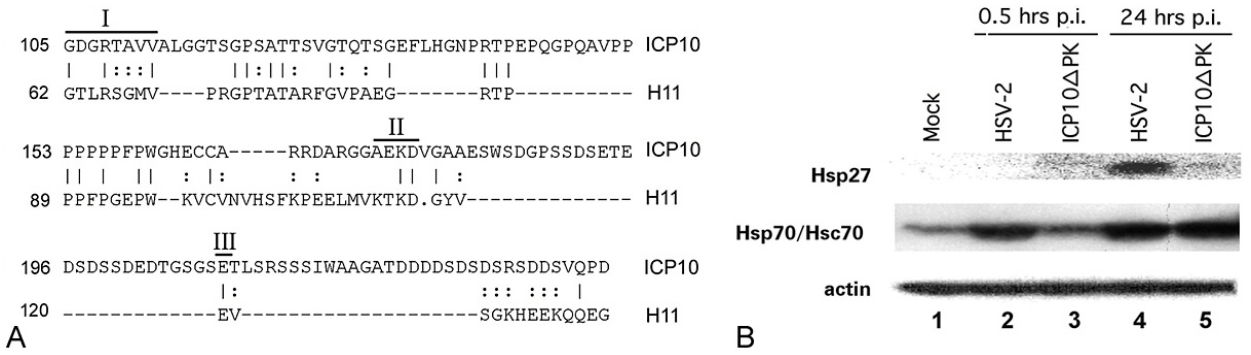

Figure 5. ICP10PK is homologous to H11 and induces the Hsp expression in primary cortical cultures. (A) Alignment of the catalytic cores of ICP10 and H11 with anchored PK motifs (roman numerals) using the ALIGN gene analysis software (http://www2.igh.cnrs.fr/bin/align-guess.cgi). (B) Primary cortical cultures were infected (0.5 or 24 hrs) with HSV-2 (lanes 2,4) or ICP10deltaPK (lanes 3,5) (10 pfu/cell) or mock infected with growth medium (lane 1). Extracts were immunoblotted with HSP27 antibody (top panel). The blot was stripped and re-probed sequentially with antibodies to Hsp70/Hsc70 (middle panel) or actin used as a loading control (bottom panel).

min after infection (Figure 5, lane 2). At this time, Hsp70 was not induced by a mutant deleted in ICP10PK (ICP10deltaPK) (Figure 5, lane 3), indicating that ICP10PK upregulates Hsp70 expression early in infection. Hsp27 was also induced by ICP10PK, but not until late in infection (24 hrs p.i.) (Figure 5, lanes 4,5). The sequential induction of different Hsp, suggests that they may function in a multiprotein complex in which client proteins are shifted from one chaperone to another in the process of their disposition. Hsp upregulation may contribute to the anti-apoptotic activity of ICP10PK by insuring removal of damaged and deleterious proteins. The upregulation of Hsp27 late in infection, when most of the protein is involved with its ribonucleotide function and virus progeny is generated, may insure energy-independent anti-apoptotic activity, that may also be related to the removal of undesirable (toxic) proteins (53, 57). Interestingly, Hsp70 expression is also induced by viral genes other than ICP10PK late in infection (Figure 5), supporting the conclusion that Hsp play an important role in virus infection.

5.5. ICP10PK commandeers all the apoptosis regulatory families

ICP10PK induces cell proliferation and survival, likely due to its anti-apoptotic activity which involves the activation of the Ras/Raf/MEK/ERK pathway, upregulation of the Hsp70 co-chaperone Bag-1 [which also activates the kinase Raf-1 (111)], stabilization of the anti-apoptotic protein Bcl-2, and activation of the transcription factor CREB. (8). These properties are similar to those of the H11 mutant H11-W51C, which shares the same sequence identity to ICP10PK as the wild type H11, activates the MEK/ERK pathway and has anti-apoptotic activity (1). We conclude that virus acquisition of the cellular protein is advantageous from the standpoint of virus survival, because ICP10PK is required for virus growth, particularly in non-dividing cells, such as neurons (93). In this context, it is particularly significant that H11-W51C compensates for ICP10PK as evidenced by single step growth curves in HEK293 cells and HEK293 cells that constitutively express H11-W51C (TAG51). Indeed, the growth of the ICP10PK deleted mutant (ICP10deltaPK) in HEK293 cells, did not begin until 10 hrs p.i., as compared to 2 hrs p.i. for HSV-2 (Figure 6A). By contrast, growth onset was not delayed in TAG51 cells (Figure 6B). In these cells, H11-W51C compensates for ICP10PK by activating the ERK pathway, as evidenced by the finding that the growth of both HSV-2 and ICP10deltaPK was delayed when the cells were treated with the MEK-specific inhibitor PD98059 (Figure 6C).

6. CONCLUSION AND PERSPECTIVE

Viruses hijack cells by commandeering signaling cascades that determine the cell's life and death decisions. Apoptotic cascades are major targets of virus modulation. Protein families that regulate apoptosis and are altered by virus infection include the Bcl-2, IAP and signaling PK families. Hsp are an emerging family of apoptosis regulatory proteins that function as molecular chaperones in regulating homeostasis. Virtually all family members have been associated with thermotolerance and cytoprotection, attributed, at least in part, to apoptosis inhibition. However, in some cases of extreme stress, Hsp overload was shown to contribute to apoptosis and Hsp were shown to enhance certain apoptotic signals. A recent report that an Hsp family member (H11) has independent pro-apoptotic activity (1) establishes the Hsp as a *bona fide* family of apoptosis regulators consisting of both pro- and anti-apoptotic members. H11 is unique among Hsp in that it is constitutively associated with the cell surface, does not translocate to the nucleus upon heat shock, has intrinsic auto- and trans- phosphorylating kinase activity, and has pro-apoptotic activity in neuronally differentiated PC12 cells and cardiac myocytes and in certain tumor cells, notably melanoma (1,2,59,60). In tumor cells, its pro-apoptotic activity is circumvented through gene silencing by aberrant promoter hypermethylation or by mutation that ablates its pro-apoptotic activity. Indeed, a single amino acid mutation at residue 51 (H11-W51C) can lead to a reversal of its apoptosis modulatory activity from pro- to anti-apoptotic (1).

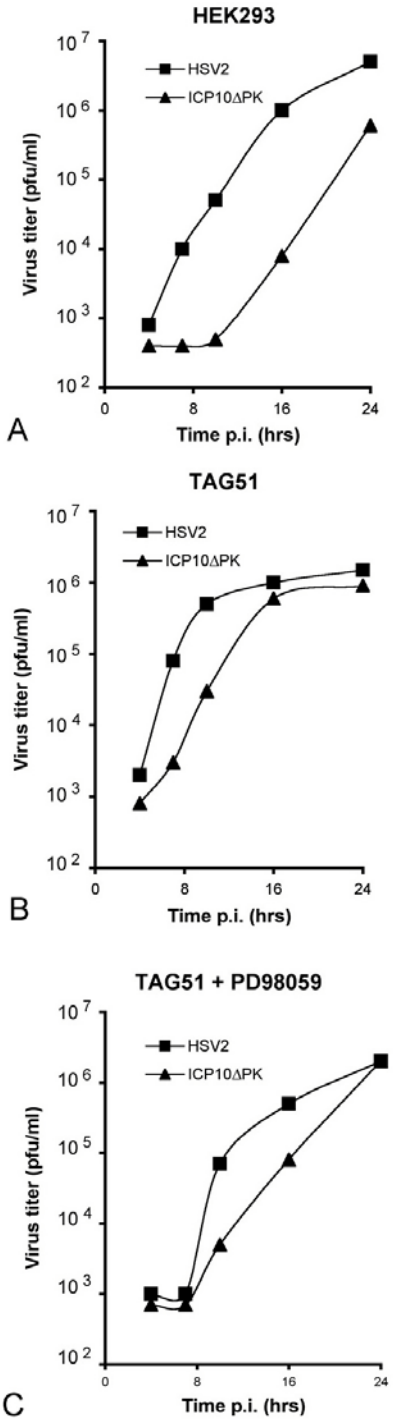

Figure 6. H11-W51C compensates for ICP10PK during virus replication. Single step growth curves of HSV-2 and ICP10deltaPK in HEK293 cells (A), HEK293 cells that constitutively express H11-W51C (TAG51) (B), and TAG51 cells treated with the MEK inhibitor PD98059 (50 μM, added 1hr prior to virus infection and maintained throughout the course of the experiment) (C). Infection was in medium with 1% fetal bovine serum. Virus titers were quantified by plaque assay (2) and results are expressed as plaque forming units/ml (pfu/ml).

The exact mechanism of Hsp anti-or pro-apoptotic activity is still unclear. An interesting question is whether Hsp modulate apoptosis strictly by virtue of their chaperone activity or whether they can also regulate apoptosis by a chaperone-unrelated protein interaction. Hsp70, for example, complexes with nascent or damaged proteins and chaperones them for refolding and function resumption, or for degradation by the proteosome complex. The co-chaperone Bag-1 plays a crucial role in the decision whether client proteins will be refolded for resumed function or targeted to the proteosome for degradation (18, 19). However, Bag-1 also has Hsp70-independent functions in apoptosis modulation including its ability to activate the Raf-1 kinase and stabilize Bcl-2 (111). In this context, it is particularly significant that Bag-1 is expressed as distinct isoforms that arise from a common transcript through alternative in-frame translational start sites. One of these, Bag-1M inhibits the refolding reaction of Hsp70, while the other, Bag-1S displays stimulating activity (112). Both isoforms could have anti-apoptotic activity, depending on whether refolding or degradation of the Hsp70 client protein is desirable for cell survival. However, Hsp function in apoptosis regulation is also chaperone-independent (21). Hsp90 has also been implicated in proteosomal degradation of client proteins, often in a multiprotein complex in which the client protein is transferred from one to the other Hsp (18). The versatility of the Hsp in apoptosis regulation makes them particularly attractive targets for virus modulation.

HSV-2 appears to be unique among human viruses that modulate apoptosis in that it encodes proteins that are Hsp homologues and modulate apoptosis. One of these, ICP10PK, likely originated from H11 or its mutant H11-W51C through an ancestral recombination event. The similarity of ICP10PK and H11-W51C (both are anti-apoptotic involving activation of the ERK survival pathway) and the ability of H11-W51C to compensate for ICP10PK during HSV-2 replication support this conclusion. However, unlike cellular Hsp, the ICP10PK promoter does not retain heat shock factor elements and its expression is regulated by AP-1 (113, 114). By inserting the co-opted Hsp downstream of a strong viral promoter, HSV-2 gained the Hsp derived apoptosis-regulatory activity, while avoiding cellular regulation of gene expression. This is especially advantageous for virus replication during latency reactivation. In neurons, ICP10PK expression is induced by factors, such as AP-1, that are upregulated by reactivation stimuli (115, 116) and its anti-apoptotic activity is important for increasing the number of live neurons that can support virus replication. Indeed, previous studies have implicated ICP10PK in HSV-2 latency reactivation by showing that: (i) an HSV-2 mutant deleted in the PK domain of ICP10 is severely compromised for latency reactivation (117, 118), and (ii) HSV-2 reactivation from explanted ganglia is inhibited by an ICP10-specific antisense oligonucleotide (119).

Since Bag-1 transfection of primary hippocampal cultures can account for over 90% of the ICP10PK anti-apoptotic activity (8), the significance of the early upregulation of Hsp70 is not immediately apparent. A

possible interpretation is that Hsp70 expression early during virus infection is important for chaperoning damaged proteins to the proteasome. The early upregulation of Bag-1 is consistent with this interpretation. The significance of Hsp27 upregulation by ICP10PK late during infection is also unclear. Because Hsp27 can chaperone ubiquitinated proteins to the proteasome in an ATP-independent manner (57), its upregulation late in infection, when the cell is likely to be energy depleted, may be advantageous. Another possibility is that ICP10 may be occupied with ribonucleotide reductace activity during the late phase of infection and thus Hsp27 may serve as a supplement to ICP10PK anti-apoptotic activity.

Targeting the Hsp family has an important additional advantage for the virus in that it allows for immune modulation. Indeed, HSV-2 infection induces both antiviral Th1 and immune downregulatory Th2 responses. A virus mutant deleted in ICP10PK shifts the balance of the virus-specific T cell response in favor of the Th1 component (120), indicating that ICP10PK functions in immune evasion by favoring the more virus-friendly Th2 response. This presumably involves the upregulation of Th2 polarizing and inflammatory cytokines, including IL-10, IL-6, IL-13, MCP-1 and RANTES in infected keratinocytes (Aurelian et al, in preparation). ICP10PK may be able to induce these cytokines because it is homologous to an Hsp and/or it upregulates Hsp70 expression. While additional studies are needed in order to better understand the role of Hsp in virus infection., it seems reasonable to conclude that by encoding an Hsp homologue, HSV-2 gains the advantage of targeting a wide range of apoptosis modulatory proteins that ensure host cell survival and thereby contribute to the virus life cycle.

7. ACKNOWLEDGEMENT

The studies in our laboratory were supported by NINDS, National Institutes of Health (NIH) public health service grant NS45169

8. REFERENCES

1. Gober MD, Smith CC, Ueda K, Toretsky JA, and Aurelian L. Forced expression of the H11 heat shock protein can be regulated by DNA methylation and trigger apoptosis in human cells. *J Biol Chem* 278, 37600-37609 (2003)

2. Smith CC, Yu YX, Kulka M, Aurelian L. A novel human gene similar to the protein kinase (PK) coding domain of the large subunit of herpes simplex virus type 2 ribonucleotide reductase (ICP10) codes for a serine-threonine PK and is expressed in melanoma cells. *J Biol Chem* 275, 25690-25699 (2000)

3. Aurelian L, Smith CC, Winchurch R, Kulka M, Gyotoku T, Zaccaro L, Chrest FJ, and Burnett JW. A novel gene expressed in human keratinocytes with long-term in vitro growth potential is required for cell growth. *J Invest Dermatol* 116, 286-295 (2001)

4. Cory S, Huang DC and Adams JM. The Bcl-2 family: roles in cell survival and oncogenesis. *Oncogene* 22, 8590-8607 (2003)

5. Tobiume K, Matsuzawa A, Takahashi T, Nishitoh H, Morita K, Takeda K, Minowa O, Miyazono K, Noda T and Ichijo H. ASK1 is required for sustained activations of JNK/p38 MAP kinases and apoptosis. *EMBO Rep* 2, 222-228 (2001)

6. Scheid MP, Schubert KM and Duronio V. Regulation of bad phosphorylation and association with Bcl-x(L) by the MAPK/Erk kinase. *J Biol Chem* 274, 31108-31113 (1999)

7. del Peso L, Gonzalez-Garcia M, Page C, Herrera R and Nunez G. Interleukin-3-induced phosphorylation of BAD through the protein kinase Akt. *Science* 278, 687-9 (1997)

8. Perkins D, Pereira EF and Aurelian L. The herpes simplex virus type 2 R1 protein kinase (ICP10 PK) functions as a dominant regulator of apoptosis in hippocampal neurons involving activation of the ERK survival pathway and upregulation of the antiapoptotic protein Bag-1. *J Virol* 77, 1292-1305 (2003)

9. Franke TF, Hornik CP, Segev L, Shostak GA, and Sugimoto C. PI3K/Akt and apoptosis: size matters. *Oncogene* 22, 8983-8998 (2003)

10. Schimmer AD. Inhibitor of apoptosis proteins: translating basic knowledge into clinical practice. *Cancer Res* 64, 7183-7190 (2004)

11. Verhagen AM and Vaux DL. Cell death regulation by the mammalian IAP antagonist Diablo/Smac. *Apoptosis* 7, 163-166 (2002)

12. Mosser DD and Morimoto RI. Molecular chaperones and the stress of oncogenesis. *Oncogene* 23, 2907-2918 (2004)

13. Samali A and Orrenius S. Heat shock proteins: regulators of stress response and apoptosis. *Cell Stress Chaperones* 3, 228-236 (1998)

14. Soti C, Sreedhar AS and Csermely P. Apoptosis, necrosis and cellular senescence: chaperone occupancy as a potential switch. *Aging Cell* 2, 39-45 (2003)

15. Feng H, Zeng Y, Graner MW and Katsanis E. Stressed apoptotic tumor cells stimulate dendritic cells and induce specific cytotoxic T cells. *Blood* 100, 4108-4115 (2002)

16. Liossis SN, Ding XZ, Kiang JG and Tsokos GC. Overexpression of the heat shock protein 70 enhances the TCR/CD3- and Fas/Apo-1/CD95-mediated apoptotic cell death in Jurkat T cells. *J Immunol* 158, 5668-5675 (1997)

17. Chen G, Cao P and Goeddel DV. TNF-induced recruitment and activation of the IKK complex require Cdc37 and Hsp90. *Mol Cell* 9, 401-410 (2002)

18. Doong H, Rizzo K, Fang S, Kulpa V, Weissman AM, and Kohn EC. CAIR-1/BAG-3 abrogates heat shock protein-70 chaperone complex-mediated protein degradation: accumulation of poly-ubiquitinated Hsp90 client proteins. *J Biol Chem* 278, 28490-28500 (2003)

19. Luders J, Demand J, and Hohfeld J. The ubiquitin-related BAG-1 provides a link between the molecular chaperones Hsc70/Hsp70 and the proteasome. *J Biol Chem* 275, 4613-4617 (2000)

20. McClellan AJ, and Frydman J. Molecular chaperones and the art of recognizing a lost cause. *Nat Cell Biol* ;3, E51-53 (2001)

21. Ravagnan L, Gurbuxani S, Susin SA, Maisse C, Daugas E, Zamzami N, Mak T, Jaattela M, Penninger JM, Garrido C, and Kroemer G. Heat-shock protein 70 antagonizes apoptosis-inducing factor. *Nat Cell Biol* 3, 839-843 (2001)

22. Gullo CA and Teoh G. Heat shock proteins: to present or not, that is the question. *Immunol Lett* 94, 1-10 (2004)

23. Srivastava P. Roles of heat-shock proteins in innate and adaptive immunity. *Nat Rev Immunol* 2, 185-194 (2002)

24. Asea A, Kraeft SK, Kurt-Jones EA, Stevenson MA, Chen LB, Finberg RW, Koo GC, and Calderwood SK. HSP70 stimulates cytokine production through a CD14-dependant pathway, demonstrating its dual role as a chaperone and cytokine. *Nat Med* 6, 435-442 (2000)

25. Wang Y, Kelly CG, Singh M, McGowan EG, Carrara AS, Bergmeier LA, and Lehner T. Stimulation of Th1-polarizing cytokines, C-C chemokines, maturation of dendritic cells, and adjuvant function by the peptide binding fragment of heat shock protein 70. *J Immunol* 169, 2422-2429 (2002)

26. Beere HM and Green DR. Stress management - heat shock protein-70 and the regulation of apoptosis. *Trends Cell Biol* 11, 6-10 (2001)

27. Song J, Takeda M and Morimoto RI. Bag1-Hsp70 mediates a physiological stress signalling pathway that regulates Raf-1/ERK and cell growth. *Nat Cell Biol* 3, 276-282 (2001)

28. Stuart JK, Myszka DG, Joss L, Mitchell RS, McDonald SM, Xie Z, Takayama S, Reed JC and Ely KR. Characterization of interactions between the anti-apoptotic protein BAG-1 and Hsc70 molecular chaperones. *J Biol Chem* 273, 22506-22514 (1998)

29. Schett G, Steiner CW, Groger M, Winkler S, Graninger W, Smolen J, Xu Q and Steiner G. Activation of Fas inhibits heat-induced activation of HSF1 and up-regulation of hsp70. *FASEB J* 13, 833-842 (1999)

30. Ahn JH, Ko YG, Park WY, Kang YS, Chung HY and Seo JS. Suppression of ceramide-mediated apoptosis by HSP70. *Mol Cells* 9, 200-206 (1999)

31. Meriin AB, Yaglom JA, Gabai VL, Zon L, Ganiatsas S, Mosser DD, Zon L, Sherman MY. Protein-damaging stresses activate c-Jun N-terminal kinase via inhibition of its dephosphorylation: a novel pathway controlled by HSP72. *Mol Cell Biol* 19, 2547-2555 (1999)

32. Klein SD and Brune B. Heat-shock protein 70 attenuates nitric oxide-induced apoptosis in RAW macrophages by preventing cytochrome c release. *Biochem J* 362, 635-641 (2002)

33. Mosser DD, Caron AW, Bourget L, Denis-Larose C and Massie B. Role of the human heat shock protein hsp70 in protection against stress-induced apoptosis. *Mol Cell Biol* 17, 5317-5327 (1997)

34. Beere HM, Wolf BB, Cain K, Mosser DD, Mahboubi A, Kuwana T, Tailor P, Morimoto RI, Cohen GM and Green DR. Heat-shock protein 70 inhibits apoptosis by preventing recruitment of procaspase-9 to the Apaf-1 apoptosome. *Nat Cell Biol* 2, 469-475 (2000)

35. Park HS, Cho SG, Kim CK, Hwang HS, Noh KT, Kim MS, Huh SH, Kim MJ, Ryoo K, Kim EK, Kang WJ, Lee JS, Seo JS, Ko YG, Kim S, and Choi EJ. Heat shock protein hsp72 is a negative regulator of apoptosis signal-regulating kinase 1. *Mol Cell Biol* 22, 7721-7730 (2002)

36. Park HS, Lee JS, Huh SH, Seo JS and Choi EJ. Hsp72 functions as a natural inhibitory protein of c-Jun N-terminal kinase. *EMBO J* 20, 446-456 (2001)

37. Yaglom JA, Gabai VL, Meriin AB, Mosser DD and Sherman MY. The function of HSP72 in suppression of c-Jun N-terminal kinase activation can be dissociated from its role in prevention of protein damage. *J Biol Chem* 274, 20223-20228 (1999)

38. Jaattela M, Wissing D, Kokholm K, Kallunki T, and Egeblad M. Hsp70 exerts its anti-apoptotic function downstream of caspase-3-like proteases. *EMBO J* 17, 6124-6134 (1998)

39. Pratt WB and Toft DO. Regulation of signaling protein function and trafficking by the hsp90/hsp70-based chaperone machinery. *Exp Biol Med* 228, 111-133 (2003)

40. Schulte TW, Blagosklonny MV, Ingui C and Neckers L. Disruption of the Raf-1-Hsp90 molecular complex results in destabilization of Raf-1 and loss of Raf-1-Ras association. *J Biol Chem* 270, 24585-24588 (1995)

41. Sato S, Fujita N, and Tsuruo T. Modulation of Akt kinase activity by binding to Hsp90. *Proc Natl Acad Sci U S A* 97, 10832-10837 (2000)

42. Pandey P, Saleh A, Nakazawa A, Kumar S, Srinivasula SM, Kumar V, Weichselbaum R, Nalin C, Alnemri ES, Kufe D and Kharbanda S. Negative regulation of cytochrome c-mediated oligomerization of Apaf-1 and activation of procaspase-9 by heat shock protein 90. *EMBO J* 19, 4310-4322 (2000)

43. Lewis J, Devin A, Miller A, Lin Y, Rodriguez Y, Neckers L and Liu ZG. Disruption of hsp90 function results in degradation of the death domain kinase, receptor-interacting protein (RIP), and blockage of tumor necrosis factor-induced nuclear factor-kappaB activation. *J Biol Chem* 275, 10519-10526 (2000)

44. Galea-Lauri J, Richardson AJ, Latchman DS and Katz DR. Increased heat shock protein 90 (hsp90) expression leads to increased apoptosis in the monoblastoid cell line U937 following induction with TNF-alpha and cycloheximide: a possible role in immunopathology. *J Immunol* 157, 4109-4118 (1996)

45. Garrido C, Gurbuxani S, Ravagnan L and Kroemer G. Heat shock proteins: endogenous modulators of apoptotic cell death. *Biochem Biophys Res Commun* 286, 433-442 (2001)

46. Mehlen P, Schulze-Osthoff K and Arrigo AP. Small stress proteins as novel regulators of apoptosis. Heat shock protein 27 blocks Fas/APO-1- and staurosporine-induced cell death. *J Biol Chem* 271, 16510-16514 (1996)

47. Samali A and Cotter TG. Heat shock proteins increase resistance to apoptosis. *Exp Cell Res* 223, 163-170 (1996)

48. Concannon CG, Orrenius S and Samali A. Hsp27 inhibits cytochrome c-mediated caspase activation by sequestering both pro-caspase-3 and cytochrome c. *Gene Expr* 9, 195-201 (2001)

49. Garrido C, Bruey JM, Fromentin A, Hammann A, Arrigo AP, Solary E. HSP27 inhibits cytochrome c-dependent activation of procaspase-9. *FASEB J* 13, 2061-2070 (1999)

50. Bruey JM, Ducasse C, Bonniaud P, Ravagnan L, Susin SA, Diaz-Latoud C, Gurbuxani S, Arrigo AP, Kroemer G, Solary E and Garrido C. Hsp27 negatively regulates cell death by interacting with cytochrome c. *Nat Cell Biol* 2, 645-652 (2000)

51. Paul C, Manero F, Gonin S, Kretz-Remy C, Virot S and Arrigo AP. Hsp27 as a negative regulator of cytochrome C release. *Mol Cell Biol* 22, 816-834 (2002)

52. Chauhan D, Li G, Hideshima T, Podar K, Mitsiades C, Mitsiades N, Catley L, Tai YT, Hayashi T, Shringarpure R, Burger R, Munshi N, Ohtake Y, Saxena S and Anderson KC. Hsp27 inhibits release of mitochondrial protein Smac in multiple myeloma cells and confers dexamethasone resistance. *Blood* 102, 3379-3386 (2003)

53. Charette SJ, Lavoie JN, Lambert H, and Landry J. Inhibition of Daxx-mediated apoptosis by heat shock protein 27. *Mol Cell Biol* 20, 7602-7612 (2000)

54. Konishi H, Matsuzaki H, Tanaka M, Takemura Y, Kuroda S, Ono Y, Kikkawa U. Activation of protein kinase B (Akt/RAC-protein kinase) by cellular stress and its association with heat shock protein Hsp27. *FEBS Lett* 410, 493-498 (1997)

55. Preville X, Salvemini F, Giraud S, Chaufour S, Paul C, Stepien G, Ursini MV and Arrigo AP. Mammalian small stress proteins protect against oxidative stress through their ability to increase glucose-6-phosphate dehydrogenase activity and by maintaining optimal cellular detoxifying machinery. *Exp Cell Res* 247, 61-78 (1999)

56. Geum D, Son GH and Kim K. Phosphorylation-dependent cellular localization and thermoprotective role of heat shock protein 25 in hippocampal progenitor cells. *J Biol Chem* 277, 19913-19921 (2002)

57. Parcellier A, Schmitt E, Gurbuxani S, Seigneurin-Berny D, Pance A, Chantome A, Plenchette S, Khochbin S, Solary E, and Garrido C. HSP27 is a ubiquitin-binding protein involved in I-kappaBalpha proteasomal degradation. *Mol Cell Biol* 23, 5790-5802 (2003)

58. Chowdary TK, Raman B, Ramakrishna T, and Rao CM. Mammalian Hsp22 is a heat-inducible small heat-shock protein with chaperone-like activity. *Biochem J* 381, 379-387 (2004).

59. Depre C, Hase M, Gaussin V, Zajac A, Wang L, Hittinger L, Ghaleb B, Yu X, Kudej RK, Wagner T, Sadoshima J, and Vatner SF. H11 kinase is a novel mediator of myocardial hypertrophy in vivo. *Circ Res* 91, 1007-1014 (2002)

60. Hase M, Depre C, Vatner SF, and Sadoshima J. H11 has dose-dependent and dual hypertrophic and proapoptotic functions in cardiac myocytes. *Biochem J* [Epub ahead of print] (2005)

61. la Cour T, Gupta R, Rapacki K, Skriver K, Poulsen FM, and Brunak S. NESbase version 1.0: a database of nuclear export signals. *Nucleic Acids Res* 31, 393-396 (2003)

62. Tawil NJ, Houde M, Blacher R, Esch F, Reichardt LF, Turner DC and Carbonetto S. Alpha 1 beta 1 integrin heterodimer functions as a dual laminin/collagen receptor in neural cells. *Biochemistry* 29, 6540-6544 (1990)

63. Liang YL, Fu Y, Chen SG, Cai XM, Su JM, Jin JW, Ma DZ, Li ZX, Zhang W, and Zha X. Integrin beta1 subunit overexpressed in the SMMC-7721 cells regulates the promoter activity of p21(CIP1) and enhances its transcription. *FEBS Lett* 558, 107-113 (2004)

64. Henriet P, Zhong ZD, Brooks PC, Weinberg KI and DeClerck YA. Contact with fibrillar collagen inhibits melanoma cell proliferation by up-regulating p27KIP1. *Proc Natl Acad Sci USA* 97, 10026-10031 (2000).

65. Wang C, Deng L, Hong M, Akkaraju GR, Inoue J, and Chen ZJ. TAK1 is a ubiquitin-dependent kinase of MKK and IKK. *Nature* 412, 346-351 (2001)

66. Owen-Schaub LB, Zhang W, Cusack JC, Angelo LS, Santee SM, Fujiwara T, Roth JA, Deisseroth AB, Zhang WW, Kruzel E, and Radinsky R. Wild-type human p53 and a temperature-sensitive mutant induce Fas/APO-1 expression. *Mol Cell Biol* 15, 3032-3040 (1995)

67. Selvakumaran M, Lin HK, Miyashita T, Wang HG, Krajewski S, Reed JC, Hoffman B, and Liebermann D. Immediate early up-regulation of bax expression by p53 but not TGF beta 1: a paradigm for distinct apoptotic pathways. *Oncogene* 9, 1791-1798 (1994)

68. Kang JH, Kim SJ, Noh DY, Park IA, Choe KJ, Yoo OJ, and Kang HS. Methylation in the p53 promoter is a supplementary route to breast carcinogenesis: correlation between CpG methylation in the p53 promoter and the mutation of the p53 gene in the progression from ductal carcinoma in situ to invasive ductal carcinoma. *Lab Invest* 81, 573-579 (2001)

69. Lo PK, Huang SZ, Chen HC and Wang FF. The prosurvival activity of p53 protects cells from UV-induced apoptosis by inhibiting c-Jun NH2-terminal kinase activity and mitochondrial death signaling. *Cancer Res* 64, 8736-8745 (2004)

70. Liu J, Li C, Ahlborn TE, Spence MJ, Meng L and Boxer LM. The expression of p53 tumor suppressor gene in breast cancer cells is down-regulated by cytokine oncostatin M. *Cell Growth Differ* 10, 677-683(1999)

71. Ferreira A and Kosik KS. Accelerated neuronal differentiation induced by p53 suppression. *J Cell Sci* 109, 1509-1516 (1996)

72. Soloveva V and Linzer DI. Differentiation of placental trophoblast giant cells requires downregulation of p53 and Rb. *Placenta* 25, 29-36 (2004)

73. Zalcenstein A, Stambolsky P, Weisz L, Muller M, Wallach D, Goncharov TM, Krammer PH, Rotter V and Oren M. Mutant p53 gain of function: repression of CD95(Fas/APO-1) gene expression by tumor-associated p53 mutants. *Oncogene* 22, 5667-5676 (2003)

74. Everett H and McFadden G. Poxviruses and apoptosis: a time to die. *Curr Opin Microbiol* 5, 395-402 (2002)

75. Djerbi M, Screpanti V, Catrina AI, Bogen B, Biberfeld P and Grandien A. The inhibitor of death receptor signaling, FLICE-inhibitory protein defines a new class of tumor progression factors. *J Exp Med* 190, 1025-1032 (1999)

76. White E. Regulation of the cell cycle and apoptosis by the oncogenes of adenovirus. *Oncogene* 20, 7836-7846 (2001)

77. Perez D and White E. TNF-alpha signals apoptosis through a bid-dependent conformational change in Bax that is inhibited by E1B 19K. *Mol Cell* 6, 53-63 (2000)

78. Cheng EH, Nicholas J, Bellows DS, Hayward GS, Guo HG, Reitz MS and Hardwick JM. A Bcl-2 homolog encoded by Kaposi sarcoma-associated virus, human herpesvirus 8, inhibits apoptosis but does not heterodimerize with Bax or Bak. *Proc Natl Acad Sci U S A* 94, 690-694 (1997)

79. Nava VE, Cheng EH, Veliuona M, Zou S, Clem RJ, Mayer ML and Hardwick JM. Herpesvirus saimiri encodes a functional homolog of the human bcl-2 oncogene. *J Virol* 71, 4118-4122 (1997)

80. Cuconati A and White E. Viral homologs of BCL-2: role of apoptosis in the regulation of virus infection. *Genes Dev* 16, 2465-2478 (2002).

81. Cleary ML, Smith SD and Sklar J. Cloning and structural analysis of cDNAs for bcl-2 and a hybrid bcl-2/immunoglobulin transcript resulting from the t(14;18) translocation. *Cell* 47, 19-28 (1986)

82. Huang Q, Petros AM, Virgin HW, Fesik SW and Olejniczak ET. Solution structure of a Bcl-2 homolog from Kaposi sarcoma virus. *Proc Natl Acad Sci U S A* 99, 3428-3433 (2002)

83. Huang Q, Petros AM, Virgin HW, Fesik SW and Olejniczak ET. Solution structure of the BHRF1 protein from Epstein-Barr virus, a homolog of human Bcl-2. *J Mol Biol* 332, 1123-1130 (2003)

84. Cheng EH, Kirsch DG, Clem RJ, Ravi R, Kastan MB, Bedi A, Ueno K and Hardwick JM. Conversion of Bcl-2 to a Bax-like death effector by caspases. *Science* 278, 1966-1968 (1997)

85. Ojala PM, Yamamoto K, Castanos-Velez E, Biberfeld P, Korsmeyer SJ and Makela TP. The apoptotic v-cyclin-CDK6 complex phosphorylates and inactivates Bcl-2. *Nat Cell Biol* 2, 819-825 (2000)

86. Bellows DS, Chau BN, Lee P, Lazebnik Y, Burns WH, and Hardwick JM. Antiapoptotic herpesvirus Bcl-2 homologs escape caspase-mediated conversion to proapoptotic proteins. *J Virol* 74, 5024-5031 (2000)

87. Clem RJ and Miller LK. Control of programmed cell death by the baculovirus genes p35 and iap. *Mol Cell Biol* 14, 5212-5222 (1994)

88. Menzo S, Clementi M, Alfani E, Bagnarelli P, Iacovacci S, Manzin A, Dandri M, Natoli G, Levrero M and Carloni G. Trans-activation of epidermal growth factor receptor gene by the hepatitis B virus X-gene product. *Virology* 196, 878-882 (1993)

89. Miller WE, Earp HS and Raab-Traub N. The Epstein-Barr virus latent membrane protein 1 induces expression of the epidermal growth factor receptor. *J Virol* 69, 4390-4398 (1995)

90. Cohen BD, Goldstein DJ, Rutledge L, Vass WC, Lowy DR, Schlegel R, and Schiller JT. Transformation-specific interaction of the bovine papillomavirus E5 oncoprotein with the platelet-derived growth factor receptor transmembrane domain and the epidermal growth factor receptor cytoplasmic domain. *J Virol* 67, 5303-5311 (1993)

91. Miller WE and Raab-Traub N. The EGFR as a target for viral oncoproteins. *Trends Microbiol* 7, 453-458 (1999)

92. Rodems SM and Spector DH. Extracellular signal-regulated kinase activity is sustained early during human cytomegalovirus infection. *J Virol* 72, 9173-9180 (1998)

93. Smith CC, Peng T, Kulka M, and Aurelian L. The PK domain of the large subunit of herpes simplex virus type 2 ribonucleotide reductase (ICP10) is required for immediate-early gene expression and virus growth. *J Virol* 72, 9131-9141 (1998)

94. Dawson CW, Tramountanis G, Eliopoulos AG and Young LS. Epstein-Barr virus latent membrane protein 1 (LMP1) activates the phosphatidylinositol 3-kinase/Akt pathway to promote cell survival and induce actin filament remodeling. *J Biol Chem* 278, 3694-3704 (2003)

95. Swart R, Ruf IK, Sample J and Longnecker R. Latent membrane protein 2A-mediated effects on the phosphatidylinositol 3-Kinase/Akt pathway. *J Virol* 74, 10838-10845 (2000)

96. Darr CD, Mauser A and Kenney S. Epstein-Barr virus immediate-early protein BRLF1 induces the lytic form of viral replication through a mechanism involving phosphatidylinositol-3 kinase activation. *J Virol* 75, 6135-6142 (2001)

97. Lee YI, Kang-Park S, Do SI and Lee YI. The hepatitis B virus-X protein activates a phosphatidylinositol 3-kinase-dependent survival signaling cascade. *J Biol Chem* 276, 16969-16977 (2001)

98. Yurochko AD, Hwang ES, Rasmussen L, Keay S, Pereira L and Huang ES. The human cytomegalovirus UL55 (gB) and UL75 (gH) glycoprotein ligands initiate the rapid activation of Sp1 and NF-kappaB during infection. *J Virol* 71, 5051-5059 (1997)

99. Milani D, Mazzoni M, Borgatti P, Zauli G, Cantley L and Capitani, S. Extracellular human immunodeficiency virus type-1 (HIV-1) Tat protein activates PI 3-Kinase in PC12 neuronal cells. *J Biol Chem* 271,22961-22964 (1996)

100. Geleziunas R, Xu W, Takeda K, Ichijo H and Greene WC. HIV-1 Nef inhibits ASK1-dependent death signalling providing a potential mechanism for protecting the infected host cell. *Nature* 410, 834-838 (2001)

101. Agranovsky AA, Boyko VP, Karasev AV, Koonin EV and Dolja VV. Putative 65 kDa protein of beet yellows closterovirus is a homologue of HSP70 heat shock proteins. *J Mol Biol* 217, 603-610 (1991)

102. Karasev AV, Kashina AS, Gelfand VI and Dolja VV. HSP70-related 65 kDa protein of beet yellows closterovirus is a microtubule-binding protein. *FEBS Lett* 304, 12-14 (1992)

103. Agranovsky AA, Folimonova SY, Folimonov AS, Denisenko ON and Zinovkin RA. The beet yellows closterovirus p65 homologue of HSP70 chaperones has ATPase activity associated with its conserved N-terminal domain but does not interact with unfolded protein chains. *J Gen Virol* 78, 535-542 (1997)

104. Yamauchi Y, Wada K, Goshima F, Daikoku T, Ohtsuka K and Nishiyama Y. Herpes simplex virus type 2 UL14 gene product has heat shock protein (HSP)-like functions. *J Cell Sci* 115, 2517-2527 (2002)

105. Yamauchi Y, Daikoku T, Goshima F and Nishiyama Y. Herpes simplex virus UL14 protein blocks apoptosis. *Microbiol Immunol* 47, 685-689 (2003)

106. Aurelian L. Herpes simplex virus type 2: unique biological properties include neoplastic potential mediated by the PK domain of the large subunit of ribonucleotide reductase. *Front Biosci* 3, d237-d249 (1998)

107. Chabaud S, Lambert H, Sasseville AM, Lavoie H, Guilbault C, Massie B, Landry J, and Langelier Y. The R1 subunit of herpes simplex virus ribonucleotide reductase has chaperone-like activity similar to Hsp27. *FEBS Lett* 545, 213-218 (2003)

108. Williams GT, McClanahan TK and Morimoto RI. E1a transactivation of the human HSP70 promoter is mediated through the basal transcriptional complex. *Mol Cell Biol* 9, 2574-2587 (1989)

109. Kobayashi K, Ohgitani E, Tanaka Y, Kita M and Imanishi J. Herpes simplex virus-induced expression of 70 kDa heat shock protein (HSP70) requires early protein synthesis but not viral DNA replication. *Microbiol Immunol* 38, 321-325 (1994)

110. Sainis I, Angelidis C, Pagoulatos G, and Lazaridis I. The hsc70 gene which is slightly induced by heat is the main virus inducible member of the hsp70 gene family. *FEBS Lett* 355, 282-286 (1994)

111. Wang HG, Takayama S, Rapp UR, and Reed JC Bcl-2 interacting protein, BAG-1, binds to and activates the kinase Raf-1. *Proc Natl Acad Sci U S A* 93, 7063-7068 (1996)

112. Luders J, Demand J, Papp O, and Hohfeld J. Distinct isoforms of the cofactor BAG-1 differentially affect Hsc70 chaperone function. *J Biol Chem* 275, 14817-14823 (2000)

113. Wymer JP, Chung TD, Chang Y-N, Hayward GS, and Aurelian L. Identification of immediate-early-type cis-response elements in the promoter for the ribonucleotide reductase large subunit from herpes simplex virus type 2. *J Virol* 63, 2773-2784 (1989)

114. Zhu J, and Aurelian L. AP-1 cis-response elements are involved in basal expression and vmx110 transactivation of the large subunit of herpes simplex virus type 2 ribonucleotide reductase (ICP10). *Virology* 231, 301-312 (1997)

115. Valyi-Nagy T, Deshmane S, Dillner A, and Fraser NW. Induction of cellular transcription factors in trigeminal ganglia of mice by corneal scarification, herpes simplex virus type 1 infection, and explantation of trigeminal ganglia. *J Virol* 65, 4142-4152 (1991)

116. Maroni P, Bendinelli P, Tiberio L, Rovetta F, Piccoletti R, and Schiaffonati L. In vivo heat-shock response in the brain: signalling pathway and transcription factor activation. *Mol Brain Res* 119, 90-99 (2003)

117. Aurelian L, Kokuba H, and Smith CC. Vaccine potential of a herpes simplex virus type 2 mutant deleted in the PK domain of the large subunit of ribonucleotide reductase (ICP10). *Vaccine* 17, 1951-1963 (1999)

118. Wachsman M, Kulka M, Smith CC, and Aurelian L. A growth and latency compromised herpes simplex virus type 2 mutant (ICP10-delta-PK) has prophylactic and therapeutic protective activity in guinea pigs. *Vaccine* 19, 1879-1890 (2001)

119. Aurelian L and Smith CC. Herpes simplex virus type 2 growth and latency reactivation by cocultivation are inhibited with antisense oligonucleotides complementary to the translation initiation site of the large subunit of ribonucleotide reductase (RR1). *Antisense Nucleic Acid Drug Dev* 10, 77-85 (2000)

120. Gyotoku T, Ono F, and Aurelian L. Development of HSV-specific CD4+ Th1 responses and CD8+ cytotoxic T lymphocytes with antiviral activity by vaccination with the HSV-2 mutant ICP10DeltaPK.*Vaccine* 20,2796-2807 (2002)

Key Words: Heat shock protein, Hsp, H11, ICP10, Cell Death, Apoptosis, Virus, HSV-2, Review

Send correspondence to: Michael D. Gober, University of Maryland, Department of Pharmacology & Experimental Therapeutics, Bressler Bldg. 4-045, 655 W. Baltimore St., Baltimore, MD 21201, Tel. 410-706-3895, Fax 410-706-2513, E-mail: mgobe001@umaryland.edu