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THE HERPES SIMPLEX VIRUS TYPE 2 PROTEIN ICP10PK: A MASTER OF VERSATILITY

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1. ABSTRACT

Herpes simplex viruses types 1 (HSV-1) and 2 (HSV-2) are associated with a wide range of diseases related to infection of epithelial or neuronal tissues. The two viruses evidence distinct pathogenesis aspects, which are likely mediated by distinct viral genes. One such gene is UL39, which codes for the large subunit of ribonucleotide reductase (R1, also known as ICP6 and ICP10 for HSV-1 and HSV-2 respectively). The HSV-2 R1 has serine-threonine protein kinase (PK) activity, which is located within the first 411 amino acids (ICP10PK). ICP10PK is a constitutively activated growth factor receptor (GFR) that signals through the Ras/MEK/ERK pathway. It has transforming activity in immortalized cells, mitogenic (but not transforming) activity in normal diploid cells, and anti-apoptotic (survival) activity in post mitotic neurons in the central nervous system (CNS). In addition to the Ras/MEK/ERK, ICP10PK also activates the PI3-K/Akt pathway, upregulates the Ras family member Rap-1 and adenylate cyclase and activates the B-Raf kinase activity. ICP10 PK appears to have a cellular origin. Its conservation is most likely to reflect the ability to impart an evolutionary advantage, particularly in the face of pro-apoptotic viral genes. Indeed, activation of the Ras/MEK/ERK pathway by ICP10PK is required for virus growth.

2. INTRODUCTION

Herpes simplex viruses types 1 (HSV-1) and 2 (HSV-2) are associated with a wide range of diseases related to infection of epithelial or neuronal tissues. Both viruses initially infect skin and mucous membranes. They replicate in epithelial cells at these sites, causing clinically overt disease (primary infection), which is characterized by vesicular lesions of different severity that often coalesce. The virus is then transported by retrograde axonal transport to trigeminal and cervical (HSV-1) or sacral (HSV-2) sensory ganglia, where a latent infection is established. During latency the viral genome persists as an episome, in a largely transcriptionally silent state. Transcription resumes upon patient exposure to a wide range of stimuli, including immunosuppression, nerve damage, psychological stress, or UV exposure. It results in virus reactivation and transport back to the initial site of infection. Contrary to the widely held belief that HSV-1 and HSV-2 are indistinguishable, the two viruses evidence distinct pathogenesis, notably the ability of HSV-1, but not HSV-2, to cause encephalitis in adult subjects (1,2). These differences point to the existence of serotype-specific functions, which are likely mediated by distinct viral genes.

The overall homology between HSV-1 and HSV-2 is 50%, and it is considerably lower for some genes or sequences within them. One viral gene that is structurally and functionally different in HSV-1 and HSV-2 is UL39, which codes for the large subunit of ribonucleotide reductase (R1, also known as ICP6 and ICP10 for HSV-1 and HSV-2 respectively). In HSV-2, the R1 protein has a serine-threonine protein kinase (PK) activity, which is located within the first 411 amino acids (ICP10PK) (3). The ICP10PK protein has a relatively low level of homology to its HSV-1 counterpart (<40%), while the R1 domain that binds the small subunit of ribonucleotide reductase, is highly conserved (90% homology). The HSV-

1 counterpart of ICP10PK does not have enzymatic activity and its function is dramatically different. This review discusses the different functions of ICP10PK, with particular emphasis on the mechanism involved in its proliferative and anti-apoptotic activities and the contribution of the cell type and its mitotic state.

3. ICP10PK IS A CONSTITUTIVELY ACTIVATED GROWTH FACTOR RECEPTOR (GFR) SERINE-THREONINE KINASE

The HSV UL39 gene product is R1, the large subunit of the viral ribonucleotide reductase (4,5). Unlike R1 counterparts in eukaryotic and prokaryotic cells and in other herpesviruses, the HSV-1/2 R1 proteins have a unique amino terminal domain that is poorly conserved relative to the carboxy-terminal domain. In HSV-2, the R1 amino-terminal domain has serine-threonine kinase activity (ICP10PK) with both auto- and trans-phosphorylating functions (6-11). The targets of ICP10PK include calmodulin, histones, ras-GAP, and the HSV-2 proteins RR2, VP16, and vhs (7-13). Philogenetic studies indicated that this amino terminal domain, which is located at amino acids 1-411, might have originated from a cellular protein with atypical serine-threonine PK activity (12). This interpretation is supported by subsequent findings that ICP10PK is homologous to a heat shock protein with atypical serine-threonine PK activity (14).

Most orthodox eukaryotic PKs have 12 catalytic motifs and function with only one ATP binding site. However, recent studies have identified eukaryotic proteins with demonstrated kinase activity that shares little or no recognizable sequence similarity with the canonical PKs (15,16). ICP10PK, which was originally considered uniquely distinct, is now recognized as a member of the growing family of atypical kinases (15). Indeed, ICP10PK has only 8 catalytic motifs. The catalytic core is localized within the first 270 amino acids (29kDa protein) (7), and two lysine residues that bind ATP are required for enzymatic activity. Kinase activity was significantly reduced by replacement of Lys^{176} (Km = 1.2 and 6.6 μM for the wild type and mutant respectively) or Lys^{259} (Km = 1.2 and 9.4 μM for the wild type and mutant respectively), both of which bind ATP, as evidenced by FSBA-binding and competition studies. Enzymatic activity was abolished only when both lysine residues were mutated (8,9).

The ICP10 PK catalytic core is preceded by a single transmembrane (TM) helical segment, which is followed by a basic amino acid that is responsible for anchoring the TM into the cell membrane. There is a short (88 amino acids) extracellular domain, which initiates with a signal peptide (6,13). In HSV-2 infected and stably transfected cells, ICP10PK is localized on the cell surface (11) and it is internalized by the endocytic pathway (17). The ICP10 kinase activity is constitutively activated, and ligand binding does not regulate it. However, it is ligand inducible, as shown with a chimera in which the ICP10 extracellular domain was replaced with that of the epidermal growth factor receptor (18). Thus, ICP10PK is an activated growth factor receptor that can transmit extracellular signals. Significantly, the HSV-1 R1 protein (also known as ICP6) retained the unique amino-terminal sequences that were likely co-opted from a cellular gene (14). However, ICP6 does not have kinase activity (6), it is not located on the cell surface (19) and it does not function as a growth factor receptor. The evolutionary pressures responsible for these differences are unknown, and may be reflect the distinct tissues infected by the two HSV serotypes and/or the ganglia in which they establish latent infection.

4. ICP10PK HAS TRANSFORMING ACTIVITY IN IMMORTALIZED CELLS

Following up on the original report that HSV-2, the lytic function of which had been inactivated, causes morphological transformation of immortalized cells (20), early studies from Aurelian's laboratory sought to identify the transforming viral DNA sequences using restriction endonuclease mapping. DNA sequences within the BglIIC restriction endonuclease fragment were shown to cause multistep neoplastic transformation of primary diploid rodent cells. The first step was cellular immortalization and it was mediated by sequences located within the left hand region of the BglIIC fragment. The mechanism of cellular immortalization is still unclear. It may involve the functional disruption of tumor suppressor genes and/or activation of telomerase activity (21). Immortalization was followed by transformation mediated by sequences within the right hand region of the BglIIC fragment that code for ICP10PK (22-26). ICP10PK also transformed immortalized human cells, but it did not transform normal diploid cells.

Immortalization is required for transformation by ICP10PK. However, transformation is independent of the mechanism of cellular immortalization, and it is equally effective in cells immortalized by spontaneous genetic alterations, HSV-2 immortalizing DNA sequences or other immortalizing viral genes (viz. adenovirus E1a) (11,22). Cell proliferation and the maintenance of the transformed/tumorigenic phenotype depends on the constitutive expression of ICP10PK, as evidenced by inhibition with antisense oligonucleotides that specifically inhibit ICP10PK expression. As will be discussed below, the mechanism of ICP10PK-mediated cell proliferation is activation of the Ras/MEK/ERK signaling pathway. This is a relatively unusual mechanism for the regulation of cell life/death decisions by a viral gene, but it is common for cellular genes that regulate such decisions, notably Ras. Indeed, enforced Ras expression converts immortalized cells to a neoplastic phenotype, and the adenovirus immortalizing early region gene (E1a) collaborates with both ICP10 PK (11) and Ras (27) to convert cells to a full neoplastic phenotype.

5. ICP10PK HAS MITOGENIC ACTIVITY IN DIPLOID CELLS

Although ICP10PK does not transform normal diploid cells, it can stimulate their proliferation. Thus, a complication of HSV-2 infection is the development of

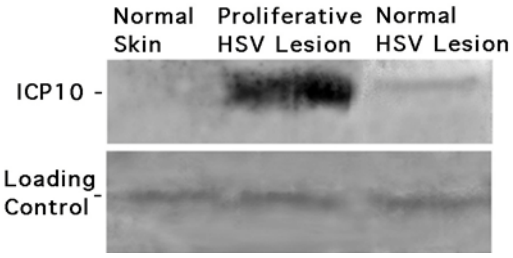

Figure 1. ICP10 expression is higher in HSV-2 hyperproliferative than vesicular lesions. Extracts from normal skin (lane 1), HSV-2 hyperproliferative (lane 2), and vesicular (lane 3) lesions were immunoblotted with ICP10 specific antibody (upper panel). Loading control protein (actin) (lower panel).

hyperproliferative lesions, which can be difficult to treat. We reported the case of a patient with common variable immunodeficiency that presented with large hyperproliferative lesions caused by an acyclovir resistant HSV-2 mutant. Histopathology revealed pseudo-epitheliomatous hyperplasia, but the lesion did not contain malignant cells and the patient was successfully treated with foscarnet (28). The lesions recurred and were similarly controlled with foscarnet therapy. Immunoblotting of extracts from hyperproliferative and vesicular HSV lesions using antibody specific for ICP10 PK indicated that the levels of ICP10 PK were significantly higher in the hyperproliferative (Figure 1, lane 2) than the vesicular (Fig 1, lane 3) lesion. Normal skin tissue did not express ICP10 PK (Fig 1 lane 1), and the loading control protein showed that equivalent amounts of extracts were analyzed. Because the patient suffered from common variable immunodeficiency, which may favor virus replication due to immune evasion, the increased levels of ICP10 PK in the hyperproliferative lesion may be due to higher levels of replicating virus. Virus titers were not directly compared in these studies. However, an alternative interpretation that is not excluded by our studies is that the increased levels of ICP10 PK in the hyperproliferative lesions are indicative of impaired regulation of viral gene expression related to acyclovir resistance. HSV-induced hyperproliferative lesions were also described by others (29). Collectively, the data indicate that ICP10 PK overexpression is associated with benign cellular hyperproliferation, a conclusion supported by the results of experimental studies (unpublished observation).

6. ICP10PK ACTIVATES THE RAS/MEK/ERK SIGNALING PATHWAY

6.1. Ras signaling defines cell life and death decisions

Cell proliferation is induced by GFR activation and/or binding of cell adhesion molecules (viz. integrins) to specific extracellular matrix (EM) molecules. Their stimulation results in a cascade of phosphorylation events which lead to the upregulation of transcription factors, increased expression of genes that regulate cell cycle progression (viz. cyclin D1) and downregulation of cyclin dependent kinase inhibitors (CDK). Ras activation initiates the cascade of protein-protein interactions known as the ERK signaling pathway. It also activates the PI3-K/Akt cascade (Figure 2B).

Ras is a membrane bound G protein, which is activated by binding of GTP as a result of growth factor-mediated activation of cognate receptors. Exchange factors, such as Sos are involved in the conversion of Ras-GDP to Ras-GTP. Once activated, Ras triggers activation of a cascade of serine/threonine PKs beginning with Raf-1 and followed by MAP kinase kinase (MEK 1/2) and mitogen activated kinase (ERK1/2). It culminates in increased expression/ activation of transcription factors, such as c-Fos, which is important for cell cycle progression into S phase. This conserved Ras-activated PK cascade regulates cell growth, proliferation and differentiation in response to growth factors, cytokines and hormones. Under normal conditions, Ras is downregulated by the GTPase activating protein Ras-GAP, which enhances the weak GTPase activity of Ras, and hydrolyzes the bound GTP to GDP. The transcription factors that are upregulated by the Ras activated pathways are likely to contribute to the cell type specificity of the response and its outcome. Determinant roles are played by the cell type, its mitotic state (position on the cell cycle) and the nature and duration of the external stimulus. For example, c-Fos is upregulated/stabilized as a result of the ICP10PK-mediated activation of the Ras/MEK/ ERK pathway in mitotic cells (30). By contrast, in post-mitotic neurons, pathway activation by ICP10PK results in the upregulation of the transcription factor CREB (31).

C-Raf-1 activation is initiated by the activated Ras (Ras-GTP), which binds to the Ras-binding domain (RBD) within the Raf-1 N-terminal regulatory region. Concomitant conformational changes and recruitment to the cell membrane promote changes in Raf phosphorylation that combine to stimulate its serine-threonine kinase activity. Raf activation is followed by the sequential phosphorylation (activation) of MEK and ERK (32). In humans, there are 3 functional Raf proteins (A-Raf, B-Raf and C-Raf) but only A-Raf and C-Raf require additional serine and tyrosine phosphorylation within the N region of the kinase domain for full activity (33). Communication between Raf, MEK and ERK requires the scaffolding protein KSR1, a Raf homolog that lacks kinase activity (34).

The Ras/MEK/ERK signaling pathway is constitutively activated in many cancers and these have been associated with mutations in Ras or B-Raf (35-37). The mechanism of oncogenic activation by B-Raf differs fundamentally from that of v-Raf, a retroviral oncogene derived from C-Raf. The constitutive activity and high transforming potential of v-Raf is likely to result from loss of the auto inhibitory N-terminal region combined with targeting to the plasma membrane, while most of the mutations in B-Raf are located at other sites within the catalytic domain. In most cases this stimulates the activity of B-Raf toward MEK. However, in some cases the B-Raf mutants adopt a conformation that stimulates the wild type C-Raf that in turn signals to ERK (38). These considerations underscore the cross talk between members

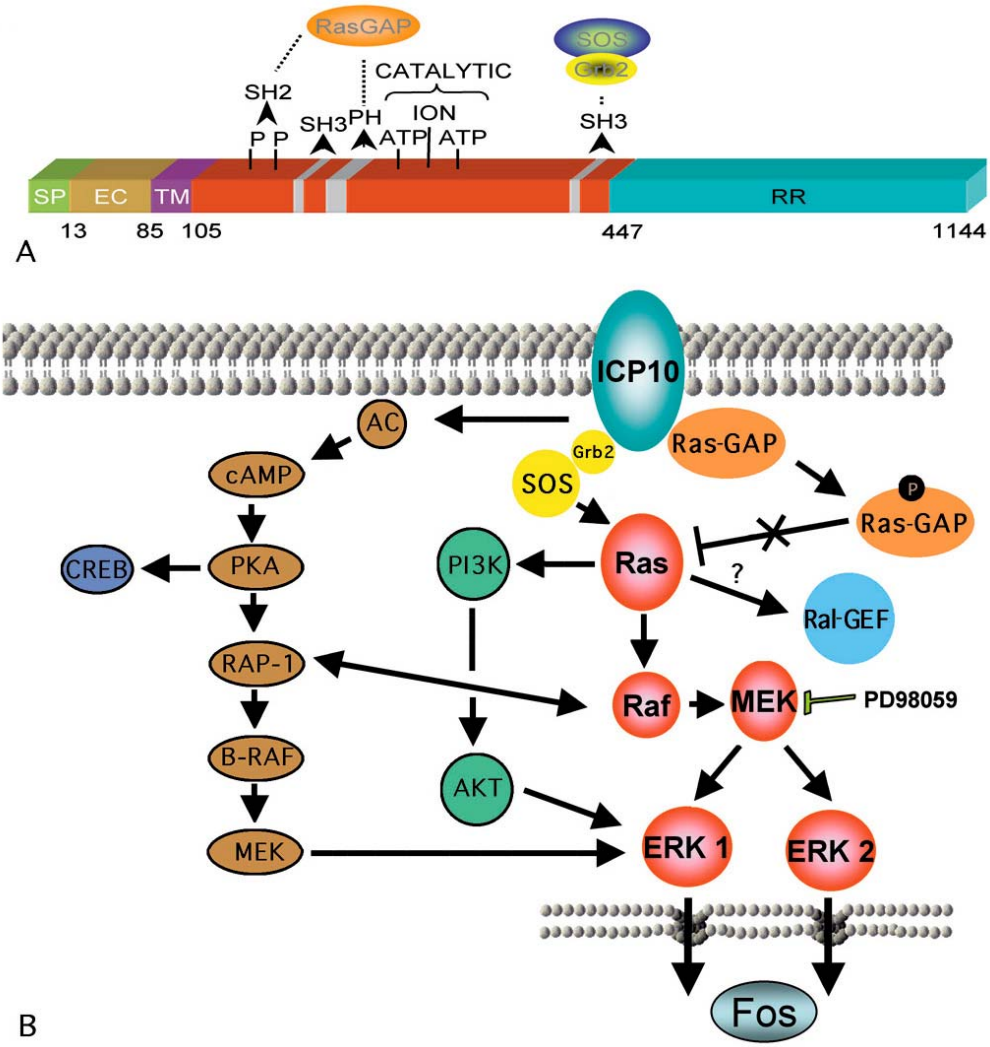

Figure 2. ICP10PK and the signaling cascades it activates. (A). Schematic representation of the ICP10PK map includes the signal peptide (SP, aa 1-13), extracellular domain (EC, aa14-85), transmembrane domain (TM, aa 86-105), and catalytic core (aa 106-270). The catalytic core contains two ATP binding sites (ATP) and an ion-binding site (Ion). The major Ras-GAP binding site is a WD40-like motif that interacts with the PH domain of Ras-GAP PH (PH). Two phosphorylated threonine residues (P) bind the Ras-GAP N-SH2 domain. The major Grb_2-hSOS-major binding site is the proline rich motif at position 396-405. A minor binding site is at position 149-159. They bind the SH3 domain on Grb_2 (SH3). (B). Schematic representation of signaling pathways activated by ICP10PK. ICP10PK functions as a constitutively activated GFR. It activates the Ras/c-Raf-1/MEK/ERK, PI3-K/Akt, and Rap-1/B-Raf pathways. Ral-GEF may also be activated. In addition, ICP10PK binds and phosphorylates Ras-GAP, thereby inhibiting its Ras downregulatory activity. PD98059 is a MEK specific inhibitor.

of signaling pathways, which enables them to regulate the cell response to extracellular stimuli.

The expression of cyclin D1 is controlled by MEK/ERK signaling (39). At least in part, this induction is likely to be mediated by the activation of the AP-1 transcription factor (40). However, the intensity and duration of the signaling through the MEK/ERK pathway determines whether the cell undergoes proliferation, differentiation or cell-cycle arrest (41). For example in response to a strong Raf signal, G1-specifc cell cycle arrest leads to the inhibition of cyclin D and E dependent kinases, and this results in cell cycle arrest (42). By contrast, moderate Raf activity is sufficient to induce cyclin D expression and DNA synthesis (43). The transcription factors c-Fos, Myc and c-Jun have been shown to dictate biological outcome (44), in a cell-type specific fashion. For example, sustained ERK signaling that results in c-Fos phosphorylation leads to its stabilization, thereby positively impacting entry into the cell cycle (45). Given the role of the MEK/ERK signaling pathway in mediating both cell cycle progression and arrest, it is particularly surprising that the Raf/MEK/ERK pathway is constitutively activated in most tumors. A possible interpretation that is gaining increasing support, is that tumor cells use this pathway to promote their survival by inhibiting apoptosis. Indeed,

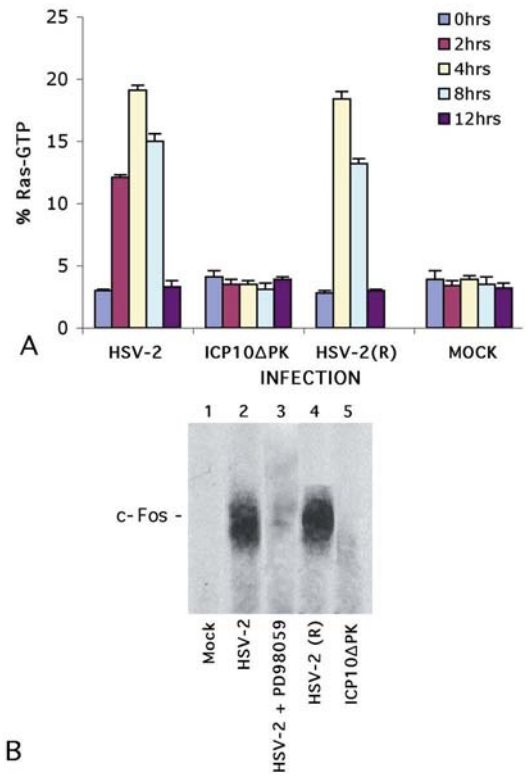

Figure 3. ICP10PK activates Ras and upregulates/stabilizes c-Fos. (A). Vero cells were labeled with [^{32}P]-orthophosphate for 18h and infected with HSV-2, the ICP10PK deleted mutant ICP10deltaPK, the revertant virus HSV-2(R), or mock infected with PBS. Cell extracts obtained at various times p.i. were precipitated with Ras antibody and the levels of Ras-GTP and Ras-GDP in the precipitates were determined after separation on PEI cellulose thin layer plates. Ras was activated by HSV-2 and HSV-2(R), but not ICP10deltaPK. (B). Extracts from Vero cells mock-infected with PBS (lane 1), or infected (18h) with HSV-2 (lane 2), HSV-2 in the presence of the MEK inhibitor PD98059 (50μM)(lane 3), HSV-2 (R) or ICP10deltaPK were immunoprecipitated/ immunoblotted with c-Fos antibody. C-Fos is induced/stabilized as a result of the MEK/ERK pathway activated by ICP10PK.

members of the Raf family are important mediators of MEK-dependent and independent anti-apoptotic activity (46).

6.2. ICP10PK activates the Ras/MEK/ERK signaling pathway

ICP10 PK is a constitutively activated GFR kinase that signals by activating the Ras/Raf-1/MEK/ERK pathway in order to induce cell proliferation and block apoptosis (9,11,30). The autophosphorylated (constitutively activated) ICP10 PK receptor binds the Grb2-hSOS complex through a direct interaction between the Grb-2 adaptor protein and proline rich SH3 binding modules on ICP10 PK (Figure 2A). The result of this interaction is to localize the guanine nucleotide exchange factor, hSOS, to the vicinity of Ras at the plasma membrane (Figure 2B), where it converts inactive Ras-GDP to the active Ras-GTP. ICP10 PK also binds and phosphorylates (and, thereby, inactivates) Ras-GAP (11), a GTPase activating protein that downregulates Ras activation by converting Ras-GTP to Ras-GDP. Ras-GAP is bound at both its N- SH-2 and PH modules (30). Interaction with the PH module is independent of ICP10PK phosphorylation, whereas binding to the N-SH2 module depends on the phosphorylation of two threonine residues, (pThr117 and pThr141) on ICP10 PK. Binding of both Ras-GAP sites may function to stabilize the interaction and/or improve presentation to the adjacent ICP10 PK catalytic core (30), thereby favoring its inactivation by ICP10PK-mediated phosphorylation. The combined effect of hSOS binding and Ras-GAP inactivation is a significant increase in the levels of activated Ras (Figure 3A) and the increased expression/stabilization of c-Fos (Figure 3B).

Kinase activity and cell membrane localization are required for the mitogenic function of ICP10PK, as evidenced by studies of ICP10PK mutants. These include a transmembrane deleted mutant (p139^{TM}) and a mutant deleted in the entire ICP10PK domain, both of which are kinase negative. The TM deleted ICP10 PK mutant (p139^{TM}) failed to anchor into the plasma membrane, resulting in the inhibition of PK activity. It did not bind Grb2-SOS nor phosphorylate Ras-GAP, thereby failing to activate Ras (11). This presumably reflects a stringent requirement for presentation of its ATP binding sites that is only achieved through relative structural rigidity imparted by protein anchorage into the plasma membrane. Mutants in various residues required for enzymatic activity had different levels of kinase reduction (11) and they were studied in parallel. There was a direct correlation between the ICP10PK kinase activity of the various mutants and their mitogenic/transforming function (Figure 4), supporting the conclusion that the ICP10PK mitogenic potential depends on its kinase activity and membrane localization, both of which are required for Ras activation. The involvement of the TM in PK activity and transforming potential has also been reported for other constitutively activated GFRs (47-49).

We conclude that Ras-GAP inactivation contributes to the mitogenic activity of ICP10 PK because an ICP10PK mutant that is deleted in the Ras-GAP binding site but retains PK activity increased Ras activation, and had a 10-20 fold higher transforming activity than the wild type ICP10PK (3). The levels of activated Ras (Ras-GTP) were significantly increased in cells infected with HSV-2, while a similar increase was not seen in cells with an HSV-2 mutant deleted in ICP10PK (ICP10deltaPK). This correlated with significantly reduced GTPase activity in HSV-2 infected cells, in which Ras-GAP was bound and phosphorylated by ICP10PK, as compared to ICP10deltaPK infected cells in which Ras-GAP was not similarly altered. The contribution of Ras-GAP regulation to ICP10PK-mediated mitogenic activity is consistent with independent reports that Ras-GAP is inactivated by phosphorylation on serine/threonine residues (50).

Ras activation by ICP10 PK stimulates a downstream kinase cascade, which results in increased

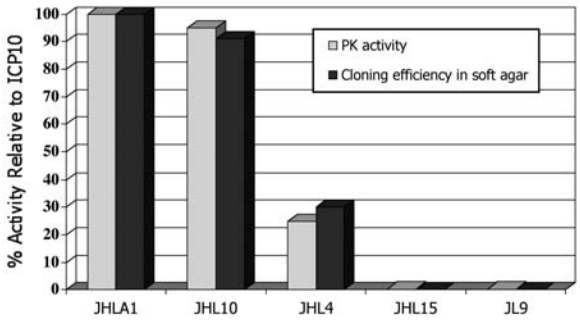

Figure 4. ICP10PK kinase activity correlates with its mitogenic/transforming potential. HEK293 cells were stably transfected with wild type ICP10PK (JHLA1), an ICP10PK mutant deleted in the extracellular domain (JHL10), a site directed mutant in Lys^{176} (to Leu^{176}) (JHL4) the transmembrane domain deleted mutant $p139^{TM}$ (JHL15), or a mutant deleted in the entire PK domain (JL9). They were assayed for ICP10PK kinase activity and cloning efficiency in soft agar. Because ICP10PK is constitutively activated, the extracellular domain is not required for PK and mitogenic activities.

phosphorylation of Raf-1, MEK1/2, and ERK. This cascade was not activated by the PK negative mutant $p139^{TM}$ that is deleted in the ICP10PK transmembrane domain nor by the ICP10deltaPK mutant in which the entire ICP10PK domain was deleted (30). ICP10 PK-induced cell proliferation depends on the activation of the Ras/Raf-1/MEK/ERK pathway because the $p139^{TM}$ mutant, who fails to activate the Ras pathway, does not induce proliferation (11,17). Consistent with previous reports about Ras effectors and their role in mitogenesis (51), it is likely that other pathways activated by ICP10PK also contribute to cell proliferation. Indeed, in addition to Raf-1, the ICP10 PK-activated Ras in turn activates phosphatidylinositol 3-kinase (PI3-K) (unpublished). It may also activate Ral-GEFs, which are guanine nucleotide exchange factors for another Ras family protein, Ral GTPase (Figure 2). PI3-K and Ral-GEFs synergize with Raf-1 in Ras-mediated transformation of rodent cells and Ral-GEFs are essential for Ras-mediated transformation of human cells (21).

7. ICP10PK HAS ANTI-APOPTOTIC, BUT NOT PROLIFERATIVE, ACTIVITY IN CNS NEURONS

Apoptosis (programmed cell death) is an important cellular response that controls cell number by removing damaged cells. In tumor cells, disruption of normal apoptotic pathways due to genomic damage protects from cell death and provides a selective advantage for tumor cell growth. Oncogenes, such as Ras, Bcr/Abl, and protein kinase B (PKB) have antiapoptotic activity, likely related to their mitogenic functions (52-54). This property is shared by ICP10PK. Thus, HEK293 cells that constitutively express ICP10PK were protected from treatment with staurosporine (induces apoptosis by PK inhibition) or D-mannitol (induces apoptosis by osmotic shock due to oxidative stress). Protection was not seen in cells that constitutively expressed the PK negative mutant $p139^{TM}$ (55), suggesting it depends on the ICP10PK kinase activity. Anti-apoptotic activity required activation of the Ras/Raf/MEK/ERK pathway (56,57).

7.1. ICP10PK is neuroprotective

Having seen that ICP10PK has anti-apoptotic activity, the question that arises is whether this activity is cell-type specific and depends on the mitotic state of the cells. HSV-1 triggers apoptosis in CNS neurons (31,55,58). In primary hippocampal cultures apoptosis involves activation of the JNK/c-Jun pathway. This results in the activation of caspase-3, destabilization of the anti-apoptotic protein Bcl-2 and increased expression of the proapoptotic protein Bad (31). Significantly, JNK/c-Jun activation was also observed in the brains from patients with HSV-1-induced encephalitis (HSE), where it was located at the sites of HSV infection, suggesting that JNK-c-Jun apoptosis plays a significant role in HSE pathogenesis (58). HSV-2 does not cause apoptosis in primary hippocampal cultures. Because the ICP10PK deleted HSV-2 mutant (ICP10deltaPK) also triggers apoptosis in these cells, the failure of HSV-2 to cause HSE is likely due to the anti-apoptotic activity of ICP10 PK. Indeed, apoptosis of primary hippocampal cultures induced by HSV-1 or ICP10deltaPK was blocked by ectopic delivery of ICP10PK, indicating that ICP10PK has dominant anti-apoptotic activity, which overrides virus-induced apoptosis. The anti-apoptotic activity of ICP10 PK is c-Raf-1 dependent, as evidenced by loss of neuroprotection in hippocampal cultures transfected with a dominant negative c-Raf-1 mutant, or by treatment with a pharmacologic inhibitor of c-Raf kinase. Anti-apoptotic activity was also blocked by the MEK inhibitor U0126, indicating that it is Raf-1/MEK/ERK dependent (31).

The contribution of the cell's mitotic state towards the outcome of Ras activation by ICP10PK is significant, with cell survival as the outcome in terminally differentiated neurons, proliferation as the outcome in diploid cells and transformation as the outcome in immortalized cells, in which the cell cycle is de-regulated. These different outcomes may reflect the upregulation/activation of distinct transcription factors. Indeed c-Fos is upregulated stabilized in immortalized cells transformed by ICP10PK. C-Fos has growth promoting activity, which is mediated by the repression of tumor suppressor genes (59) and by the upregulation of cell cycle regulators, such as cyclin D1 (59,60). However AP-1 does not have a mitogenic effect in post-mitotic cells, such as hippocampal neurons. Moreover, in these cells, ICP10PK induces/activates/ stabilizes CREB, a transcription factor that is involved in neuronal cell survival. CREB is the primary transcription activator of Bcl-2 (61) and it has been implicated in neurotrophin-mediated gene transcription associated with neuronal survival (62). Either the PI-3K/Akt or the MEK/ERK pathways can activate CREB, which is required for basal maintenance of hippocampal neurons (63). Since activation of the PI-3K/AKT pathway is not involved in ICP10 PK-mediated protection from virus infection (55), any involvement of CREB in ICP10 PK protection in this paradigm would most likely be due to

the MEK/ERK pathway. However, the exact role played by CREB in inhibition of virus-induced apoptosis, is still unclear.

In post-mitotic neurons, Bag-1 is another factor that is upregulated as the result of ICP10PK-mediated activation of the Ras/MEK/ERK pathway (31). The activated Bag-1 isoform (Bag-1S) has anti-apoptotic activity and is involved both in the upregulation of the Raf-1 kinase and in the stabilization of the anti-apoptotic protein Bcl-2 (64). The increased stability of Bcl-2 in HSV-2, but not ICP10deltaPK, infected hippocampal cultures, may be due to its interaction with Bag-1, which is known to modulate chaperone activity (65). The pro-apoptotic protein BAD, which is associated with glutamate-induced neuronal apoptosis and neurodegenerative disorders (66-68), was induced in HSV-1 and ICP10deltaPK infected hippocampal neurons, as the outcome of JNK/c-Jun activation by HSV-1 pro-apoptotic genes. Its upregulation/activation, which is induced by other HSV pro-apoptotic genes, was blocked by ICP10PK (31).

Bag-1 is a major factor in ICP10PK-mediated neuroprotection accounting for over 90% of its neuroprotective activity. When ectopically delivered Bag-1 blocked HSV-1 or ICP10deltaPK-induced apoptosis just as well as the ectopically delivered ICP10PK. Moreover, both Bag-1 and ICP10PK had a bystander trophic effect exemplified by a higher reduction in the percentage of of apoptotic cells (80%) than either the ICP10PK or Bag-1 expressing cells (38-45%) (31).

7.2. ICP10PK is neuroprotective in paradigms other than virus infection

ICP10PK has anti-apoptotic (neuroprotective) activity also in paradigms other than virus infection. These include trophic factor deprivation, oxidative stress, and genetic alterations (mutation). Thus, neuronally differentiated [(NGF)-dependent)] PC12 cells die by apoptosis upon NGF withdrawal (69). Transient expression of ICP10PK, but not its PK negative mutant p139^{TM}, protected differentiated PC12 cells from apoptosis due to NGF withdrawal (70). Oxidative stress is also associated with apoptosis in many neurological disorders, and 3-4% of these cases are associated with mutations in the gene encoding superoxide dismutase (SOD1) (71). ICP10PK is also neuroprotective under these conditions. Transient expression of ICP10PK in differentiated mouse neuroblastoma cells that constitutively express the wild type or mutated SOD1 (72) protected these cells from apoptosis induced by mutant SOD1 and oxidative stress resulting from xanthine/xanthine oxidase treatment (73). Among the genetic defects associated with apoptosis, ts16 is believed to confer increased vulnerability to neurodegeneration (74). The ts16 mouse is considered to be a model of Down's syndrome (DS; trisomy 21) and primary hippocampal neurons from these mice exhibit increased cell death even in the presence of adequate trophic support (75). Transfection of ts16 hippocampal neurons with an ICP10PK expression vector, increased survival and blocked apoptosis as compared to the untransfected cells. Cells transfected with an ICP10 mutant that lacks PK activity, were not protected. In all these paradigms, protection was associated with ERK activation. The signaling pathways leading to ERK activation in the various paradigms are currently under investigation. Protection by ICP10PK delivery may be due to compensation for the innate defects in receptor signaling which are known to exist in these cells (76).

8. OTHER PROTEINS AND SIGNALING CASCADES ACTIVATED BY ICP10PK

In addition to the activation of the Ras/Raf-MEK/ERK signaling pathway, ICP10PK also upregulates expression of the Ras family member, Rap-1 GTPase, and activates the Rap-1/B-Raf module, as evidenced by activation of B-Raf kinase activity in HSV-2, but not in ICP10deltaPK infected hippocampal neurons (submitted for publication). B-Raf kinase activation can be mediated by direct binding to activated Ras (77,78). However, in HSV-2 infected hippocampal cultures, activation appears to be related to Rap-1 upregulation. Indeed, Rap-1 co-precipitated with the activated B-Raf, but it did not co-precipitate with the non-activated B-Raf from mock or ICP10deltaPK infected cells (Smith, in preparation). This was particularly intriguing, since both ICP10PK (31,55,70,79,80) and activated Rap-1/B-Raf (81,82) are associated with neuro-protection. However, ICP10PK activates the Ras/Raf-1 signaling module (31,55), and it appears to play a major (>85%) role in the neuroprotective activity of ICP10PK (31). Therefore, involvement of the activated Rap-1/B-Raf activation in neuroprotection, if any, is probably due to cross talk between the two pathways. This was also implied by independent studies which indicate that Ras and Rap-1 can be activated by the same survival signal (83,84), and the observation that B-Raf mutants can stimulate the wild type c-Raf which in turn signals ERK (35), and adenylate cyclase (submitted for publication). Finally, ICP10PK upregulates adenylate cyclase that activates ERK through cAMP and protein kinase A (PKA)-dependent activation of Rap-1 (82).

Although the interaction of the activated Ras/Raf-1 and Rap-1/B-Raf pathways is still poorly defined, it has been reported that c-Raf and B-Raf can herodimerize (84) and B-Raf may play a role in Ras-induced Raf-1 activation (85). Additional studies are needed, using dominant negative mutants or siRNAs to Rap-1 or B-Raf kinase, in order to further define the potential cross talk between the c-Raf and B-Raf modules in ICP10 PK-mediated protection of CNS neurons. Since Ras and Rap-1 have distinct functions and regulate unique cellular processes in response to various extracellular ligands (86), it seems reasonable to assume that the two modules may also be independently regulated by ICP10 PK in response to various apoptotic stimuli.

9. ICP10PK-MEDIATED ACTIVATION OF THE ERK PATHWAY IS REQUIRED FOR VIRUS REPLICATION

Why does HSV-2 need a function that induces cell proliferation? As stated earlier, signaling pathways

determine the cell's ability to respond to external stimuli, with transduced signals being necessary for cell proliferation, differentiation, or apoptosis, depending on the cell type and the nature of the stimulus. Because viruses depend on cells for replication, they take advantage of pre-existing signaling pathways or activate them in order to enhance their replicative potential. The activated Ras/MEK/ERK pathway can cause a variety of outcomes, including increased cell proliferation, and activation of both pro- and antiapoptotic signaling pathways determined by cell type and context (52).

Studies of an HSV-2 mutant deleted in ICP10PK (ICP10deltaPK) indicated that optimal virus replication depends on the ability of ICP10PK to activate the Ras/MEK/ERK signaling pathway. Single step growth curves indicated that, by comparison to HSV-2, onset of ICP10deltaPK growth was was significantly delayed (10 hrs), both in replicating (10% serum) and non-replicating (1% serum) cells. A similar delay was seen in cells infected with HSV-2 in the presence of the MEK inhibitor PD98059, suggesting that activation of the Ras/MEK/ERK pathway is required for the timely onset of virus growth. Immediate early (IE) proteins regulate optimal virus growth and their synthesis is delayed in ICP10deltaPK, as compared to HSV-2 infected cells (87). Therefore, it is likely that ICP10PK-mediated activation of the Ras/MEK/ERK pathway is required for timely onset of IE gene expression and virus growth, due to its effect on cell proliferation. Indeed, both ICP10deltaPK and HSV-2 grown in the presence of PD98059 eventually begin to replicate and virus titers ultimately reach levels similar to those seen for untreated HSV-2, in replicating (10% serum) cells (87). In non-replicating cells (1% serum), the titers of both ICP10deltaPK and HSV-2 grown in the presence of PD98059 are significantly lower than those of untreated HSV-2, suggesting that a MEK-independent cellular function is ultimately induced by virus infection and it provides the environment that is conducive for virus growth (87). The identity of this compensatory function is under investigation.

The culmination of the Ras/Raf-1/ERK signaling pathway activated by ICP10PK is the increased expression of one of the AP1 transcription factors, c-Fos (17,30). AP-1 is involved in cellular proliferation, transformation and cell death depending on the physiological stimuli and environmental insults as well as the cell type. It is likely to regulate distinct genes in each one of these circumstances. Growth factors and oncoproteins (viz. Ha-Ras) induce c-fos: c-jun dimerization in dividing cells, which is mediated by the ERK signaling cascade (59). This suggests that the c-fos: c-jun heterodimers are involved in cell proliferation and play a key role in transformation. The ability of ICP10PK, but not the PK negative mutant $p139^{TM,}$ or the ICP10PK deleted virus, ICP10deltaPK, to activate AP-1 correlates with its ability to induce cell proliferation, suggesting that AP-1 is involved in this process, as previously described for Ras (51). Significantly, however, in addition to its growth promoting effects, c-fos functions in a positive feedback loop to increase ICP10 expression (30), which is mediated through AP-1 binding sites on the ICP10 promoter (88). The net result is increased ICP10 expression, further facilitating virus replication. This regulatory function may be particularly relevant for the support of virus growth in cells that do not replicate such as terminally differentiated neurons, potentially explaining the ability of HSV-2 to replicate in these cells during latency reactivation.

9. CONCLUSION

The cumulative impact of novel information about molecular mechanisms of disease pathogenesis has led to increasing realization that HSV-1 and HSV-2 are biologically distinct. One viral gene that is responsible for some of these differences is the large subunit of ribonucleotide reductase that has PK activity in HSV-2, but not HSV-1. The HSV-2 RR1 PK (ICP10 PK) functions by activating the Ras/MEK/ERK signaling pathway. Other viruses can also activate Ras signaling pathways to enhance their virus growth. However, this is a rather unique function for a viral protein and the activating mechanism used by ICP10PK is different. Thus, Vaccinia virus activates Ras by tyrosine phosphorylation of the epidermal growth factor receptor (89), human cytomegalovirus activates ERK (90) and Coxsackievirus B3 activates Ras by hydrolyzing Ras-GAP (91).

ICP10PK can induce cell proliferation, transformation or survival depending on the cell type and its mitotic or post-mitotic state, presumably by virtue of its ability to activate Ras, which has the same properties. At the molecular level, the distinct biological outcome probably reflects the activation of a range of transcription factors, which is determined by the cell type and its position along the cell cycle and results in increased expression of different target genes. For example, ICP10 PK-mediated activation of the Ras/Raf-1/MEK/ERK pathway in immortalized cells leads to upregulation of the AP1 transcription factor c-fos. This transcription factor is responsible for increased expression of positive cell cycle regulators and downregulation of tumor suppressor genes that inhibit cell cycle progression. However, in post mitotic neuronal cells, activation of the Ras/Raf-1/MEK/ERK pathway by ICP10 PK increases the expression of neuroprotective anti-apoptotic genes, such as CREB and Bag-1. It will be interesting to see whether other survival pathways are responsible for the ability of ICP10PK to protect from apoptosis caused by other stress conditions.

In summary, ICP10PK is a remarkably versatile protein with a variety of biological activities. Given its likely cellular origin, it is tempting to conclude that the virus has conserved it because it imparts an evolutionary advantage particularly in the face of other pro-apoptotic viral genes. Its mitogenic activity is beneficial because stimulation of cellular machinery is likely to aid in virus growth. Indeed, ICP10 PK is required for immediate early gene expression and timely onset of virus growth (3). The antiapoptotic function of ICP10PK in post mitotic neurons may promote survival of neuronal cells after HSV-2 infection, which would allow for latency establishment, and

allow for virus reactivation induced by stimuli, which normally would be apoptotic. An interesting question that arises from these considerations, is why does the HSV-1 homologue of ICP10 PK lack similar functions and by implication why did HSV-1 retain this co-opted cellular gene?

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