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The herpes simplex virus type 2 gene ICP10PK protects from apoptosis caused by NGF deprivation through inhibition of caspase-3 activation and XIAP upregulation

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Abstract

The herpes simplex virus type 2 (HSV-2) protein ICP10PK has anti-apoptotic activity in virus-infected hippocampal cultures through activation of the Ras/Raf-1/MEK/ERK pathway (Smith et al. 2000; Perkins et al. 2002; Perkins et al. 2003). To exclude the possible contribution of other viral proteins to cell fate determination, we examined the survival of primary hippocampal cultures and neuronally differentiated PC12 cells transfected with ICP10PK from apoptosis caused by NGF withdrawal. NGF deprivation caused apoptosis in cultures mock-transfected or transfected with the kinase-negative ICP10 mutant p139TM, but not in ICP10PK-transfected cultures. In one clone (PC47), ICP10PK inhibited caspase-3 activation through upregulation/stabilization of adenylate cyclase (AC), activation of PKA and MEK, and the convergence of the two pathways on ERK activation. The anti-apoptotic proteins Bag-1 and Bcl-2 were stabilized and the pro-apoptotic protein Bad was phosphorylated (inactivated). In another clone (PC70), ICP10PK inhibited apoptosis through MEK-dependent upregulation of the anti-apoptotic protein XIAP (that inhibits the activity of processed caspase-3) and downregulation of the apoptogenic protein Smac/DIABLO. This may be cell-type specific, but the baculovirus p35 protein did not potentiate the neuroprotective activity of ICP10PK in PC12 cells, suggesting that ICP10PK inhibits both caspase activation and activity. The data indicate that ICP10PK inhibits apoptosis independent of other viral proteins and is a promising neuronal gene therapy platform.

Keywords

ICP10PK; apoptosis; gene therapy; MEK; PKA; XIAP

INTRODUCTION

Apoptosis is a self-destruct program that constitutes a significant component of acute and chronic neurodegenerative diseases. It is primarily mediated by caspases, which are cysteine proteases with aspartate specificity that are activated by the cleavage of inactive zymogens (procaspases). The cascade begins with initiator caspases (viz. caspase-9). These, in turn activate the executioner caspases (viz. caspase-3) that cause proteolytic degradation of proteins required for normal cell function and internucleosomal DNA fragmentation (Friedlander 2003; Aurelian 2005).

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In neuronal cells, including neuronally differentiated rat pheochromocytoma (PC12), growth factor withdrawal triggers caspase-dependent apoptosis (McCarthy et al. 1997; Schulz et al. 1997; Rong et al. 1999). NGF overrides the apoptotic cascade by activating survival pathways (viz. MEK/ERK and AC/cAMP/PKA) (Rukenstein et al. 1991; Vaudry et al. 2002; Jiang et al. 2005) that alter the balance of apoptosis regulatory proteins. These include members of the Bcl-2 family that have anti- (viz. Bcl-2) or pro- (viz. Bad) apoptotic activity and are differentially mobilized by various stimuli (Tsujimoto 1998), the anti-apoptotic heat shock protein chaperone Bag-1 that stabilizes Bcl-2 (Townsend et al. 2005), and the inhibitor of apoptosis proteins (IAPs), that inhibit the activity of processed (activated) caspases -9 and -3 (Deveraux and Reed 1999). The X-linked IAP (XIAP) protein also targets the apoptogenic protein Smac/DIABLO for proteolytic degradation by functioning as its ubiquitin ligase (Morizane et al. 2005). It is becoming increasingly evident that in order to be clinically efficacious, a neuroprotective platform must function at multiple levels of the apoptotic cascade, including those downstream of caspase activation.

Viruses depend on cells for their replication and they have evolved various strategies to prevent apoptosis. These include expression of IAP homologues, such as the baculovirus p35 protein, that forms a stable complex with caspase precluding its subsequent protease activity (Callus and Vaux 2007) and preventing neuronal degeneration (Miagkov et al. 2004). The herpes simplex virus type 2 (HSV-2) protein ICP10PK is another viral protein with neuroprotective activity. It activates survival pathways that inhibit caspase-3 activation in virus infected and excitotoxin-treated primary and organotypic hippocampal cultures and in animal models treated with the growth defective ICP10PK vector, Δ RR (Gober et al. 2005, 2006; Laing et al. 2006; Golembewski et al. 2007). However, studies of virus-infected cells do not fully exclude the potential contribution of other viral proteins to cell fate determination. Also, the ability of ICP10PK to inhibit caspase-3 after it has been activated, a function that is likely to impact the therapeutic window, is still unknown. The studies described in this report were designed to address these limitations. They indicate that ICP10PK is neuroprotective independent of other HSV genes and it functions through inhibition of both caspase-3 activation (by MEK/ERK and AC/PKA activation) and the protease activity of processed caspase-3 (by XIAP upregulation). This is the first report that ICP10PK activates the AC/PKA pathway and interferes with the activity of caspase-3.

MATERIALS AND METHODS

Cells

Vero cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS, Gemini Bioproducts, Calabasas, CA) and used for virus growth. 2-2 cells are Vero cells stably transfected with the HSV IE2 gene. They were cultured in DMEM-10% FBS. Primary hippocampal cultures were established from 16- to 19-day-old fetuses of Sprague-Dawley rats as described (Perkins et al. 2002; Perkins et al. 2003). They were plated at a density of approximately 750,000/2 ml on collagen-coated 35-mm dishes (Nunc, Rochester, NY) and used at 6 days in culture, when most (>85%) cells are not dividing, as determined with the 5-bromo-2'-deoxyuridine labeling and detection kit (Roche Molecular Bioproducts, Indianapolis, IN). The hippocampal cultures were established on glass coverslips etched with a grid of 175 $\times$ 175 μ m squares (CELLocate; Eppendorf, Madison, WI) and grown (2 days) in MEM with B27 supplement (Gibco) which contains optimized concentrations of neuron survival factors, as described (Bambrick and Krueger 1999). PC12 cells were grown in DMEM/F12 medium (Media Tech, Herndon, VA) with 10% FBS, 0.36% D-glucose (Sigma, St. Louis, MO), and 0.009% gentamicin (Invitrogen-Gibco-BRL, Gaithersburg, MD).

Plasmids, amplicons and viruses

The large subunit of HSV-2 ribonucleotide reductase (R1, also known as ICP10) is a chimera that consists of an amino-terminal domain with serine-threonine protein kinase (PK) activity and a carboxy-terminal domain with R1 activity that function independently of each other. The construction and properties of the expression vectors for ICP10PK (pJW17N) and its PK-negative mutant p139TM (pJHL15N) were previously described (Luo and Aurelian 1992). The amplicon DNA vector pHSV-1005 which is based on HSV-1 and is modified to express EGFP, was the gift of Dr. R.L. Neve (McLean Hospital, Harvard Medical School). Construction of the amplicon vectors for ICP10PK (HSV-ICP10PK) or the ICP10PK/baculovirus p35 fusion protein (HSV-ICP10PK/p35) was as described (Neve et al. 2005). Briefly, HSV-ICP10PK consists of the ICP10PK coding sequence (Hind III/Stu I fragment from pJW17N) inserted into the EcoRV site of pHSV-1005 downstream of the IE4/5 promoter. To construct the amplicon vector HSV-ICP10PK/p35, the XhoI/EcoR I fragment from the pCMVp35neo vector [gift of Dr. S.E. Hasnain (Aparna et al. 2003)] was fused in-frame with ICP10PK by cloning into the Stu I site of pJW17N. The resulting ICP10PK/p35 fragment (Hind III/EcoR I) was filled in at both ends and cloned into the EcoRV site of pHSV-1005 downstream of the IE4/5 promoter. Packaging of the respective DNA constructs used the IE2 deletion mutant 5dl1.2 (helper virus) and culture was in 2-2 cells. The titers of the HSV-ICP10PK and HSV-ICP10PK/p35 amplicon stocks were 1×10^8 infectious units/ml. HSV-2 (G strain) and the ICP10PK deleted mutant Δ PK were as previously described (Smith et al. 1998; Smith et al. 2000; Perkins et al. 2002; Perkins et al. 2003).

Cell transfection, neuronal differentiation, and NGF deprivation

Primary hippocampal cultures were transiently transfected with the expression vectors for ICP10PK (pJW17N) or its kinase negative mutant p139TM (pJHL15N) (Luo and Aurelian 1992), the medium was replaced with MEM free of serum and growth factors (0 h) and the cultures were maintained in this medium for 72 h. Neuronal survival was determined by counting live cells (phase-dark bodies and fine neurites) in seven randomly chosen squares (Bambrick and Krueger 1999) and the results expressed as % surviving cells $\pm$ SEM relative to 0 h. PC12 cells were stably transfected with pJW17N or pJHL15N using the Fugene 6 transfection reagent, according to the manufacturer's instructions (Roche), and selection was with G418 (Invitrogen; 400 μ g/ml) as described (Smith et al. 1994). Two randomly selected ICP10 clones (PC47 and PC70) and one randomly selected clone transfected with p139TM (PC139 cells) were used in all studies. For neuronal differentiation the cells were cultured (4 days) on rat tail collagen-coated glass slides or polystyrene flasks, in NeuroBasal medium with 2 mM L-glutamine, B-27 supplement, 0.009% gentamicin (all Invitrogen-Gibco-BRL), and 100 ng/ml NGF (2.5S; Roche). Fresh NGF was supplied every other day. For NGF deprivation, cells were extensively rinsed with serum- and NGF-free MEM with 0.009% gentamicin and cultured (0–3 days) with MEM free of serum and NGF.

Antibodies and reagents

The generation and specificity of the rabbit ICP10 antibody was described (Luo and Aurelian 1992; Smith et al. 1994; Perkins et al. 2003). It recognizes an epitope located within amino acid residues 13–26 that are retained by both ICP10PK and p139TM. The following antibodies were purchased and used according to manufacturer's instructions. Antibodies to caspase-3 (recognizes both the zymogen and its cleavage products), adenylate cyclase (AC), ERK1/2, Bag-1, Bcl-2, Bad, GAPDH, MAP-2, XIAP and Smac/DIABLO were purchased from Santa Cruz Biotechnology, Santa Cruz, CA. Antibody to phosphorylated (activated) ERK 1/2 (pERK1/2) was purchased from Promega, Madison, WI. Antibodies to phosphorylated PKA (pPKA), PKA and phosphorylated Bad (pBad) were obtained from Cell Signaling Technologies (Beverly, MA). The antibodies to GAP-43 and Bad were respectively purchased

from Chemicon (Temecula, CA) and Calbiochem (San Diego, CA). Alexa Fluor 350-labeled anti-rabbit antibody was purchased from Molecular Probes (Eugene OR). Vectashield with DAPI was purchased from Vector (Burlingame, CA). Pharmacological inhibitors for MEK (U0126, Promega) and PKA (H89, Sigma), were used according to manufacturers' instructions. The pan-caspase inhibitor ZVAD-fmk (Sigma) was reconstituted (1000x stock solution) in dimethyl sulfoxide (DMSO), according to manufacturer's instructions. Phosphorothioate antisense (TCAAACTGTAAAAGTCAT) and sense (ATGACTTTTAACAGTTTTGA) oligonucleotides specific for XIAP were synthesized by the Biopolymer/Genomics Core Facility at the University of Maryland School of Medicine, reconstituted in sterile water, and used at a final concentration of 10 μ M.

RT-PCR

RNA (2 μ g) from mock and virus-infected cells was reverse transcribed with oligo(dT)₁₅, as described (Imafuku et al. 1997; Kulka et al. 2003). Primers were as described in the Clontech AtlasTM and obtained from Genset Corporation (La Jolla, CA). cDNA was amplified in PCR buffer, 2.5mM MgCl₂ 100 pmol primers, 0.5 mM dNTP and 2.5 units of Taq polymerase (Promega Corp, Madison WI). Thermal cycles included 1.5 min denaturation (94°C) followed by 35 cycles consisting of 94°C for 1.5 min, annealing for 90 seconds and extension for 2 min at 72°C. Final extension was for 10 min at 72°C. Products were visualized by electrophoresis on 1% agarose gels containing 1 μ g/ml ethidium bromide.

TUNEL

The *In situ* Cell Death Detection kit (Roche) was used according to the manufacturers' instructions. Briefly, cells grown on glass slides were fixed in 4% paraformaldehyde in PBS, pH 7.4 [1 h, room temperature (RT)] followed by permeabilization in 0.1% Triton-X (in 0.1% sodium citrate) for 2 min on ice. DNA breaks were labeled by incubation (60 min at 37°C) with terminal deoxynucleotidyl transferase and nucleotide mixture containing fluorescein isothiocyanate (FITC)-conjugated dUTP (TUNEL reagent), followed by anti-FITC antibody conjugated to alkaline phosphatase (30 min at 37°C). Chromogenic reaction was carried out by adding alkaline phosphatase substrate solution containing 0.4 mg of nitroblue tetrazolium chloride per ml and 0.2 mg of 5-bromo-4-chloro-3-indolylphosphate toluidine salt (NBT-BCIP, Roche) per ml in 0.1 M Tris-HCl (pH 9.5)-0.05 M MgCl₂-0.1 M NaCl-1 mM levamisole (10 min, RT). Apoptotic cells (characterized by a dark nuclear precipitate) and non-apoptotic cells (unstained or displaying a diffuse, light, and uneven staining) were counted in five randomly chosen microscopic fields (at least 250 cells), and results are expressed as % TUNEL⁺ (apoptotic) cells $\pm$ SEM (Perkins et al. 2002; Perkins et al. 2003; Golembewski et al. 2006; Laing et al. 2006). In one experiment, the *In Situ* Cell Death Detection Kit, TMR was used.

Immunoblotting

Immunoblotting was as described (Smith et al. 1998; Smith et al. 2000). Briefly, cells were lysed with radioimmunoprecipitation buffer [RIPA; 20 mM Tris-HCl (pH 7.4), 0.15 M NaCl, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate] supplemented with protease and phosphatase inhibitor cocktails (Sigma) and sonicated twice for 30 seconds at 25% output power with a Sonicator ultrasonic processor (Misonix, Inc., Farmingdale, NY). Protein concentrations were determined by the bicinchoninic assay (Pierce, Rockford, IL), and 100 μ g protein samples were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. The blots were incubated (1 h, RT) in TNT buffer (0.01 M Tris-HCl [pH 7.4], 0.15 M NaCl, 0.05% Tween 20) containing either 5% nonfat dried milk or 1% bovine serum albumin (BSA) to block nonspecific binding. Blots were exposed overnight at 4°C to appropriate antibodies diluted in

TNT buffer with either milk or BSA, washed in TNT buffer, and incubated (1 h; RT) with anti-rabbit IgG conjugated to horseradish peroxidase (HRP; Cell Signaling). After extensive washing, bands were detected using enhanced chemiluminescence reagents (ECL, Amersham Pharmacia, Piscataway, NJ) and exposure to high-performance film (Hyperfilm ECL, Amersham). Quantitation was by densitometric scanning with the Bio-Rad GS-700 imaging densitometer (Bio-Rad, Hercules, CA) and results are expressed as densitometric units $\times 100$.

Immunocomplex kinase assay

Kinase assays were as previously described (Smith et al. 1994; Smith et al. 1998). Briefly, cell extracts in lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1% NP-40 and protease and phosphatase inhibitor cocktails) were standardized for protein concentration and incubated with 10 μ l of ICP10 antibody (1 h, 4°C) and 100 μ l of protein A-sepharose CL4B beads (50% v/v) (30 min, 4°C). The beads were washed (3 $\times$) with RIPA buffer followed by TS buffer [20 mM Tris-HCl (pH 7.4), 0.15 M NaCl], resuspended in 50 μ l kinase reaction buffer consisting of 10 μ Ci [³²P]-ATP (0.1 μ M, 3000 Ci/mmol, NEN), 5 mM MgCl₂, 2 mM MnCl₂, 20 mM Tris-HCl (pH 7.4), and incubated at 30°C for 30 min. Samples were washed in 20 mM Tris-HCl (pH 7.4) with 0.15 M NaCl and boiled for 5 min after addition of 100 μ l denaturing solution. Proteins were resolved by SDS-PAGE.

RESULTS

ICP10PK inhibits neuronal cell death caused by loss of trophic growth support independent of other viral proteins

We have previously shown that ICP10PK has anti-apoptotic activity in virus-infected primary and organotypic hippocampal cultures and animal models of excitotoxicity through ICP10PK-mediated activation of the Ras protein, which in turn, activates the downstream Raf-1/MEK/ERK and PI3-K/Akt survival pathways (Perkins et al. 2002; Perkins et al. 2003; Gober et al. 2006; Golembewski et al. 2006; Laing et al. 2006). To activate Ras, ICP10PK functions as a constitutively activated growth factor receptor that: (i) phosphorylates (and thereby inactivates) the negative Ras regulator Ras-GAP, and (ii) binds the GDP/GTP exchange factor SOS (complexed to the adaptor protein Grb2), thereby favoring its ability to convert Ras to its GTP (active) form (Smith et al. 1994; Smith et al. 2000). However, an important caveat, is that apoptosis inhibition was examined in virus-infected cells, in which the possible contribution of other viral genes cannot be fully excluded, as various viral proteins upregulate cellular genes that may contribute to cell fate determination (Taddeo et al. 2002; Esclatine et al. 2004; Prechtel et al. 2005).

To examine whether ICP10PK can protect from cell death independent of any other viral genes, we used hippocampal cultures transiently transfected with the expression vectors for ICP10PK or p139TM and examined their ability to survive the loss of trophic growth support. The results are expressed as % surviving (live) cells $\pm$ SEM relative to 0 h, as described in Materials and Methods. Untransfected cultures maintained in medium with B27 supplement (+B27) or without the supplement (–B27) served as control. Neuronal identity was confirmed by staining with the neuron-specific antibody to class III β tubulin (TuJ1) (Ferreira and Caceres 1992). Neuronal cell death was seen in untransfected cultures (Fig. 1A) and in cultures transfected with p139TM (Fig. 1B) maintained without B27, but not in cultures transfected with ICP10PK and similarly maintained in medium without the B27 supplement (Fig. 1C). The % surviving cells were respectively 52.6 ± 7.2 and 56 ± 3.1 at 48 h and 34.3 ± 7.6 and 34.8 ± 3.8 % at 72 h for untransfected and p139TM-transfected cells cultured without B27. The viability of the ICP10PK-transfected cells was significantly ($p < 0.05$ by ANOVA) higher (80.6 ± 2.7 and 67.7 ± 2.4 % at 48 and 72 h, respectively) and similar to that of untransfected cells cultured with B27 (88.7 ± 3.1 and 80.6 ± 2.7 % at 48 and 72 h, respectively) (Fig. 1D). The data indicate that

ICP10PK promotes the survival of hippocampal neurons in the absence of growth factors, independent of other HSV proteins.

ICP10PK protects neuronally differentiated PC12 cells from apoptosis caused by NGF deprivation

Transiently transfected primary hippocampal cultures are not amenable to accurate and detailed analysis of the molecular mechanism of apoptosis inhibition. To address this question, we used PC12 cells, which are an established model of neuronal survival after NGF deprivation (Batistatou and Greene 1991; Francois et al. 2001; Valavanis et al. 2001). PC12 cells, two randomly selected clones of PC12 cells stably transfected with ICP10PK (PC47 and PC70) and PC12 cells stably transfected with the ICP10 kinase negative mutant p139TM (PC139) were studied. The transgenes (ICP10PK and p139TM) were expressed equally well (150, 160, 180 densitometric units for PC139, PC70, and PC47, respectively) (Fig. 2A) and ICP10 retained kinase activity (Fig. 2B). Robust expression and kinase activity were seen throughout the study interval (passages 15–40). Transgene expression did not alter the NGF-induced differentiation. Thus, PC139, PC70, and PC47 cells cultured (4 or 12 days) with NGF evidenced virtually identical neurite formation as PC12 cells studied in parallel (Fig. 2C), and there was no visible difference between the 4 and 12 day cultures. We conclude that neurite formation reflects differentiation, because the microtubule-associated protein (MAP-2), which is an established marker of mature neurons (Sano et al. 1990), was only expressed in cultures grown with NGF. Its levels were similar in PC12, PC139, PC70, and PC47 cells (Fig. 2D). These results are not an artifact caused by the selection of a unique differentiation marker, as similarly robust expression was seen in the NGF-differentiated PC12, PC139, PC47 and PC70 cells immunoblotted with antibody to GAP-43 (Fig. 2E), another marker of neuronal differentiation (Das et al. 2004). The NGF-differentiated cells were largely non-dividing, as determined by 5-bromo-2'-deoxyuridine incorporation.

To examine whether ICP10PK can protect from apoptosis caused by NGF withdrawal, also in this system, neuronally differentiated PC12, PC139, PC47, and PC70 cells were deprived of NGF and stained by TUNEL, a stringent marker of DNA fragmentation (Gold et al. 1994). A time-dependent increase in the % TUNEL⁺ cells was seen in PC12 cells (Fig. 3A), with maximal levels (54±9%) reached on day 3 after NGF withdrawal (Fig. 3B). Consistent with previous reports that apoptosis caused by NGF withdrawal is caspase-dependent (McCarthy et al. 1997; Schulz et al. 1997; Rong et al. 1999), the % TUNEL⁺ cells was significantly ($p<0.01$) decreased by culture of the PC12 cells with the pancaspase inhibitor ZVAD-fmk (9±5%). Similar results were obtained for PC139 cultures in which the % TUNEL⁺ cells on day 3 after NGF withdrawal was 50±4% and decreased to 6±4% in the presence of ZVAD-fmk. By contrast, PC47 and PC70 cultures had a significantly ($p<0.01$) lower % TUNEL⁺ (apoptotic) cells, even at 72 h after NGF withdrawal (9±6 and 14±4%, respectively) (Fig. 3A,B) and positivity was not altered by ZVAD-fmk (11±6 and 11±3%, respectively). The data indicate that ICP10PK inhibits caspase-dependent apoptosis caused by loss of trophic growth support.

ICP10PK has a dual function on caspase-3 activity

To confirm the role of caspase-3 in apoptosis caused by NGF deprivation and its inhibition by ICP10PK, extracts of PC12, PC139, PC70 and PC47 cells were immunoblotted with an antibody that recognizes both procaspase-3 and its cleavage product caspase-3p20. NGF deprivation caused caspase-3 activation in PC12 and PC139 cells, as evidenced by the loss of procaspase-3 and the concomitant appearance of caspase-3p20. Although both PC47 and PC70 cells were protected from DNA fragmentation (Fig. 3A,B), caspase-3 activation was only inhibited in PC47 cells (Fig. 3C). Because NGF withdrawal causes caspase-3 dependent apoptosis (McCarthy et al. 1997; Schulz et al. 1997; Rong et al. 1999), a finding confirmed by

our studies, the data suggest that in PC70 cells, ICP10PK-mediated neuroprotection is through interference with the protease activity of processed caspase-3.

ICP10PK upregulates and stabilizes AC

We have previously shown that in virus-infected cells, ICP10PK activates Ras and its downstream effectors, Raf-1/MEK/ERK and PI3-K/Akt (Smith et al. 2000; Perkins et al. 2002; Perkins et al. 2003; Gober et al. 2006; Golembewski et al. 2006; Laing et al. 2006). However, recent array studies confirmed independent reports (Taddeo et al. 2002; Esclatine et al. 2004; Prechtel et al. 2005) that distinct virus proteins upregulate various cellular genes, and comparison of cells infected with HSV-2 or its ICP10PK-deleted mutant (Δ PK) suggested that AC is one of the genes upregulated by ICP10PK (Goswami and Aurelian, unpublished).

Because AC initiates the cAMP/PKA pathway that is associated with NGF-induced differentiation and neuronal survival (Rukenstein et al. 1991; Vaudry et al. 2002), we considered the possibility that AC may contribute to the anti-apoptotic activity of ICP10PK. In a first series of experiments, we used RT-PCR and immunoblotting with AC antibody, to confirm the results of the array studies. Specifically, Vero cells were mock-infected with PBS or infected with HSV-2 or Δ PK (10^6 pfu) and assayed by RT-PCR at 10 and 18 h p.i. AC RNA was seen in cells infected with HSV-2, both at 10 and 18 h p.i., but it was not seen in mock or Δ PK-infected cells. This is shown in Fig. 4A for 10 h infected cells. In a second series of experiments, neuronally differentiated PC12, PC47 and PC70 cells were immunoblotted with AC antibody at the time of NGF withdrawal (0 h) and at 24 h after NGF deprivation. Consistent with the conclusion that AC is upregulated by ICP10PK, its levels at 0 h after NGF withdrawal were higher in PC47 and PC70 than PC12 cells. However, by 24 h after NGF withdrawal, AC was barely detectable in PC12 and PC70 cells (Fig. 4B), but its levels were still elevated in PC47 cells. The data indicate that ICP10PK also causes AC stabilization after NGF withdrawal, albeit in a cell-type specific fashion. As found for Δ PK-infected cells, AC was not upregulated nor stabilized in PC139 cells (data not shown).

The anti-apoptotic activity of ICP10PK in PC47 cells is PKA- and MEK-dependent

Having seen that ICP10PK upregulates/stabilizes AC in NGF-deprived PC47 cells, we wanted to know whether the AC/PKA pathway is associated with the ICP10PK anti-apoptotic activity in these cells. Neuronally differentiated PC12, PC139, PC70 and PC47 cells were cultured in NGF- and serum-free medium supplemented or not with the PKA inhibitor H89 (20 μ M) and assayed for TUNEL at 24 and 48 h after NGF withdrawal. Because we had previously associated the ICP10PK anti-apoptotic activity with its ability to activate Ras and the resulting MEK/ERK activation (Smith et al. 2000; Perkins et al. 2002; Perkins et al. 2003; Gober et al. 2006), cells cultured with the MEK inhibitor U0126 (20 μ M) were studied in parallel. In PC12 and PC139, U0126 caused a time-dependent increase in the % TUNEL⁺ cells, with maximal levels (88–100%) seen at 48 h after withdrawal. A similar pattern was seen for PC70 cells, suggesting that their survival is MEK-dependent. The activity of U0126 is not due to non-specific toxicity, because cell death was minimal in all 3 cultures at 24 h after NGF withdrawal and the % TUNEL⁺ cells in PC47 cultures was not increased by treatment with U0126 for 24 or 48 h. In these cells, the % TUNEL⁺ cells was only increased (99% at 48 h) by culture with both H89 and U0126 (Fig. 5), indicating that either one of the two survival pathways (MEK or PKA) prevents apoptosis. Consistent with previous reports (Perkins et al., 2002; 2003), similar results were obtained for U0126 at 10 μ M and for another MEK inhibitor (PD98059; 50 μ M) (data not shown).

PKA is activated in PC47 cells

To confirm that PKA was activated in PC47 cells, neuronally differentiated PC12, PC139, PC70 and PC47 cells were grown in NGF-free medium with or without H89 and

immunoblotted with antibody to phosphorylated (activated) PKA (pPKA) at 0 and 4 h post withdrawal. Antibody to total PKA served as control. The levels of pPKA at 0 h were relatively high in all cultures, consistent with previous reports that the AC/PKA pathway is involved in NGF-induced differentiation (Yao et al. 1998; Vaudry et al. 2002; Obara et al. 2004). At 4 h after NGF withdrawal, the levels of pPKA in PC12, PC139 and PC70 cells were not significantly altered, but in PC47 cells, they were significantly higher than 0 h after NGF withdrawal (Fig. 6). Similar results were obtained at 24 h after NGF withdrawal (data not shown). Densitometric scanning and data analysis as pPKA/PKA ratios indicated that the levels of pPKA in PC47 cells were 7-fold higher than in PC12 cells. PKA activation was inhibited in PC47 cells cultured with H89 (Fig. 6B).

ICP10PK increases ERK1/2 activation, but activation is prolonged only in PC47 cells

Having seen that U0126 contributes to the inhibition of survival in PC47 cells, we wanted to confirm that its effect is associated with the activation of ERK, which is downstream of MEK. Neuronally differentiated PC12, PC139, PC70 and PC47 cells were cultured in NGF-free medium with (or without) H89, U0126 or both, and assayed for ERK1/2 activation by immunoblotting with antibody to phosphorylated ERK 1/2 (pERK1/2) at 0, 4 and 24 h post NGF withdrawal. Antibody to total ERK1/2 was used as control. At 0 and 4 h after NGF withdrawal, pERK1/2 were seen in all cultures, presumably related to NGF-induced differentiation (Cowley et al. 1994; Grewal et al. 1999; Klesse et al. 1999; Hetman and Xia 2000). However, the levels of pERK1/2 were significantly higher in PC70 and PC47 than in PC12 and PC139 cells, indicating that ICP10PK increases the levels of activated ERK1/2, relative to those associated with NGF-induced differentiation. By 24 h after NGF withdrawal, pERK1/2 were virtually undetectable in PC12, PC139 and PC70 cells, while activation was still robust in PC47 cells (Fig. 7B). In these cells, activation was only inhibited by treatment with both U0126 and H89, while in PC12, PC139 and PC70 cells, activation was inhibited by U0126 alone (as shown in Fig. 7A,B for 4 h after NGF withdrawal). The data indicate that ERK1/2 activation in PC47 cells reflects the convergence of the activated MEK and PKA pathways, potentially explaining its longer duration.

Bag-1 and Bcl-2 are stabilized and Bad is phosphorylated in PC47 cells through MEK and PKA activation

In virus-infected hippocampal cultures ICP10PK upregulates Bag-1, which plays a crucial role in its anti-apoptotic activity (Perkins et al. 2003). To examine whether Bag-1 is also involved in the neuroprotective activity of ICP10PK in our system, neuronally differentiated PC12, PC139, PC70 and PC47 cells were examined for Bag-1 expression at 0 – 72 h after NGF withdrawal. Duplicate samples were also immunoblotted with antibodies to the anti-apoptotic protein Bcl-2, the pro-apoptotic protein Bad or Bad phosphorylated on Serine¹¹² (pBad), which no longer has pro-apoptotic activity (Harada et al. 1999) in order to verify whether ICP10PK alters the homeostatic balance of these apoptosis regulatory proteins. Bag-1 and Bcl-2 were expressed in all cultures at 0 h after NGF withdrawal, presumably related to NGF-induced differentiation/neuronal survival. However, in PC12, PC139 and PC70 cells, their levels decreased with time post NGF withdrawal and they were no longer seen by 72 h. By contrast, expression of both Bag-1 and Bcl-2 was still robust in PC47 cells (Fig. 8A) and it decreased only by treatment with the combination of U0126 and H89, as shown for Bag-1 in Fig. 8B. The levels of pBad were also significantly higher in PC47 than PC12, PC139 and PC70 cells after NGF withdrawal, and this increase was also virtually abrogated by the U0126 and H89 combination. Bad levels were similar in all cultures (Fig. 8C,D). Thus, survival of PC47 cells after NGF deprivation is associated with ICP10PK-mediated stabilization of the anti-apoptotic proteins Bag-1 and Bcl-2 and inhibition (phosphorylation) of the pro-apoptotic protein Bad through PKA and MEK activation. These apoptosis regulatory proteins were not associated with the survival of PC70 cells.

XIAP is upregulated and Smac/DIABLO is inhibited in PC70 cells

Having seen that PC70 cells are protected from caspase-dependent apoptosis after NGF withdrawal although caspase-3 is activated, we reasoned that the apoptotic process is halted after it has begun. This is particularly promising from a therapeutic standpoint and was not previously described for ICP10PK. Neuronally differentiated PC70 cells were cultured in NGF-free medium with or without U0126 (24 h) and cell extracts were immunoblotted with antibody to XIAP, which is a potent inhibitor of the activity of the already processed (activated) caspases -9 and -3 (Deveraux and Reed 1999). The blots were stripped and re-probed with GAPDH antibody (control). PC12, PC139 and PC47 cells were studied in parallel and served as controls. Low levels of XIAP were seen in PC12, PC139 and PC47 cells at 0 h post withdrawal and they were further reduced or not altered at 24 h after NGF withdrawal. The levels of XIAP were significantly higher in PC70 cells both at 0 and 24 h after NGF withdrawal, and they were decreased by U0126 (Fig. 9A), indicating that XIAP is upregulated in these cells, through a MEK-dependent process. The levels of the apoptogenic protein Smac/DIABLO were also significantly lower in PC70, than PC12, PC139 and PC47 cells at 24 h after NGF withdrawal (Fig. 9B). This is consistent with previous reports that XIAP functions as an ubiquitin ligase towards Smac/DIABLO, causing its proteolytic degradation (Morizane et al. 2005).

XIAP upregulation is required for apoptosis inhibition in PC70 cells

Having seen that XIAP is upregulated in PC70 cells through the activation of the MEK pathway, which is also required for apoptosis (TUNEL) inhibition, we wanted to confirm that cell survival is XIAP-dependent. Neuronally differentiated PC70 cells were incubated with XIAP antisense (or sense) oligonucleotides (10 μ M) at the time of NGF withdrawal (0 h) and duplicate samples were immunoblotted with XIAP antibody or stained by TUNEL 24 h later. XIAP expression was significantly decreased (Fig. 9C) and the % TUNEL⁺ cells was significantly ($p < 0.05$) increased (Fig. 9D) by the XIAP antisense oligonucleotide, but sense oligonucleotide had no effect on XIAP expression or cell survival. The data confirm that the survival of PC70 cells is XIAP-dependent.

The baculovirus protein p35 does not potentiate the ICP10PK anti-apoptotic activity in transduced PC12 cells

The use of stably transfected clonal lines allowed us to document the ability of ICP10PK to inhibit the activity of the processed caspase-3, a function that was masked in the total cell population by the activation of survival pathways, which inhibit caspase-3 activation (Perkins et al. 2002; Perkins et al. 2003; Gober et al. 2006; Laing et al. 2006; Golembewski et al. 2007). To examine whether inhibition of caspase-3 protease activity contributes to ICP10PK neuroprotection in total cell populations, we asked whether the baculovirus p35 protein potentiates the anti-apoptotic activity of ICP10PK. Because p35 is known to inhibit processed caspase-3 (Miagkov et al. 2004; Callus and Vaux 2007), we reasoned that if ICP10PK only functions by inhibiting caspase-3 activation, p35 will further decrease the % TUNEL⁺ cells by interfering with the activity of the enzyme that escaped ICP10PK. By contrast, if ICP10PK functions both by inhibiting caspase-3 activation and disrupting its activity, the % TUNEL⁺ cells will be similar for ICP10PK and an ICP10PK/p35 fusion protein. Also, because PC47 and PC70 are clonal derivatives, we used PC12 cells transduced with amplicon vectors in order to measure global effects on the entire cell population.

Neuronally differentiated PC12 cells were transduced with the EGFP-labeled vectors HSV-ICP10PK, HSV-ICP10PK/p35 or the empty vector, cultured (48 h) in NGF-free medium and examined for TUNEL as in Materials and Methods. Duplicate cultures grown in NGF-containing medium served as control. All the cultures were positive for EGFP, but only those transduced with HSV-ICP10PK or HSV-ICP10PK/p35 stained with Alexa-Fluor 350-labeled

ICP10 antibody, indicative of transgene expression. Significantly, NGF deprivation caused a similarly low level of TUNEL⁺ cells in both HSV-ICP10PK and HSV-ICP10PK/p35 transduced cultures (9.5 ± 1 and $10.1 \pm 2\%$, respectively), indicating that p35 does not increase the anti-apoptotic activity of ICP10PK. The % TUNEL⁺ cells in PC12 cells transduced with the empty vector and subjected to NGF deprivation was significantly ($p < 0.001$) higher ($33 \pm 2\%$) (Fig. 10).

DISCUSSION

The salient feature of our data is the finding that ICP10PK inhibits neuronal cell apoptosis caused by loss of trophic growth support independent of other viral genes, by inhibiting caspase-3 activation and its protease activity. ICP10PK was previously shown to inhibit neuronal cell death through activation of survival pathways that initiate with Ras activation, also in animal models (Perkins et al. 2002; Perkins et al. 2003; Gober et al. 2006; Laing et al. 2006; Golembewski et al. 2007). However, this is the first report that it: (i) functions independently of other viral genes, (ii) activates the AC/cAMP/PKA pathway, and (iii) interferes with the protease activity of processed caspase-3 through XIAP upregulation. The following comments seem pertinent with respect to these findings.

MEK/ERK activation is consistent with previous reports that ICP10PK activates Ras by: (i) phosphorylating (inhibiting) the negative regulator Ras-GAP and (ii) binding the Grb2/SOS complex, thereby favoring the SOS/Ras interaction (Smith et al. 1994; Smith et al. 2000). Indeed, Ras activates the downstream Raf-1/MEK/ERK and PI3-K/Akt pathways (Rubio et al. 1997; Yang et al. 2004), and we have previously shown that these pathways are activated by ICP10PK and they override the caspase-dependent apoptotic cascade induced by virus infection or excitotoxic injury (Perkins et al. 2002; Perkins et al. 2003; Gober et al. 2006; Laing et al. 2006; Golembewski et al. 2007). However, these studies used virus infection and a caveat is that they do not fully exclude the possible contribution of other viral genes which affect cell fate determination (Taddeo et al. 2002; Esclatine et al. 2004; Prectel et al. 2005). Also, because the activated survival pathways are likely to mask the potential of ICP10PK to function downstream of caspase activation, it is unclear whether ICP10PK can also inhibit the activity of processed caspase-3. Finally, the neuroprotective activity of ICP10PK in other paradigms of apoptotic cell death is still poorly understood.

To address these questions, we studied cells transfected with ICP10PK or its kinase negative mutant p139TM, which fails to activate Ras (Luo and Aurelian 1992; Smith et al. 1994; Smith et al. 2000; Smith 2005). We found that ICP10PK, but not p139TM, protected transiently transfected hippocampal cultures and stably transfected neuronally differentiated PC12 cells from death caused by loss of trophic growth support. Because transient transfection is not an appropriate model for mechanistic studies, these were done in the stably transfected PC12 cells, which are an established and widely used model of cell survival after NGF deprivation (Batistatou and Greene 1991; Francois et al. 2001; Valavanis et al. 2001). Two clones of ICP10PK-transfected cells (PC47 and PC70) and one clone of cells transfected with p139TM (PC139) were studied in all assays, together with the parental PC12 cells. Similar differentiation patterns were evidenced by the transfected and parental PC12 cells, as determined by neurite formation and expression of the differentiation-specific genes MAP-2 and GAP-43. These findings are consistent with previous reports that ICP10PK activates the classical Ras pathway, which functions through c-Raf-1 (Smith et al. 2000; Perkins et al. 2002), while NGF causes differentiation through activation of M-Ras (Sun et al. 2006) or Rap-1 (York et al. 1998), both of which function through B-Raf. Our data suggest that the NGF-induced pathway is dominant, consistent with previous reports that Rap-1 functions as an antagonist of Ras signaling (Vossler et al. 1997; Schmitt and Stork 2001).

ICP10PK-mediated survival was likely caused by inhibition of apoptosis, as measured by TUNEL, a stringent marker of DNA fragmentation/apoptosis (Gold et al. 1994). The study of clonal lines allowed us to verify that ICP10PK can inhibit caspase activity (as evidenced by PC70 cells), a function that was masked in total cell populations by the inhibition of caspase activation (Perkins et al. 2002; Perkins et al. 2003; Gober et al. 2006; Laing et al. 2006; Golembewski et al. 2007). As in hippocampal cultures and animal models, the neuroprotective activity of ICP10PK in PC47 cells was through activation of survival pathways that inhibit caspase-3 activation. Neuroprotection was blocked only by inhibiting both the MEK/ERK and AC/PKA survival pathways, indicating that either one of these two ICP10PK-activated pathways can override the apoptotic cascade induced by NGF withdrawal. MEK/ERK activation was in line with our previous reports (Smith et al. 2000; Perkins et al. 2002; Perkins et al. 2003). However, this is the first report that ICP10PK upregulates/stabilizes AC and activates the AC/PKA pathway. Because AC was only stabilized in PC47 cells (while upregulation was also seen in PC70 cells), we conclude that PKA activation is associated with AC stabilization. The exact mechanism responsible for AC upregulation/stabilization by ICP10PK is still unclear. It may involve AP-1 activation through Ras/Raf-1/MEK/ERK (Smith et al. 2000; Gober et al. 2005) because the AC promoter contains AP-1 response elements (Abdel-Halim et al. 1998). The PACAP-preferring receptor (PAC1) that activates AC in PC12 cells, may also be involved in an AP-1 amplification loop (Vaudry et al. 2002). Consistent with this interpretation, AC was not upregulated/stabilized in cells infected with Δ PK [or transfected with p139TM (data not shown)] in which Ras/Raf-1/MEK/ERK and AP-1 are not activated (Smith et al. 2000; Gober et al. 2005; Laing et al. 2006).

As previously reported (Xing et al. 1998; Yao et al. 1998; Klesse et al. 1999; Hetman and Xia 2000), ERK1/2 were activated in all cultures at 0 h after NGF withdrawal. However, prolonged activation (24 h after NGF withdrawal) was only seen in PC47 cells. Activation was inhibited by the combination of U0126 and H89, but not each inhibitor alone, suggesting that the MEK/ERK and AC/PKA pathways converge on ERK activation. Both pathways were also involved in neuroprotection (TUNEL inhibition) and in the stabilization of Bag-1 and Bcl-2 and the inactivation of Bad through phosphorylation. This is schematically represented in Fig. 11. However, the exact contributions of these proteins to cell survival, is still unclear. Bag-1 overexpression reduces neuronal injury (Kermer et al. 2003) and mediates ICP10PK neuroprotection in virus-infected hippocampal cultures (Perkins et al. 2003). The mechanism of the Bag-1 anti-apoptotic activity is still poorly understood, but it may include Bcl-2 stabilization and activation of c-Raf-1 kinase (Wang et al. 1996). Bag-1 was stabilized in PC47 cells, with robust expression still seen at 72 h after NGF withdrawal, potentially reflecting the ability of ICP10PK to inhibit proteolytic pathways involved in Bag-1 degradation. The anti-apoptotic protein Bcl-2 was also stabilized in PC47 cells, either through interaction with Bag-1 or at the transcriptional level. Bcl-2 proteins interact with Ca²⁺ release channels and pumps and may inhibit Ca²⁺ stores below the threshold needed to trigger apoptosis (Dremina et al. 2004). The pro-apoptotic protein Bad was phosphorylated, an alteration that appears to be essential for neuronal survival (Tan et al. 2000). The alteration of all these proteins likely shifts the homeostatic balance in favor of apoptosis inhibition.

An exciting observation is the finding that ICP10PK can also inhibit the protease activity of caspase-3, underscoring its redundant mechanism of neuroprotection, and presumably explaining why ICP10PK has a wide activity spectrum and therapeutic window both in culture and in animal models (Perkins et al., 2002, 2003; Gober et al. 2006; Laing et al., 2006; Golembewski et al. 2007). ICP10PK interfered with the protease activity of caspase-3 through upregulation of XIAP, a potent inhibitor of the activity of processed (activated) caspases (Deveraux and Reed 1999). Both XIAP upregulation and cell survival were inhibited with U0126, consistent with previous reports that XIAP is upregulated through MEK/ERK activation (Pardo et al. 2003). We conclude that XIAP is required for cell survival, because the

latter was inhibited with XIAP antisense, but not sense, oligonucleotides. Smac/DIABLO, which is known to inhibit the anti-apoptotic activity of the IAPs (Srinivasula et al. 2001), was also downregulated in PC70 cells, consistent with independent reports that XIAP is a ubiquitin ligase for Smac/DIABLO (Morizane et al. 2005). It should be pointed out that the rapid (0 and 4 h after NGF withdrawal) MEK-dependent ERK1/2 activation seen in PC70 cells, was also seen in PC12 and PC139 cells, that were not protected from apoptosis (DNA fragmentation) caused by NGF withdrawal. Because the levels of activated ERK1/2 were higher in PC70 than PC12 and PC139 cells, it seems reasonable to assume that XIAP upregulation (and thereby PC70 cell survival) are determined by the higher levels of activated ERK1/2. Indeed, adaptor molecules used to recruit and activate Ras, the cross-talk between distinct signaling pathways that converge on ERK, growth factor receptor turnover rates, and the resulting levels and duration of ERK activation, were implicated in the different outcomes of ERK activation on cell fate determination (Marshall 1995; Vaudry et al. 2002; Colucci-D'Amato et al. 2003).

The finding that ICP10PK functions either through inhibition of caspase-3 activation or disruption of its protease activity, presumably reflects the use of transfected clonal lines. However, we conclude that both mechanisms are involved in neuroprotection in total cell populations, because the baculovirus p35 protein did not potentiate apoptosis inhibition over that seen for ICP10PK in PC12 cells transduced with HSV amplicon vectors. The failure of p35 to potentiate the activity of ICP10PK is not an artifact resulting from increased toxicity caused by double doses of amplicon vector or because different cells were transduced with ICP10PK or p35, because p35 was fused to ICP10PK in HSV-ICP10PK/p35. It is also not due to the failure of p35 to be expressed because both EGFP and ICP10PK (which is upstream of p35) were expressed equally well for both HSV-ICP10PK and HSV-ICP10PK/p35. We do not exclude the possibility that the failure of p35 to potentiate neuroprotection is due to the efficient inhibition of caspase-3 activation by ICP10PK and ongoing studies are designed to address this question and define the conditions that determine whether ICP10PK inhibits caspase-3 activation, its activity, or both. Collectively, our data confirm that ICP10PK differs from most other gene therapy platforms designed to interrupt the apoptotic cascade induced by neurodegenerative stimuli, in that it has a broad spectrum anti-apoptotic activity, which is made possible by the use of distinct molecular strategies characterized by remarkable functional redundancy.

The abbreviations used are

MEK, mitogen activated protein kinase kinase
 ERK, mitogen-activated protein kinase/extracellular signal-regulated kinase
 AC, adenylate cyclase
 PKA, cAMP-dependent protein kinase
 PC12, rat pheochromocytoma
 NGF, nerve growth factor
 GAPDH, glyceraldehyde-3-phosphate dehydrogenase
 GAP-43, growth-associated protein 43
 XIAP, X-linked inhibitor of apoptosis protein
 FITC, fluorescein isothiocyanate
 TUNEL, terminal deoxynucleotidyl transferase FITC-dUTP nick end labeling
 SDS, sodium dodecyl sulfate

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Fig. 1. ICP10PK transfection protects hippocampal cultures from apoptosis induced by growth factor withdrawal

Hippocampal cultures were established on glass coverslips etched with a grid of $175 \times 175 \mu\text{m}$ squares and grown (2 days) as described in Materials and Methods. At this time they were transfected with the expression vectors for ICP10PK (pJW17) or p139TM (pJHL15), the medium was replaced with MEM free of serum and growth factors (0 h) and the cultures were maintained in this medium for 72 h. Untransfected cultures maintained in medium with (+B27) or without (–B27) B27 supplement served as control. Untransfected cells (A) and cells transfected with p139TM (B) or ICP10PK (C), are shown at 48 h p.w.d. Neuronal survival was determined daily by counting live cells (phase-dark bodies and fine neurites) (confirmed by TUJ1 staining) in seven randomly chosen squares, and the results expressed as % surviving cells $\pm$ SEM relative to 0 h (D).

Fig. 2. Transgene expression and ICP10 kinase activity in stably transfected PC12 cells

A, Extracts of PC12, PC139, PC70, and PC47 cells were immunoblotted with ICP10 antibody and the blots were stripped and reprobed with GAPDH antibody, used as loading control. Similar results were obtained throughout the study interval (passages 15–40). **B**,

Immunocomplex PK assay of extracts from PC12, PC139, PC70 and PC47 cells with ICP10 antibody, done as described in Materials and Methods. **C**, Neurite formation in cultures grown (4 days) in medium containing 100 ng/ml NGF (panels 1–4). Similar results were obtained throughout the study interval (passages 15–40). **D**, Cell extracts were immunoblotted with MAP-2 antibody before differentiation (0) or after growth (4 days) in NGF-containing medium (4). The blot was stripped and re-probed with GAPDH antibody. **E**, PC12, PC139, PC70, and PC47 cell extracts were immunoblotted with GAP-43 antibody after 4 days of differentiation with NGF. The blot was stripped and re-probed with GAPDH antibody.

Fig. 3. ICP10PK inhibits apoptosis caused by NGF withdrawal

A, PC12, PC139, PC47 and PC70 cells were differentiated by culture (4 days) with medium containing NGF (100 ng/ml) and examined for apoptosis by TUNEL at 24–72 h post NGF withdrawal, as described in Materials and Methods. Results are shown for the 72 h cultures. B, TUNEL⁺ cells were counted as described in Materials and Methods and results are expressed as %TUNEL⁺ ± SEM (**p < 0.01, ***p < 0.001 vs. PC12 by 2-tailed student t-test). C, Extracts of neuronally differentiated PC12, PC139, PC70 and PC47 cells collected at 24 or 72 h after NGF withdrawal (pwd) were immunoblotted with a caspase-3 antibody that recognizes the zymogen (procaspase-3) and its cleavage product (p20). The blots were stripped and re-probed with antibody to GAPDH.

Fig. 4. ICP10PK induces adenylate cyclase upregulation

A, Vero cells were infected with PBS (mock), HSV-2, or ICP10 Δ PK for 10 h, at which time RNA was collected as described in Materials and Methods. The RT-PCR product is shown. Similar results were obtained at 18 h p.i. **B**, Extracts of neuronally differentiated PC12, PC47 and PC70 cells collected on day 4 of differentiation (0) or 24 h post NGF withdrawal (24) were immunoblotted with antibody to adenylate cyclase (AC).

Fig. 5. ICP10PK-mediated inhibition of DNA fragmentation caused by NGF withdrawal is PKA and/or MEK-dependent

NGF-differentiated PC12, PC139, PC70 and PC47 cells were cultured in NGF-free medium with or without inhibitors of PKA (H89, 20 μ M) or MEK (U0126, 20 μ M), or their combination (H89+U0126) and assayed for apoptosis by TUNEL at 24 and 48 h post NGF withdrawal. Results are expressed as % TUNEL⁺ cells $\pm$ SEM. (***, $p < 0.001$ vs. MEM by 2-tailed student t-test).

Fig. 6. ICP10PK activates PKA

A, Extracts of NGF-differentiated PC12, PC139, PC70 and PC47 cells cultured for 4 h in NGF-free medium with (4+H) or without H89 were immunoblotted with antibody to activated (phosphorylated) PKA (pPKA). The blot was stripped and re-probed with antibody to total PKA. **B**, The blots in **A** were analyzed by densitometric scanning and the results are expressed as pPKA/PA ratios.

Fig. 7. ICP10PK activates ERK

A, extracts of NGF-differentiated PC12, PC139, PC70 and PC47 cells were cultured (4 h) in NGF-free medium with or without H89 (20 μ M), U0126 (20 μ M) or both inhibitors (H89+U0) and cell extracts were immunoblotted with antibody to activated (phosphorylated) ERK1/2 (pERK1/2). The blots were stripped and re-probed with antibody to total ERK1/2. **B**, Cells cultured as in **A** were collected at 4 and 24 h after NGF withdrawal and examined for ERK activation. Results are expressed as pERK/ERK ratios obtained by densitometric scanning for ERK1 or ERK2.

Fig. 8. ICP10PK causes Bcl-2 and Bag-1 stabilization and Bad inhibition through phosphorylation after NGF withdrawal

A, Neuronally differentiated PC12, PC139, PC70 and PC47 cells were collected at 0 and 72 h post NGF withdrawal and cell extracts were immunoblotted with antibody to Bag-1, stripped and sequentially re-blotted with antibodies to Bcl-2, followed by GAPDH used as loading control. **B**, Extracts of NGF-differentiated PC47 cells cultured (24 h) in NGF-free medium with or without H89, U0126 or H89+U0126 were immunoblotted with antibody to Bag-1 followed by GAPDH antibody. Results obtained with Bcl-2 antibody were similar to those seen for Bag-1 antibody. **C**, Extracts of PC12, PC139, PC70 and PC47 cells collected at 24 h after NGF withdrawal were immunoblotted with antibody to Bad phosphorylated on Ser¹¹² (pBad) followed by antibody to total Bad. **D**, Neuronally differentiated PC47 cells were cultured (24 h) in NGF-free medium without or with H89, U0126 or both and immunoblotted with antibody to pBad, followed by antibody to total Bad.

Fig. 9. XIAP is upregulated and Smac/DIABLO is downregulated in PC70 cells

A, Neuronally differentiated PC12, PC139, PC70, and PC47 cells collected at 0 and 24 h post NGF withdrawal without and with the MEK inhibitor U0126 were immunoblotted with antibody for XIAP, with GAPDH used as loading control. **B**, Extracts of neuronally differentiated PC12, PC139, PC70, and PC47 cells collected 24 h post withdrawal were immunoblotted with antibody to Smac/DIABLO, stripped and re-blotted with GAPDH antibody used as loading control. **C**, Extracts of PC70 cells differentiated for 4 days were incubated with or without XIAP-specific antisense or sense (control) oligonucleotides before and after NGF withdrawal and immunoblotted with antibody specific for XIAP. GAPDH was used as loading control. **D**, Duplicate cultures treated as in **C** were examined for TUNEL as described in Materials and Methods. PC70 cells incubated with antisense oligonucleotides 24 h post NGF withdrawal had significantly (*, $p < 0.05$) more TUNEL⁺ cells.

Fig. 10. Baculovirus p35 does not potentiate ICP10PK-mediated neuroprotection

A, PC12 cells transduced with empty vector, pHSV-ICP10PK, or pHSV-ICP10PK/p35 were examined by immunofluorescence for transduction (EGFP), TUNEL (TMR) and ICP10PK expression (Alexa Fluor 350 staining). B, A minimum of 3 randomly selected fields (250 cells) were counted for each treatment. Results are expressed as % TUNEL⁺ cells $\pm$ SEM (***, $p < 0.001$)

Fig. 11. Schematic representation of the mechanism of ICP10PK anti-apoptotic activity in PC12 cells undergoing NGF withdrawal

We have previously shown that ICP10PK activates Ras by phosphorylation (inactivation) of the negative regulatory GTPase Ras-GAP (Smith et al. 2000), as well as recruitment of the Grb2/Sos complex (Nelson et al. 1996) and it, in turn, activates the Raf-1/MEK/ERK downstream pathway components (Perkins et al. 2002, 2003; Gober et al. 2006). Our present findings indicate that ICP10PK can also activate the adenylyl cyclase/PKA pathway, which converges with MEK on ERK activation. This results in Bag-1 and Bcl-2 stabilization and Bad phosphorylation (inhibition) leading to inhibition of caspase-3 activation. In addition, ERK activation causes XIAP upregulation and Smac/DIABLO downregulation through XIAP-mediated proteosomal degradation. XIAP upregulation and Smac/DIABLO downregulation inhibit the activity of cleaved (activated) caspase-3. The outcome is apoptosis inhibition. The dotted lines represent the hypothetical involvement of AP-1, upregulated/activated by ICP10PK through the activation of the Ras/Raf-1/MEK/ERK pathway (Smith et al. 2000; Laing et al. 2006) in AC upregulation directly, or through PAC1.