

BRIEF COMMUNICATION

Expression of Herpes Simplex Virus Type 2 Protein ICP10 PK Rescues Neurons from Apoptosis Due to Serum Deprivation or Genetic Defects

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Previous studies have shown that the herpes simplex virus type 2 protein kinase ICP10 PK activates the Ras/MEK/MAPK pathway in nonneuronal cells. Here we report that ectopically expressed ICP10 PK has anti-apoptotic activity in various paradigms of neuronal cell death. Neuronally differentiated PC12 cells and primary murine hippocampal cultures transfected with an expression vector for ICP10 PK were protected from cell death resulting from growth factor withdrawal. Protection from apoptosis was also seen in ICP10 PK-transfected hippocampal neurons from the trisomy 16 mouse, a naturally occurring genetic abnormality the human analog of which is Down syndrome. Cells transfected with an expression vector for a mutant that lacks kinase activity were not protected, although it was expressed as well as ICP10 PK. The data indicate that ICP10 PK has a broad anti-apoptotic activity in neuronal cells which depends on a functional PK. © 2002 Elsevier Science (USA)

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Apoptosis is a physiological cell death program that occurs during neuronal development as a result of inadequate trophic support (11). It is thought to be responsible for neuronal cell death in neurodegenerative disorders, such as Alzheimer's disease (AD) (9). The trisomy 16 (Ts16) mouse is a naturally occurring genetic abnormality, the human analog of which (Down syndrome) leads to AD (8, 15). Cultured hippocampal neurons from Ts16 mice have an accelerated rate of apoptotic death (2–4) due to the innate genetic defects that seem to be related to neurotrophic factor signaling

(4). Neuron survival depends on the blockade of death signals by neurotrophic factors, including nerve growth factor (NGF) and BDNF, that activate survival pathways such as Ras/MEK/MAPK and P13-K/Akt (10).

Our studies were stimulated by previous findings that the large subunit of the herpes simplex virus type 2 (HSV-2) ribonucleotide reductase (RR) (also known as ICP10) contains a serine–threonine protein kinase (PK) that is located at the amino-terminus, functions independently of RR and activates the Ras/MEK/MAPK pathway in nonneuronal cells (16). Because this pathway is associated with protection of neuronal cells from apoptosis (10), we reasoned that should ICP10 PK activate Ras/MEK/MAPK also in neurons, it would promote their survival in experimental paradigms created by trophic factor deprivation or genetic defects in neurotrophin receptor signaling. As such, ICP10 PK would represent a novel therapeutic strategy for the treatment of neurodegenerative diseases.

In a first series of experiments to test this hypothesis, we took advantage of previous findings that rat pheochromocytoma (PC12) cells acquire properties of sympathetic neurons (neurite outgrowth, electrical excitability, and expression of specific neuronal markers) when grown in NGF-containing medium and die by apoptosis upon NGF withdrawal (7). We asked whether ICP10 PK can substitute for NGF in maintaining survival of differentiated PC12 cells. Cells [obtained from A. T. Campagnoni (UCLA)] were cultured in DMEM/F12 (Gibco-BRL, Gaithersburg, MD) with 10% fetal bovine serum (FBS) (Gemini, Calabasas, CA), 0.36% D-glucose (Sigma, St. Louis, MO), 0.21% sodium bicarbonate (Sigma), and 0.009% gentamycin (Sigma) and neuronally differentiated by growth (at least 12 days) in the same medium but with 100 ng/mL NGF (Roche Molecular Biochemicals, Indianapolis, IN) instead of serum. They were transfected [using FuGene 6 Transfection Reagent (Roche Molecular Bio-

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chemicals)] with vectors pJW17 or pJHL15, which respectively express ICP10 or the ICP10 PK negative mutant p139 (12, 16) and cultured for 24 h to allow for transgene expression. At this time, the cells were washed, the medium was replaced with serum and NGF-free medium (0 h after NGF withdrawal), and the cells were cultured in this medium for an additional 48 h. They were examined daily for viability using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI) that measures enzymatic activity of functional mitochondria, according to the manufacturer instructions. Results are expressed as percentage of viable cells \pm SEM relative to 0 h after NGF withdrawal.

Transgene expression was examined at 24 h post-transfection by immunoperoxidase staining with an ICP10-specific antibody (recognizes amino acids 13–26 in both proteins) or normal rabbit serum (control), as previously described (1, 12). The cultures were counterstained with Mayer's hematoxylin (Sigma), the staining cells were counted in five randomly chosen microscopic fields (containing at least 250 cells), and the average percentage of staining cells was calculated. The results are expressed as percentage of positive cells \pm SEM. Consistent with previous reports for non-neuronal cells transiently transfected with these vectors (12, 16), staining was localized in the cytoplasm and both its intensity and the proportion of stained cells (25–35%) were similar for pJW17 and pJHL15 (Fig. 1A, panels 2 and 3), suggesting that ICP10 and p139 are expressed equally well. Staining was not seen in control (nontransfected) PC12 cells (Fig. 1A, panel 1), nor with normal rabbit serum (data not shown).

The kinetics of cell death in the nontransfected cultures were similar to those previously reported for this system (14), with a respective survival of 65.7 ± 2.7 and $48.1 \pm 1\%$ at 24 and 48 h after NGF withdrawal, respectively). Similar kinetics were seen in pJHL15 transfected cultures (61.2 ± 1 and $43.1 \pm 1.4\%$ at 24 and 48 h after NGF withdrawal, respectively). By contrast, the survival of pJW17-transfected cells was 82.3 ± 3.9 and $73.2 \pm 3.3\%$ at 24 and 48 h after NGF removal ($P < 0.05$ vs control and pJHL15 transfected cells, by ANOVA) (Fig. 1B), suggesting that ICP10 PK can compensate for the absence of NGF. Similar results were obtained in an independent series of experiments with survivals (determined by counting morphologically viable cells) of 70 ± 6.3 and $90 \pm 4.7\%$ for nontransfected and pJW17-transfected cells respectively, at 48 h after NGF withdrawal.

In a second series of experiments designed to examine the role of ICP10 PK in neuronal survival, primary hippocampal cultures from embryonic (day 16) mice were established on glass coverslips etched with a grid of $175 \times 175\text{-}\mu\text{m}$ squares (CELLocate; Eppendorf, Madison, WI) and grown (2 days) in MEM with B27 supplement (Gibco) which contains optimized concen-

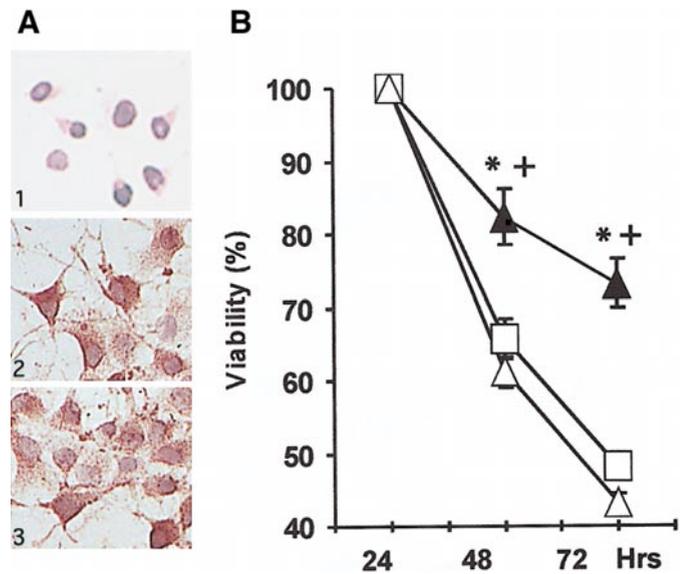


FIG. 1. ICP10 PK protects neuronally differentiated PC 12 cells and primary hippocampal neurons from death due to growth factor withdrawal. (A) PC12 cells untransfected (panel 1) or transfected with pJW17 (ICP10; panel 2) or pJHL15 (p139; panel 3) were stained with ICP10-specific antibody and counterstained with Mayer's hematoxylin. Transgene expression is cytoplasmic (red staining). (B) PC 12 cells were differentiated by growth (at least 12 days) in serum-free medium supplemented with 100 ng/mL of NGF and transfected with expression vector pJW17 (ICP10) (solid triangles) or pJHL15 (p139) (open triangles). Nontransfected cells (open squares) served as control. Viability was determined using CellTiter 96 Aqueous One Solution Cell Proliferation Assay and expressed as percentage relative to the number of cells at 0 h post-NGF withdrawal \pm SEM (* $P < 0.05$ vs control; + $P < 0.05$ vs pJHL15-transfected cells, by ANOVA with Tukey–Kramer posttest).

trations of neuron survival factors, as previously described (2). At this time the cells were transfected with pJW17 or 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. Nontransfected cultures maintained in medium with B27 supplement (Eu + B27) or without the supplement (Eu – B27) served as controls. Neuronal survival was determined daily by counting live cells (phase-dark bodies and fine neurites) in seven randomly chosen squares (2) and the results are expressed as percentage surviving cells \pm SEM relative to 0 h. Neuronal identity was confirmed by staining with the neuron-specific antibody to class III β tubulin (TuJ1) (5). The percentage surviving cells were respectively 52.6 ± 7.2 and 56 ± 3.1 for Eu-B27 and pJHL15-transfected cells at 48 h and 34.3 ± 7.6 and $34.8 \pm 3.8\%$ at 72 h. The viability of pJW17-transfected cells was significantly ($P < 0.05$ by ANOVA) higher (80.6 ± 2.7 and $67.7 \pm 2.4\%$ at 48 and 72 h, respectively) and similar to that of Eu + B27 cells (88.7 ± 3.1 and $80.6 \pm 2.7\%$ at 48 and 72 h, respectively) (Fig. 2A). The data suggest that ICP10 PK promotes survival of hippocampal neurons in the absence of growth factors.

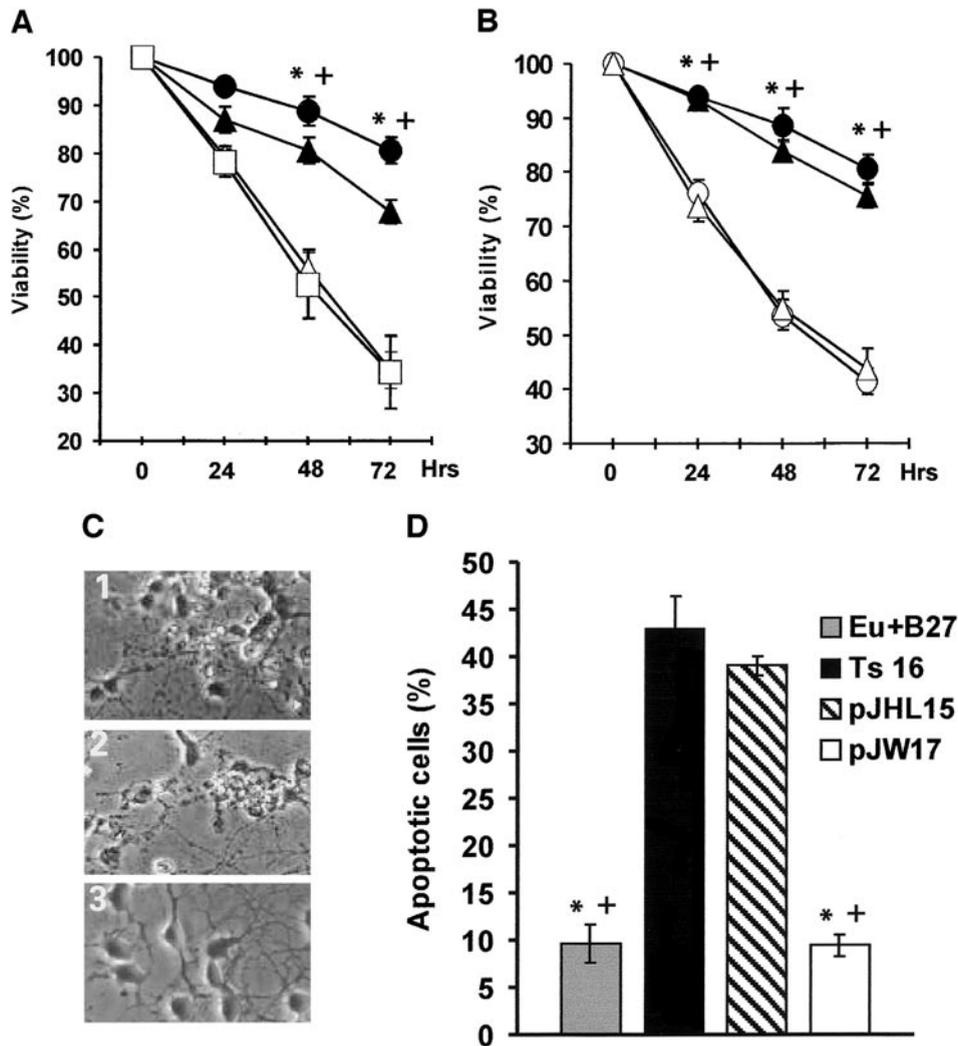


FIG. 2. ICP10 PK blocks apoptotic death of Ts16 hippocampal neurons. (A) Mouse hippocampal neurons were plated on glass coverslips etched with a grid of 175×175 -m squares, maintained for 2 days in MEM/B27 media, transfected with pJW17 (solid triangles) or pJHL15 (open triangles) and the medium was replaced with MEM free of serum or B27. Nontransfected cells were maintained in MEM (Eu-B27) (open squares) or MEM with B27 (Eu + B27) (solid circles). Viability was determined by counting live neurons and results are expressed as a percentage relative to the initial (0 h) number of viable cells \pm SEM [$*P < 0.05$ vs control (Eu-B27), $^+P < 0.05$ vs pJHL15-transfected cells, by ANOVA with Tukey-Kramer posttest]. (B) Ts16 mouse hippocampal neurons were grown on glass coverslips and transfected with pJW17 (solid triangles), pJHL15 (open triangles), or nontransfected (open circles). Nontransfected euploid littermate hippocampal cells maintained in B27-supplemented medium (Eu + B27) (solid circles) were used as control. Viability was determined by counting live neurons and expressed as a percentage relative to the initial ($t = 0$) number of viable cells \pm SEM ($*P < 0.001$ vs control Ts16 nontransfected; $^+P < 0.001$ vs pJHL15-transfected cells, by ANOVA with Tukey-Kramer posttest). (C) Photomicrographs of Ts16 cultures nontransfected (panel 1), pJHL15 transfected (panel 2), or pJW17 transfected (panel 3) at 72 h posttransfection (5 days in culture). (D) Ts16 mouse hippocampal neurons grown and transfected as in A and nontransfected euploid hippocampal cells maintained in B27-supplemented medium (Eu + B27) were assayed by TUNEL at 72 h posttransfection. Results are expressed as percentage apoptotic (TUNEL-positive) cells \pm SEM ($*P < 0.01$ vs control Ts16 nontransfected; $^+P < 0.01$ vs pJHL15-transfected cells, by ANOVA with Tukey-Kramer posttest).

The Ts16 mouse is considered to be a model of Down's syndrome (DS; trisomy 21). Ts16 is a genetic defect believed to confer increased vulnerability to neurodegeneration (8, 15). Cultured hippocampal neurons from the Ts16 mouse exhibit increased cell death relative to littermate euploid cells, even in the presence of adequate trophic support (2). To examine whether ICP10 PK can promote survival in this system, primary hippocampal cultures from Ts16 mice [estab-

lished as described (2)] were transfected with pJW17 or pJHL15 at 2 days in culture and maintained in B27-supplemented medium for the duration of the experiment. Live cells were counted as described above and the results are expressed as percentage surviving cells \pm SEM. As previously described (2, 8), nontransfected (Ts16) neurons evidenced an accelerated death rate (76.2 ± 2.3 ; 53.7 ± 2.8 , and $41.4 \pm 2.3\%$ survival at 24, 48, and 72 h, respectively) relative to euploid

neurons maintained in B27-supplemented medium (Eu + B27) (93.8 ± 1.7 , 88.7 ± 3.1 , and $80.6 \pm 2.7\%$ survival at 24, 48, and 72 h, respectively) ($P < 0.01$ by ANOVA) (Fig. 2B). Similar cell death was seen for pJHL15-transfected Ts16 neurons (73.8 ± 3.0 , 54.9 ± 3.2 , and 43.8 ± 3.7 , at 24, 48, and 72 h ($P > 0.05$ vs nontransfected Ts16 neurons by ANOVA). By contrast, the survival of pJW17-transfected Ts16 neurons (93.5 ± 1.5 , 83.9 ± 2 , and $75.6 \pm 2.2\%$ at 24, 48, and 72 h, respectively) was similar to that of euploid neurons maintained in B27-supplemented medium (Eu + B27) and significantly ($P < 0.001$ by ANOVA) higher than that of nontransfected Ts16 neurons. Morphologically, both the nontransfected (Fig. 2C, panel 1) and pJHL15-transfected (Fig. 2C, panel 2) cells showed degenerating cell bodies and "beading" of neurites starting at approximately 24 h after NGF removal, while the pJW17-transfected cultures were debris-free, with long neurites and cell bodies resembling those of embryonic cultures maintained in medium supplemented with B27 (Fig. 2C, panel 3). Transgenes were expressed equally well in all three systems (PC12, primary hippocampal neurons deprived of trophic support and Ts16 cultures), and there was virtually absolute correlation between the estimated transfection efficiency (25–35%) and the increased survival of pJW17-transfected relative to nontransfected cells (20–35%).

To examine whether the increased survival of pJW17-transfected Ts16 cultures is due to the ability of ICP10 PK to negatively regulate apoptosis, the cultures (nontransfected or transfected with pJW17 or pJHL15) were fixed with 4% paraformaldehyde at 72 h posttransfection and examined for apoptosis by TdT-mediated dUTP nick end labeling (TUNEL), an assay that is widely considered to be specific for apoptosis (6). The *In Situ* Cell Death Detection Kit-AP (Roche Molecular Biochemicals) was used according to the manufacturer instructions. Apoptotic cells (characterized by a dark nuclear precipitate) and nonapoptotic cells (unstained or displaying a diffuse, light, and uneven cytoplasmic staining) were counted in five randomly chosen microscopic fields (containing at least 250 cells). Results are expressed as percentage of apoptotic cells \pm SEM. The proportion of TUNEL-positive (apoptotic) cells in nontransfected ($43 \pm 3.4\%$) and pJHL15-transfected ($39 \pm 1\%$) cells was significantly higher ($P < 0.01$, by ANOVA) than in pJW17-transfected ($9.4 \pm 1.1\%$) or in euploid cultures maintained in MEM with B27 supplement (Eu + B27) ($9.6 \pm 2\%$) (Fig. 2D). The lower proportion of dead cells determined by TUNEL as compared to cell counting, presumably reflects cell loss during fixation. Similar results were obtained in three independent experiments, suggesting that ICP10 PK blocks apoptotic death of Ts16 neurons, presumably compensating for the innate defects in receptor signaling (4).

The salient feature of our findings is the observation that ICP10 PK blocks neuronal death in the experimental paradigms created by trophic factor deprivation or genetic defects. ICP10 has a transmembrane helical segment that serves to anchor it to the plasma membrane of HSV-2 infected and constitutively expressing cells (12). Its PK functions as an activated growth factor receptor, and both the auto- and transphosphorylating activities of a fusion protein consisting of ICP10 PK and the ligand binding extracellular domain of epidermal growth factor (EGF) receptor are ligand (EGF)-inducible (17). Because ICP10 PK activates the Ras signaling pathway in HSV-2-infected and constitutively expressing cells (13, 16, 18), the most likely interpretation is that it blocks caspase-dependent apoptosis by activating the Ras/MEK/MAPK survival pathway. The following findings are consistent with this interpretation: (i) ICP10 PK protects hippocampal neurons from apoptosis induced by virus infection (protection involves inhibition of caspase-3 activation and is dependent on MEK/MAPK activation (13)), and (ii) p139 does not activate the Ras/MEK/MAPK survival pathway (16) and does not rescue neurons from death due to apoptosis (13). However, the exact contribution of the activated pathway to the neuroprotective activity of ICP10 PK in the paradigms studied in these experiments, the role of other survival pathways and the identity of the survival genes involved in ICP10 PK mediated protection remain to be elucidated.

In DS, AD, and other neurodegenerative disorders, specific neuronal populations die by apoptosis. The widespread nature of the neuronal injury presents a considerable challenge to the development of therapeutic strategies. The development of strategies to prevent the death of hippocampal neurons is limited by the paucity of genes that block apoptosis in these cells. By promoting the survival of hippocampal neurons through activation of intracellular survival pathways, ICP10 PK represents a novel therapeutic strategy for treatment of neurodegenerative disorders that are associated with apoptosis. Apoptotic death following single stimulus, such as stroke or trauma, may be even more amenable to treatment with ICP10 PK.

REFERENCES

1. Aurelian, L., P. Terzano, C. C. Smith, T. D. Chung, A. Shamsuddin, S. Costa, and C. Orlandi. 1989. Amino-terminal epitope of herpes simplex virus type 2 ICP 10 protein as molecular diagnostic marker for cervical intraepithelial neoplasia. *Cancer Cells* 7: 187–191.
2. Bambrick, L. L., and B. K. Krueger. 1999. Neuronal apoptosis in mouse trisomy 16: Mediation by caspases. *J. Neurochem.* 72: 1769–1772.
3. Bambrick, L. L., P. J. Yarowsky, and B. K. Krueger. 1995. Glutamate as a hippocampal neuron survival factor: An inher-

- ited defect in the trisomy 16 mouse. *Proc. Natl. Acad. Sci. USA* **92**: 9692–9696.
4. Dorsey, S. G., B. K. Krueger, and L. L. Bambrick. 1999. Altered TrkB expression and the accelerated death of hippocampal neurons from the Trisomy 16 mouse. *Soc. Neurosci. Abstr.* **25**: 2023.
 5. Ferreira, A., and A. Caceres. 1992. Expression of the class III beta-tubulin isotype in developing neurons in culture. *J. Neurosci. Res.* **32**: 516–529.
 6. Gavrieli, Y., Y. Sherman, and S. A. Ben-Sasson. 1992. Identification of programmed cell death via specific labeling of nuclear DNA fragmentation. *J. Cell. Biol.* **119**: 493–501.
 7. Greene, L. A., and A. S. Tischler. 1982. PC12 pheochromocytoma cells on neurobiological research. *Adv. Cell. Neurobiol.* **3**: 373–414.
 8. Hattori, M., A. Fujiyama, T. D. Taylor, H. Watanabe, T. Yada, H.-S. Park, A. Toyoda, K. Ishii, Y. Totoki, D. K. Choi, E. Soeda, M. Ohki, T. Takagi, Y. Sakaki, S. Taudien, K. Blechschmidt, A. Polley, U. Menzel, J. Delabar, K. Kumpf, R. Lehman, D. Patterson, K. Reichwald, A. Rump, M. Schillhabel, A. Schudy, W. Zimmerman, A. Rosenthal, J. Kudoh, K. Shibuya, K. Kawasaki, S. Asakawa, A. Shintani, T. Sasaki, K. Nagamine, S. Mitsuyama, S. E. Antonarakis, S. Minoshima, N. Shimizu, G. Nordstiek, K. Hornischer, P. Brandt, M. Scharfe, O. Schon, A. Desario, J. Reichelt, G. Kauer, H. Blocker, J. Ramser, A. Beck, S. Klages, S. Hennig, L. Riesselmann, E. Dagand, S. Wehrmeyer, K. Borzym, K. Gardiner, D. Nizetic, F. Francis, H. lechrach, R. Reinhardt, and M.-L. Yaspo. 2000. DNA sequence of human chromosome 21. *Nature* **405**: 311–319.
 9. Honig, L. S., R. N. Rosenberg. 2000. Apoptosis and neurological disease. *Am.J.Med.* **108**: 317–330.
 10. Kaplan, D. and F. Miller. 2000. Neurotrophin signal transduction in the nervous system. *Curr. Opin. Neurobiol.* **10**: 381–391.
 11. Lo, A. C., L. J. Houenou, R. W. Oppenheim. Apoptosis in the nervous system: Morphological features, methods, pathology, and prevention. 1995. *Arch. Histol. Cytol.* **58**: 139–149.
 12. Luo, J. H. and L. Aurelian. 1992. The transmembrane helical segment but not the invariant lysine is required for the kinase activity of the large subunit of Herpes Simplex Virus Type 2 ribonucleotide reductase. *J. Biol. Chem.* **267**: 9645–9653.
 13. Perkins, D., E. F. R. Pereira, C. C. Smith, and L. Aurelian. 2000. ICP10 PK anti-apoptotic activity and CNS resistance to HSV-2-induced encephalitis. *Soc. Neurosci. Abstr.* **26**: 1062.
 14. Pittman, R. N., S. Wang, A. J. DiBenedetto, and J. C. Mills. 1993. A system for characterizing cellular and molecular events in programmed cell death. *J. Neurosci.* **13**: 3669–3680.
 15. Sawa, A. 1999. Neuronal cell death in Down's syndrome. *J. Neural. Transm. [Suppl]* **57**: 87–97
 16. Smith, C. C., J. H. Luo, J. C. Hunter, J. V. Ordonez, and L. Aurelian. 1994. The transmembrane domain of the large subunit of HSV-2 ribonucleotide reductase (ICP10) is required for protein kinase activity and transformation—Related signaling pathways that result in ras activation. *Virology* **200**: 598–612.
 17. Smith, C. C., J. H. Luo, and L. Aurelian, L. 1996. The protein kinase activity of the large subunit of Herpes Simplex Virus Type 2 ribonucleotide reductase (ICP10) fused to the extracellular domain of the epidermal growth factor receptor is ligand-inducible. *Virology* **217**: 425–434.
 18. Smith, C. C., J. Nelson, L. Aurelian, M. Gober, and B. B. Goswami. 2000. Ras-GAP binding/phosphorylation by HSV-2 RRIPK (ICP10) and activation of the Ras/MEK/MAPK mitogenic pathway are required for timely onset of virus growth. *J. Virol.* **74**: 10,417–10,429.