

A HERPES SIMPLEX VIRUS TYPE 2 PROTEIN (ICP10 PK) INHIBITS CASPASE-3 ACTIVATION IN HIPPOCAMPAL NEURONS

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This article describes the use of Promega's CaspACE™ FITC-VAD-FMK In Situ Marker (Cat.# G7461, G7462) to investigate caspase activation in primary hippocampal cell cultures. The cells were infected with either wildtype or mutant HSV-2 that contains a deletion of the genetic information encoding the amino terminal domain of the large subunit of ribonucleotide reductase.

Introduction

Apoptosis is a physiological cell-death program that occurs as a prominent feature of many neurological diseases, including acute brain injury (e.g., hypoxia-ischemia and trauma), neurodegenerative disorders (e.g., Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis), and infections (1). Neurological disorders involving apoptosis might be amenable to gene therapy as an alternative to more conventional pharmacological treatments. However, the progress of gene therapy for neurological diseases is limited by the paucity of genes with anti-apoptotic activity in neuronal cells.

Many of the morphological changes associated with apoptosis result from the actions of a group of cysteine aspartyl proteases (caspases) that are specifically activated in apoptotic cells by proteolytic cleavage. Virus infection both triggers and counteracts apoptosis by poorly understood direct and indirect mechanisms that are mediated by a variety of viral genes (2).

The large subunit of the Herpes Simplex Virus Type 2 (HSV-2) ribonucleotide reductase (RR1; also known as ICP10) differs from RR1 subunits in eukaryotic or prokaryotic cells and other herpes viruses in that it contains a unique amino terminal domain with serine/threonine protein kinase (PK) activity (3). ICP10 has a transmembrane helical segment and localizes to the plasma membrane, like growth factor receptors (4). In cells infected with HSV-2 or constitutively expressing ICP10 PK, this gene activates the Ras/MEK/MAPK signaling pathway (5). Recent findings indicate that activation of MEK/MAPK by virion ICP10 PK is necessary and sufficient to protect cultured hippocampal neurons from virus-

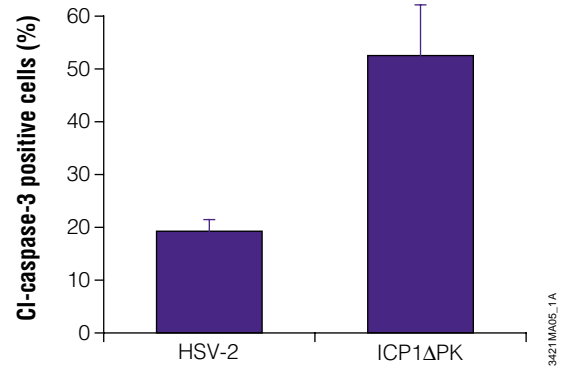


Figure 1. Comparison of activated caspase-3 in primary hippocampal cultures infected with wildtype HSV-2 or with HSV-2 containing the ICP10ΔPK mutation. Note that HSV-2 infected cells show significantly less active caspase-3.

induced apoptosis (6). Here we report that ICP10 PK inhibits caspase-3 activation in primary cultures of hippocampal neurons.

ICP10ΔPK infected cells show increased staining for active caspase-3

We performed two sets of experiments to determine the effect of ICP10 PK on caspase-3 activation in rat primary hippocampal cultures (E17-18 at day 6 in vitro) that contain over 85% neurons (7). We infected cultures with 10 plaque forming units (pfu)/cell of HSV-2, an HSV-2 mutant with the information encoding the PK domain of ICP10 deleted (designated ICP10ΔPK), or mock-infected with growth medium only. In the first series of experiments, cells were fixed at 24 hours post-infection (p.i.) with 4% paraformaldehyde (pH 7.4) and stained with an antibody specific for the 17–20kDa fragment of active caspase-3. We detected bound antibody using immunoperoxidase staining and counterstaining with hematoxylin. We counted cells in 5 randomly chosen microscopic fields and expressed the results as mean percent CL-caspase-3 (cleaved) positive cells ± SEM. In three independent experiments, background staining in mock-infected cells was less than 5%, while 52.6 ± 9.6% of cells infected with ICP10ΔPK, and 19.3 ± 2% of cells infected with HSV-2 stained with CL-caspase-3 ($p < 0.05$ for ICP10ΔPK vs. HSV-2 by Student's *t*-test; Figure 1).

ICP10ΔPK-infected cultures show increased binding of CaspACE™ FITC-VAD-FMK In Situ Marker

In the second series of experiments, we investigated caspase activation using Promega's CaspACE™ FITC-VAD-FMK In Situ Marker (Cat.# G7461 and G7462). Briefly, we treated cultures infected as above with 10μM of the FITC-conjugated cell permeable caspase inhibitor VAD-FMK and

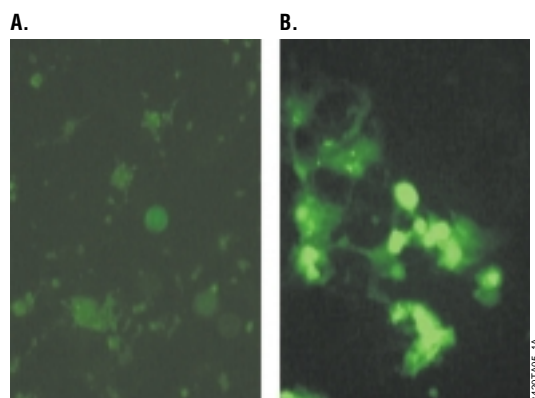


Figure 2. Primary cultures of hippocampal neurons infected with wildtype HSV-2 and ICP10ΔPK. No signal was detected in HSV-2 infected cells (**Panel A**), but cells infected with HSV-2 containing the ICP10ΔPK mutation showed increased binding of the CaspACE™ FITC-VAD-FMK In Situ Marker (Cat.# G7461) as evidenced by FITC signal in 50–55% of the cells (**Panel B**).

incubated for 20 minutes at 36.5°C, 5% CO₂, to allow binding of inhibitor to the activated caspase. We fixed the cells in 10% formaldehyde (pH 7.6) and analyzed them by fluorescence microscopy. No signal was detected in HSV-2 (Figure 2A) or in mock-infected cultures (data not shown). By contrast, ICP10ΔPK-infected cultures showed increased binding of the caspase inhibitor (Figure 2B) as evidenced by FITC signal in approximately 50–55% of cells, supporting the conclusion that ICP10 PK inhibits caspase-3 activation in primary cultures of hippocampal neurons. These results correlate well with those obtained by TdT-mediated dUTP nick end-labeling (TUNEL) (less than 6% and 65–70% TUNEL-positive cells for HSV-2 and ICP10ΔPK, respectively).

The mechanism by which the ICP10 protein kinase domain inhibits caspase-3 activation is still unknown and is the subject of ongoing studies in our laboratory.

Notwithstanding, the anti-apoptotic activity of ICP10 PK in hippocampal neurons suggests that it might provide a novel strategic approach in the treatment of neurological disorders with an apoptotic component.

References

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Ordering Information

Product	Size	Cat. #
CaspACE™ FITC-VAD-FMK In Situ Marker	50µl	G7461
	125µl	G7462
Apoptosis Detection System, Fluorescein*(a)	60 reactions	G3250
Apo-ONE™ Homogeneous Caspase-3/7 Assay*(b)	10ml	G7790
	100ml	G7791
CaspACE™ Assay System, Fluorometric*	160 assays	G3540
CaspACE™ Assay System, Colorimetric*	100 assays	G7220
	50 assays	G7351
Caspase Inhibitor Z-VAD-FMK	50µl	G7231
	125µl	G7232
Anti-ACTIVE® Caspase-3 pAb	50µl	G74810

* For Laboratory Use.

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(b) This product is covered by U.S. Pat. Nos. 4,557,862 and 4, 640,893 and is sold as a general purpose reagent for research and laboratory use only. All other uses, including by not limited to use as a clinical diagnostic or therapeutic, require a separate license. Please contact Promega Corporation for details.

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Table 1. RNA Targets and Primer Sequences (from page 5)

RNA Target	Full-Length Message	Primer Sequence 5' → 3'	Amplification Product
Human β-Actin mRNA	1,761nt	Upstream Primer: GGGAAATCGTGCGTGACATTAAG Downstream Primer: TGTGTTGGCGTACAGGCTTTG	275bp
Human γ-Actin mRNA	1,918nt	Upstream Primer: AAGTACCCATTGAGCATGGC Downstream Primer: CACAGCTTCTCCTTGATGTCGC	449bp
Human ADP Ribosylation Factor mRNA (ARF-1)	1,816nt	Upstream Primer: GCCAGTGTCCCTTCCACCTGTC Downstream Primer: GCCTCGTTCACACGCTCTCTG	336bp
Human Cysteine Protease Yama mRNA (caspase-3)	834nt	Upstream Primer: CTCGGTCTGGTACAGATGTCGATG Downstream Primer: GGTTAACCCGGTAAGAATGTGCA	533bp