Ectromelia virus virulence factor p28 acts upstream of caspase-3 in response to UV light-induced apoptosis

David J. Brick,1 Robert D. Burke,2 Aaron A. Minkley1 and Chris Upton1

Departments of Biochemistry and Microbiology1 and Biology2, University of Victoria, PO Box 3055, Victoria, BC, Canada V8W 3P6

Ectromelia virus (EV) virulence factor p28 (EVp28) is a member of a family of poxvirus proteins that are defined largely by the presence of a C-terminal RING finger motif and localization to virus factories within the cytoplasm of infected cells. Previously, overexpression of the Shope fibroma virus (SFV) homologue, N1R, in vaccinia virus (VV)-infected BGMK cells was found to inhibit virus-induced apoptosis. Here, we report that both EVp28 and overexpression of SFV N1R in poxvirus-infected HeLa cells protect specifically from UV light-induced apoptosis, but not from apoptosis induced by Fas or TNF. Further, we report that both VV and EV protect from apoptosis induced by UV, Fas and TNF. Immunoblot analysis indicates that EVp28 acts upstream of caspase-3, blocking activation of the protease in response to UV irradiation. Although no difference was found in replication of an EVp28
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mutant virus, which expresses a truncated p28 protein lacking the RING motif, compared to EV wild-type in HeLa cells, UV irradiation of infected HeLa cells reduced the replication of the EV mutant compared with wild-type EV.

Introduction

The study of virus-encoded proteins that modulate host defence mechanisms has provided important insights into both virus pathogenesis and the host cellular immune processes. Ectromelia virus (EV), the causative agent of mousepox, encodes a 28 kDa RING finger protein that is expressed early during infection (Senkevich et al., 1995). Although non-essential for replication of EV in tissue culture, expression of this protein is crucial for EV virulence in mice, the natural host (Senkevich et al., 1994). A prominent feature of the poxvirus RING finger proteins, which localize to the cytoplasmic poxvirus factories, is a zinc-binding RING finger motif. The RING finger motif (C3HC4) is a conserved, cysteine-rich amino acid sequence that has been found in more than 80 proteins of diverse evolutionary origin that function in oncogenesis, development, signal transduction and apoptosis, but the precise role of this motif remains unknown (Borden & Freemont, 1996; Freemont, 1993; Saurin et al., 1996).

Poxviruses express a variety of proteins that are non-essential for replication in tissue culture, but help the virus to evade the host response to infection. Examples include viral proteins that target and counteract immune molecules of the host such as TNF (Upton et al., 1991), interferon γ (Upton et al., 1992), interleukins (Spriggs et al., 1992) and chemokines (Cao et al., 1995; Graham et al., 1997). One particularly effective host defence mechanism is for the infected cell to self-destruct. Apoptosis is an intrinsic cell suicide mechanism that refers to a carefully orchestrated pattern of cytoplasmic and nuclear changes that result in self-destruction and clearance of individual cells from tissues without provoking an inflammatory response (Wyllie et al., 1980). Apoptosis not only acts as a defence mechanism against virus pathogenesis and DNA damage, but also plays vital roles in normal development, tissue homeostasis, immune cell development and function (Thompson, 1995; White, 1996).

Apoptosis can be initiated by a variety of extra- and intracellular factors. Examples include triggering of the TNF or Fas (CD95) receptors, UV irradiation, exposure to cytotoxic drugs and virus infection. Central to the initiation and execution of the apoptotic program, two key mediators have emerged: mitochondria and the caspases (cysteine proteases). It is apparent that several distinct routes exist to caspase activation, dependent upon the initiating stimulus. Death-receptor aggregation following FasL or TNF binding results in recruitment of caspase-8 through adapter molecules (Boldin et al., 1996; Chinnaiyan et al., 1995, 1996; Muzio et al., 1996).
Alternatively, proapoptotic signals that lead to perturbation of mitochondrial membrane integrity result in cytochrome c leakage from the intermembrane space into the cytosol, where an interaction with Apaf-1 enables recruitment and activation of caspase-9 (Kluck et al., 1997; Li et al., 1997; Liu et al., 1996; Zou et al., 1997, 1999). In both cases, activation of initiator caspases results in activation of downstream effector caspases (such as caspase-3 and caspase-6) and cleavage of various death substrates, culminating in apoptosis (Medema et al., 1997; Scaffidi et al., 1998; Slee et al., 1999).

Poxviruses have evolved a number of strategies to curtail the host’s apoptotic response to infection. These include proteins that directly reduce levels of inducers of apoptosis such as TNF (Macen et al., 1996; Schreiber et al., 1997; Sedger & McFadden, 1996), double-stranded (ds) RNA (Kibler et al., 1997; Lee & Esteban, 1994; Rivas et al., 1998) and oxidative stress (Shisler et al., 1998). Additionally, the cowpox virus crmA gene product, a serpin, acts downstream of apoptotic initiation and inhibits a number of caspases (Kettle et al., 1997; Tewari et al., 1995a; Zhou et al., 1997) and granzyme B (Quan et al., 1995) specifically to provide protection from death-receptor ligation and cytotoxic T lymphocyte killing (Dobbelstein & Shenk, 1996; Tewari et al., 1995b). Further, the molluscum contagiosum virus MC159 gene product, which interacts with receptor signalling adapter molecule FADD, blocks recruitment and activation of caspase-8 (Bertin et al., 1997).

Although the molecular mechanisms involved in receptor-mediated apoptosis have been well characterized, those involved in UV-induced apoptosis remain largely obscure (Martin & Cotter, 1991; Martin et al., 1995). A variety of components, such as protein kinase signalling pathways (Berra et al., 1998; Verhaegen et al., 1996), Myc (Sugiyama et al., 1995), have been implicated in this process, but the cell cycle-regulated components, such as protein kinase signalling pathways (Berra et al., 1998; Verhaegen et al., 1996), Myc (Sugiyama et al., 1995), have been implicated in this process, but the cell cycle-regulated tumour suppressor protein p53, a DNA-damage sensor (Lane, 1991). p53-mediated apoptosis is likely triggered in part through activation of its downstream genes such as p21Cip1/Waf1, GADD45, PAG 608, IGF-BP3 and notably bax, a proapoptotic bcl-2 family member (Buckbinder et al., 1995; Gujuluva et al., 1994; Israeli et al., 1997; Liu & Pelling, 1995; Zhan et al., 1994). In addition, p53 has been found to downregulate bcl-2 expression (Miyashita et al., 1994). p53, however, also induces apoptosis independent of its transcriptional activity (Caellen et al., 1994; Haupt et al., 1995). The critical role of p53 in the response of mammalian cells to UV-induced apoptosis is shown by p53-knockout mice, in which epidermal cells undergo reduced apoptosis after UV irradiation (Ziegler et al., 1994). Interestingly, p53 is targeted for inactivation by many DNA viruses (Havre et al., 1995; Moore et al., 1996; Okan et al., 1995; Scheffner et al., 1990; Steegenga et al., 1996; Szekely et al., 1993; Wang et al., 1995; Werness et al., 1990), a fact that suggests an anti-viral function for p53.

We have shown previously that overexpression of SFV N1R in VV-infected BGMK cells reduced virus-induced apoptosis (Brick et al., 1998). To clarify the role of N1R protein with respect to apoptosis and to examine whether the related EV virulence factor p28 might also play a role in protection against apoptosis, a p28 mutant EV and the VV-N1R virus were tested for their ability to interfere with apoptosis induced by different signals. We report that both SFV N1R and EVP28 protect infected HeLa cells from apoptosis induced by UV light, but not from apoptosis induced by TNF ligand or anti-Fas antibody. The possible implications of our findings reported here are discussed with regard to the poxviral RING finger proteins in poxvirus infection.

Methods

- **Cells and viruses.** VV strain IHDW, BGMK and HeLa cells were provided by G. McFadden (University of Western Ontario, London, ON, Canada). The Moscow strain of EV (passage 3; EV-WT) and the recombinant p28 mutant EV were generous gifts of R. M. L. Buller (St Louis University Medical School, St Louis, MO, USA). The p28 mutant EV has been described previously (Senkevich et al., 1994, 1995), as has the recombinant VV-N1R (Brick et al., 1998). Viruses and cells were grown at 37°C in Dulbecco’s minimal Eagle’s medium supplemented with 10% newborn bovine serum (Gibco BRL).

- **ELISA detection of apoptotic oligonucleosomes.** A sandwich assay was performed using a pair of MAbs specific for two nucleosomal epitopes to capture and detect cytoplasmic nucleosomes (Salgame et al., 1997). Hybridoma cell lines LG11-2 and PL2-3 were generously provided by Marc Monestier (Temple University School of Medicine, Philadelphia, PA, USA). MAbs were prepared by Immuno-Precise Antibodies (Victoria, BC, Canada). Samples of semi-confluent HeLa cells (6 × 10⁶) in 6 well dishes were infected with virus at an m.o.i. of 3. At 12 h post-infection, cells were either mock-treated or treated with TNFα (10 ng/ml; Boehringer Mannheim) and cycloheximide (CHX) (15 µg/ml) and cycloheximide (CHX) (15 µg/ml) and cycloheximide (CHX) (15 µg/ml) alone or a 2 min exposure to UV light (302 nm; UV Transilluminator TM-36, UVP-Ultraviolet Products) from underneath the dish surface. Adherent and floating cells were harvested 12 h later and processed for the ELISA (Salgame et al., 1997) with the following modifications: 1% normal goat serum (Immuno-Precise Antibodies) was used in place of 1% BSA and cytoplasmic lysates were incubated overnight at 4°C. The ELISA was performed using 3.75 × 10⁻³ cell equivalents per well in 96 well plates (Becton Dickinson).

- **DAPI analysis.** For 4′,6′-diamidino-2-phenylindole (DAPI) analysis of nuclear morphology, infections and treatments were carried out as described for the apoptotic ELISA with the following modifications: cell cultures were grown in 8 chamber slides or, for UV analysis, on coverslips in 6 well dishes. Treated and mock-treated samples were fixed at 24 h of infection with ice-cold 70% ethanol for 15 min. Cells were then rinsed in PBS, pH 7.4, and stained with 500 ng/ml DAPI (Sigma) in PBS for 5 min in the dark. After rinsing in PBS, coverslips were mounted, viewed and photographed with epifluorescence using excitation and emission filters of 365 and 420 nm, respectively.
Flow cytometric analysis. Samples of semi-confluent HeLa cells (6 x 10⁶) in 6 well dishes were infected with virus at an m.o.i. of 3. At 8 h post-infection, cells were either mock-exposed or exposed to UV light (302 nm) for 2 min. Adherent and floating cells were harvested 1 h later, washed in ice-cold PBS and resuspended in PBS containing 5% glucose and 5% glycerol. Cells were permeabilized by the dropwise addition of 95% ice-cold ethanol to a final concentration of 80% and incubated at 4°C for 15 min. Thereafter, cells were stored at -20°C until analysis. Prior to analysis, cells were washed in PBS and resuspended in PBS containing 1·25 μg/ml propidium iodide (PI) (Sigma) and 50 μg/ml RNase A (Sigma) and incubated in the dark for 15 min at 4°C. The DNA content of 10000 cells per sample was measured by using a FACScalibur flow cytometer (Becton Dickinson) equipped with an argon-ion laser (488 nm) and the data were registered on a logarithmic scale. The light-scatter characteristics were measured simultaneously and all data were acquired and analysed using CELLQuest software (Becton Dickinson).

Caspase-3 activation assay. Samples of semi-confluent HeLa cells (6 x 10⁶) in 6 well dishes were infected with viruses at an m.o.i. of 3. At 8 h post-infection, cells were either mock-exposed or exposed to UV light (302 nm) for 2 min. Adherent and floating cells were harvested 1 h later. Rabbit polyclonal antibody CPP32 (H-277) (Santa Cruz Biotechnology) recognizes the full-length precursor form of human CPP32. Protein samples for detection of CPP32 were separated on 18% SDS–PAGE, transferred to PVDF membrane and blocked with 5% Hipure liquid gelatin (Norland Products) in PBS containing 0·1% Tween 20 (Aldrich) for 90 min. Blots were incubated with polyclonal CPP32 (H-277) (1:200) in PBS containing 1% BSA and 0·1% Tween 20 overnight at 4°C. Bound antibody was detected by horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5000) (Caltag) and Supersignal chemiluminescent substrate (Pierce) as described by the manufacturer.

Effects of UV-induced apoptosis on virus titre. Semi-confluent HeLa cells (6 x 10⁶) in 6 well dishes were infected with viruses at an m.o.i. of 3. At 4 h post-infection, infected cells were mock-exposed or exposed to UV light for either 1 or 2 min. Virus was harvested 48 h later and the yield was determined in a standard plaque assay.

Statistical analysis. Results from the apoptotic ELISA, DAPI staining and virus titre experiments were analysed with Student’s t-test by using the statistical package in Microsoft Excel and are presented as means and either SEM or SD.

Results

SFV N1R protein and EVp28 protect from UV- but not from TNF- or anti-Fas-induced apoptosis

Apoptosis is characterized by a number of distinct morphological features, such as cell shrinkage, loss of cell–cell contacts, plasma membrane blebbing, chromatin condensation, endonuclease-mediated DNA fragmentation into oligonucleosomes and the formation of apoptotic bodies (small, membrane-bound vesicles containing nuclear fragments) that are destined for phagocytosis by neighbouring cells or macrophages. Previously, we showed that overexpression of SFV N1R in UV-infected BGMK cells inhibited virus-induced apoptosis significantly at late times post-infection by using two assays (chromosomal DNA laddering on agarose gels and an ELISA that quantifies cytoplasmic oligonucleosomes). Because of the critical role of EVp28 in the pathogenicity of this virus, it was of interest to determine whether the EVp28 RING homologue could also protect from apoptosis. Apoptosis was assessed in mock-infected HeLa cells and cells infected with either EV, EV-N1R, EV-WT or p28− mutant EV. Mock-infected or infected cells were either untreated, UV-irradiated or treated with CHX + anti-Fas Ab, CHX + TNF or CHX alone at 12 h post-infection. Duplicate samples were collected and each was assayed in triplicate. The means ± SEM of these determinations are shown. In some instances, error bars are too small to be seen. The y-axis represents the absorbance. Shaded columns represent mock-infected cells, cross-hatched columns represent VV-N1R-infected, hatched columns represent VV-infected, filled columns represent EV-WT-infected and open columns represent p28− mutant EV-infected cells.

![Fig. 1. Detection of apoptotic cytoplasmic oligonucleosomes by ELISA. HeLa cells were mock-infected or infected at 3 p.f.u. per cell with VV, VV-N1R, EV-WT or the p28− mutant EV. Mock-infected or infected cells were either untreated, UV-irradiated or treated with CHX + anti-Fas Ab, CHX + TNF or CHX alone at 12 h post-infection. Duplicate samples were collected and each was assayed in triplicate. The means ± SEM of these determinations are shown. In some instances, error bars are too small to be seen. The y-axis represents the absorbance. Shaded columns represent mock-infected cells, cross-hatched columns represent VV-N1R-infected, hatched columns represent VV-infected, filled columns represent EV-WT-infected and open columns represent p28− mutant EV-infected cells.](image-url)
Apoptosis was assessed further by examination of nuclear morphology by using the DNA fluorochrome DAPI. Apoptotic cells exhibit increased nuclear fluorescence with this dye. DAPI analysis (performed blind) supported the ELISA-based findings (Fig. 2). Treatment with TNF + CHX or anti-Fas + CHX induced apoptosis in approximately 60% of mock-infected HeLa cells, whereas both the EV-WT- and p28− mutant EV-infected cells exhibited approximately 20% apoptosis (data not shown). No appreciable difference was observed between EV-WT and the p28− mutant EV in response to TNF or anti-Fas, but UV treatment of the p28− mutant EV-infected cells (Fig. 2f) showed nuclear condensation, nuclear blebbing and the presence of apoptotic bodies (indicative of late stages of apoptosis) that were also present in mock-infected UV-treated cells (Fig. 2d). In contrast, UV treatment of EV-WT-infected cells showed no signs of induction of apoptosis (Fig. 2e); nuclear morphology was similar to that observed in both untreated mock-infected and untreated virus-infected cells. The mean number of apoptotic cells was determined from five randomly chosen fields (Fig. 2g). Again, EV-WT was found to inhibit UV-induced apoptosis strongly (P = 0.003) compared with the p28− mutant EV.

DAPI staining, however, relies on analysis of adherent cells. To measure apoptosis in all cells, flow cytometric analysis of PI-stained cells was employed. The fragmented DNA of apoptotic cells incorporates less PI than the intact DNA of normal cells, thus the DNA content, as determined in single cells by FACS, provides a good estimate of the degree of apoptotic (hypodiploid) cells in a population (Nicoletti et al., 1991) (Fig. 3). Cells showing decreased fluorescence after PI staining (marked by a gate in Fig. 3) appear to the left of those in G1 or G2 phase of the cell cycle. Untreated mock-infected or virus-infected cells showed no difference in the level of apoptosis at 24 h post-infection. However, UV treatment resulted in elevated levels of apoptosis in mock-infected and p28− mutant EV-infected HeLa cells (Fig. 3d, f) compared with UV-treated, EV-WT-infected cells (Fig. 3e).

Apoptotic cells undergo condensation and shrinkage, which results in altered light-scattering characteristics. The scatter profiles of the cell population were measured simultaneously.
EVp28 inhibits UV-induced apoptosis

Fig. 3. FACS analysis of the DNA content of mock-infected and virus-infected cells. HeLa cells were mock-infected or infected at 5 p.f.u. per cell with EV-WT or the p28\(^{-}\) mutant EV. Mock-infected or virus-infected cells were either mock UV-irradiated or UV-irradiated for 2 min at 10 h post-infection. Cells were harvested at 24 h post-infection, permeabilized and stained with PI. Ten thousand cells per sample were analysed by FACS and cell counts were plotted against PI fluorescence. The percentage of cells of the hypodiploid (apoptotic) population is indicated in each graph.

by FACS (Fig. 4). While the scatter plots of untreated mock-infected and virus-infected cells showed similar profiles of background levels of apoptosis at 24 h post-infection (Fig. 4a–c), UV treatment of mock-infected and p28\(^{-}\) mutant EV-infected cells (Fig. 4d, f) resulted in a shift towards decreased cell size that was accompanied by decreased PI fluorescence. Again, consistent with a role for the EVp28 RING finger protein in suppression of UV-induced apoptosis, UV treatment of EV-WT-infected HeLa cells inhibited the shift of cell size and PI fluorescence into the apoptotic region (Fig. 4e) compared with both UV-treated, mock-infected and p28\(^{-}\) mutant EV-infected cells. Thus, FACS analysis is further consistent with the ELISA- and DAPI-based findings, which indicate that, while EV-WT infection protects infected cells from UV-induced apoptosis, the EVp28 gene product has a specific involvement in suppression of UV-induced apoptosis.

**EVp28 acts upstream of caspase-3 in response to UV irradiation**

Caspase-3 is central to the execution of apoptosis in response to several stimuli (Janicke et al., 1998; Porter & Janicke, 1999; Schlegel et al., 1996; Woo et al., 1998). The enzyme is synthesized in cells as an inactive 32 kDa precursor, which is proteolytically processed into the 17 and 12 kDa subunits of the mature caspase-3 during apoptosis. Several targets of activated caspase-3 have been identified. These include DNA repair enzymes such as poly(ADP-ribose) polymerase and DNA-dependent protein kinase and ICAD, the recently identified inhibitor of caspase-activated DNase (Enari et al., 1998; Han et al., 1996; Sakahira et al., 1998; Tewari et al., 1995a). Inhibition of caspase-3 has been shown to block apoptosis (Nicholson et al., 1995) and the biological importance of this protease is demonstrated by caspase-3-knockout mice, which do not undergo normal apoptosis in certain tissues and die shortly after birth (Kuida et al., 1996).

An antibody that detects full-length human caspase-3 was used to examine caspase-3 cleavage after UV irradiation of infected cells (Fig. 5). The 32 kDa procaspase form of caspase-3 was present in similar amounts in untreated mock-infected, EV-WT- and p28\(^{-}\) mutant EV-infected samples at 24 h post-infection. UV treatment, however, resulted in greatly diminished levels of procaspase-3 in both mock-infected and p28\(^{-}\) mutant EV-infected cells. Correlating with our apoptotic assay findings of inhibition of UV-induced apoptosis by EVp28, procaspase-3 was consistently protected from cleavage in lysates prepared from UV-irradiated, EV-WT-infected cells compared with UV-irradiated, p28\(^{-}\) mutant EV-infected and mock-infected irradiated cells (Fig. 5, lanes 4–6). This indicates that EVp28 acts upstream of caspase-3 activation in response to UV irradiation. Although the immunoblot does not show...
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Fig. 4. FACS analysis of the light-scattering characteristics of mock-infected and virus-infected cells. HeLa cells were mock-infected or infected at 5 p.f.u. per cell with EV-WT or the p28\(^{−}\) mutant EV. Mock-infected or virus-infected cells were either mock UV-irradiated or UV-irradiated for 2 min at 10 h post-infection. Cells were harvested at 24 h post-infection, permeabilized and stained with PI. Ten thousand cells per sample were analysed by FACS and forward scatter (FSC; cell size) was plotted against PI fluorescence. The percentage of cells in the apoptotic (R1) and normal (R2) regions is indicated.

Fig. 5. Processing of procaspase-3 (CPP32) in extracts from mock-infected and virus-infected HeLa cells following a 2 min UV exposure at 8 h post-infection. Cells were harvested at 24 h post-infection. Lanes: 1, mock-infected; 2, EV-WT-infected; 3, p28\(^{−}\) mutant EV-infected; 4, mock-infected/UV-exposed; 5, EV-WT-infected/UV-exposed; 6, p28\(^{−}\) mutant EV-infected/UV-exposed. Positions of marker proteins (in kDa) are denoted by bars.

detection of either of the activated subunits of caspase-3 following UV irradiation, the greatly diminished levels of procaspase-3 in the p28\(^{−}\) mutant EV-infected, UV-treated cells is unlikely to be due to different rates of procaspase-3 synthesis and degradation in virus-infected cells, since in the absence of UV treatment the levels of procaspase-3 were similar between mock-infected and EV-infected samples. The cross-reacting proteins provide a useful control for the evaluation of sample loading equivalency between lanes. Further, the reduced presence of procaspase-3 in both UV-irradiated, mock-infected and p28\(^{−}\) mutant EV-infected cells is wholly consistent both with the observed role of EVp28 in protection against UV-induced apoptosis and the essential nature of procaspase-3 activation in promotion of the nuclear and morphological changes of apoptosis (Frutos et al., 1999; Kimura et al., 1998; Miller et al., 1998; Nemoto et al., 1998).

**Restricted replication of the p28\(^{−}\) mutant EV following UV-induced apoptosis**

The critical role of host cell apoptosis as an antiviral defence mechanism, restricting virus replication and titre, is supported by the growing number of virus-encoded genes that have been shown to suppress apoptosis. Previous studies suggested that p28 was critical for the replication of EV in murine resident peritoneal macrophages, based on the result that no virus factories were detected by Hoechst dye staining in most of the
Table 1. Reduction in progeny virus titre after increasing exposure to UV

<table>
<thead>
<tr>
<th>UV exposure (min)</th>
<th>Virus titre (log_{10})*</th>
<th>Unexposed virus (%)</th>
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<tr>
<td></td>
<td>EV-WT p28^- EV†</td>
<td>EV-WT p28^- EV</td>
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<tr>
<td>0</td>
<td>6.95 ± 0.36</td>
<td>6.94 ± 0.35</td>
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<tr>
<td>1</td>
<td>6.03 ± 0.34</td>
<td>5.85 ± 0.33</td>
</tr>
<tr>
<td>2</td>
<td>5.23 ± 0.34</td>
<td>4.55 ± 0.31</td>
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* Arithmetic mean±SD (n = 2).
† Values were compared with those for EV-WT by using Student’s t-test and were either not significantly different (a, t = 0.19, P = 0.43) or significantly different (b, t = 3.8, P = 0.03; c, t = 28.2, P = 0.0006).

The p28^- mutant EV-infected macrophages compared with EV-WT infection (Senkevich et al., 1995). In order to examine a correlation between suppression of UV-induced apoptosis by EVP28 and virus replication, HeLa cells were infected with either EV-WT or the p28^- mutant EV and treated with UV light for either 0, 1 or 2 min at 4 h post-infection. At 48 h post-infection, virus was harvested and titrated. UV irradiation reduced the number of progeny virus recovered from the p28^- mutant EV-infected cells compared with EV-WT-infected cells (Table 1). There was an approximately 1.4-fold difference in titre of the p28^- mutant EV compared with EV-WT after exposure for 1 min (P = 0.03), which increased to a 4.9-fold difference after 2 min UV exposure (P = 0.0006). Thus, the decrease in progeny virus formation of the p28^- mutant EV increased with the duration of UV exposure. This observed difference in UV sensitivity between the EV-WT- and p28^- mutant EV-infected cells was not due to incomplete or delayed virus infection with the p28^- mutant EV since, in the absence of UV treatment, no difference in the replication of EV and the p28^- mutant EV was found (P = 0.43).

This agrees with previous studies that found no difference in replication between EV-WT and the p28^- mutant EV following infection of a variety of cell culture lines (Senkevich et al., 1994, 1995) and with our observation that over-expression of SFV N1R in VV-infected cells does not affect replication (data not shown). We have shown that UV treatment of HeLa cells infected with the p28^- mutant EV results in apoptosis, whereas cells infected by EV-WT are protected. These data suggest that the reduced virus yield of the p28^- mutant EV following exposure to UV may be a manifestation of inefficient suppression of apoptosis by the p28^- mutant EV, rather than a direct requirement for EVP28 in promoting virus replication.

Discussion

Detection of virus-infected cells by induction of apoptosis is an important host-defence mechanism that may serve to limit virus replication and spread within tissues of the host (Teodoro & Branton, 1997). Previously, overexpression of SFV N1R RING finger protein was shown to delay the onset and reduce the extent of apoptosis in the context of VV infection of BGMK cells. We have now shown that both SFV N1R and EVP28 proteins protect virus-infected HeLa cells from UV-induced apoptosis, but not from apoptosis induced through TNF or the Fas receptor. This result not only indicates a specific role for this poxviral protein in modulation of UV-induced apoptosis pathways, but further supports the idea that the pathways involved in UV-induced apoptosis are at least distinct from those involved in Fas- or TNF-induced apoptosis (Green, 1998).

In addition, we have found that EVP28 appears to act upstream of caspase-3, since it suppresses procaspase-3 activation in response to UV treatment. Thus, EVP28 likely functions during the UV signalling phase of apoptosis at or before caspase activation. UV irradiation has been shown to promote cytochrome c release from mitochondria in coordination with caspase-3 activation, events that are prevented by Bcl-2 expression (Knuck et al., 1997). It will be interesting to determine whether EVP28 infringes on mitochondrial/Bcl-2 apoptotic pathways. Additionally, it is evident from our apoptotic assays that VV and EV infection protect the infected cell from induction of apoptosis by TNF, anti-Fas and UV light. This is testament to the combined efforts of several documented poxviral anti-apoptotic proteins to target various control molecules in apoptosis pathways (reviewed in McFadden & Barry, 1998).

Whether p28 is directly anti-apoptotic in response to UV-induced pathways is, however, currently unknown, since we have been unable to express this protein in tissue culture cells in the absence of virus infection. Another important question is whether the protection from UV-induced apoptosis observed here plays a role in protecting the virus-infected cell from environmental UV damage in vivo, or whether as yet unidentified functions of EVP28 traverse UV-induced molecular apoptotic pathways. It is, however, interesting that the defect in virulence of the p28^- mutant EV resulted in a failure of the mutant virus to spread from the skin, a major portal of poxvirus entry in natural infections (Buller & Palumbo, 1991) and notably the primary organ affected by UV light (Griffiths et al., 1998). It is possible that this poxviral RING finger protein may promote replication and spread of the virus in the skin by protecting the infected cell and/or viral DNA from the effects of UV light. EV infection and pathogenicity in mice have been shown to be affected by environmental conditions (Roberts, 1964a) and the identification of both a cyclobutane pyrimidine dimer photolyase encoded by Melanoplus sanguinipes en- tomopoxvirus (Afonso et al., 1999) and a novel molluscum...
contagiosum virus antioxidant selenoprotein (Shisler et al., 1998) that protects cells from UV- or peroxide-induced damage suggests that poxviruses have indeed evolved to cope with, and are subject to, environmentally induced genotoxic stress.

The EV RING finger protein has been described as being critical for the replication of this virus in resident murine peritoneal macrophages (Senkevich et al., 1995), cells that are prone to significant inherent levels of apoptosis (Papadimitriou et al., 1980; Papadimitriou & van Bruggen, 1993). However, several EV strains that are attenuated in vivo also undergo restricted replication (Roberts, 1964b) in these cells, as does VV (Natuk & Holowczak, 1985). It is striking that both reports of abortive virus infection of these macrophages resulted in detection of some early viral protein expression but undetectable viral DNA replication (Natuk & Holowczak, 1985; Senkevich et al., 1995). The reported defect in replication of the p28− mutant EV may be indicative of apoptosis of the infected macrophages, since the VV study reported that the VV-infected macrophages underwent a marked generalized cytopathic effect, becoming highly vacuolated, granular, rounded and detached from the substratum; in effect, displaying symptoms of apoptosis (Natuk & Holowczak, 1985). Clearly, inefficient virus-induced suppression of apoptosis of these infected cells would lead to the observed diminished virus replication of the p28− mutant EV reported in these macrophages.

Since EVp28 is not required for replication of EV in tissue culture and the gene is absent from VV-Copenhagen, it is difficult to conceive of a direct role for this protein in poxvirus replication. While the DNA-binding activity of this group of poxviral proteins may be required as an accessory factor for DNA replication or transcription in certain cell types, it is also possible that the requirement for these poxviral RING finger proteins in virus replication stems from a role in suppression of apoptosis, such as was proposed for the baculovirus apoptosis inhibitor p35 (Lu & Miller, 1995). We envisage that p28 facilitates replication of the virus by protecting the infected cell from apoptosis. This hypothesis is supported by findings reported here, which show reduced yields of the p28− mutant EV compared with EV-WT after UV treatment of virus-infected cells. Since poxvirus infection probably suppresses expression of cellular UV-protective genes, the synthesis of a virus-encoded UV-protective molecule may provide a selective advantage in those infected cells that are subject to UV stress in vivo. In this respect, several correlations between poxvirus-encoded anti-apoptosis proteins and virulence in vivo have been documented (Barry et al., 1997; Messud-Petit et al., 1998; Mossman et al., 1996; Sedger & McFadden, 1996).

There is a positive correlation between protection from UV-induced apoptosis by EVp28 and localization of this viral protein to the virus DNA replication factories, since the p28− mutant EV synthesizes a non-factory-associated RING-truncated p28 protein (Senkevich et al., 1995). While our results point to a role for the RING finger region in suppression of UV-induced apoptosis, mutagenesis studies on the localization of N1R to the virus factories indicate that the RING finger region may primarily fulfil a structural role (D. J. Brick, R. D. Burke & C. Upton, unpublished result). It is noteworthy that the UV wavelengths (peak 302 nm) used in our experiments lie within the UV-B region, which is generally believed to exert cytotoxic effects through direct DNA damage, primarily the formation of cyclobutane pyrimidine dimers, 6-4 photoproducts and thymine glycols (Griffiths et al., 1998), and that soluble extracts of SFV N1R MAb have been shown previously to bind DNA cellulose (Brick et al., 1998). It is tempting to speculate that the poxvirus RING finger proteins may function by regulating the cellular response to DNA damage rather than having a direct role in a repair process. Through binding and sequestering viral DNA, these proteins may somehow prevent the activation of cellular DNA-damage sensors such as p53 in response to the presence in the cytoplasm of poxvirus replication intermediates. In such a scenario, EVp28 may function analogously to the VV E3L dsRNA-binding protein, which inhibits apoptosis through blocking activation of interferon-induced protein kinase by dsRNA (Kibler et al., 1997; Rivas et al., 1998).

No close homologues of the poxviral RING finger protein have been found in database searches. Therefore, the origin of these proteins remains obscure; however, it is likely that they were acquired by an ancestral poxvirus from a host cell. EVp28 contains a carboxy-terminal RING finger motif but it is not a baculovirus IAP homologue (Crook et al., 1993), since it lacks the baculovirus IAP repeat sequences. Although epidemiological evidence strongly suggests a link between UV irradiation from sunlight and epidermal neoplasms (Brash et al., 1996; Elder, 1989; Rundel & Nachtwey, 1978), the molecular pathways involved in UV-induced apoptosis have unfortunately remained largely fragmented. We are hopeful that future research on this poxviral RING finger virulence protein may prove a useful tool to permit elucidation of the precise nature of these processes.

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References


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