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Protection against Apoptosis by the Vaccinia Virus SPI-2 (B13R) Gene Product

MATTHIAS DOBBELSTEIN* AND THOMAS SHENK

Howard Hughes Medical Institute, Department of Molecular Biology, Princeton University, Princeton, New Jersey 08544-1014

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Vaccinia virus contains a gene, termed SPI-2 or B13R, that is closely related in its sequence to a potent inhibitor of apoptosis from cowpox virus (*crmA*). Infection by vaccinia virus protects HeLa cells against apoptosis that is induced by an immunoglobulin M antibody against the fas receptor or by tumor necrosis factor alpha. This effect is profoundly reduced when the SPI-2 gene is deleted. The SPI-2 gene, when transiently expressed in these cells, can also protect against apoptosis mediated by these agents. Given the similarity to *crmA*, it seems likely that SPI-2 functions in an analogous fashion, inhibiting the activity of ICE protease family members and blocking the onset of apoptosis.

Apoptosis, or programmed cell death, has been characterized as an important mechanism not only in the development of multicellular organisms but also in the cytotoxic immune response. To be eliminated by a cytotoxic T cell, the target cell must actively participate in its own death (3). Consequently, a virus can interfere with the immune response by modulating the host cell's apoptotic mechanisms. Many viruses have been found to interfere with programmed cell death or apoptosis (27). Viral gene products preventing apoptosis have been identified in adenovirus (35), cytomegalovirus (37), baculovirus (2), Epstein-Barr virus (11, 12), and others. The *crmA* gene of cowpox virus is known to specifically inhibit T-cell-mediated cytotoxicity (33). *crmA* also prevents apoptosis induced by tumor necrosis factor alpha (TNFα) and by immunoglobulin M (IgM) antibodies against the fas receptor (8, 20, 30, 31), which can mimic aspects of T-cell-mediated cytotoxicity. *crmA* inhibits several proteases that are believed to be part of pathways leading to apoptosis (16, 25, 26, 32).

A gene of vaccinia virus, called SPI-2 or B13R, is highly homologous to the cowpox virus gene *crmA*. A second vaccinia virus gene, SPI-1 or B22R, is more weakly homologous to both *crmA* and SPI-2. All three belong to the class of proteins termed serpins (a family of proteins comprising serine protease inhibitors) (17, 28). However, it has been unclear whether vaccinia virus is capable of preventing apoptosis. In fact, this virus induces apoptotic cell death in nonpermissive Chinese hamster ovarian cells (13); and vaccinia virus infection can lead to lymphopenia in mice that has been ascribed to programmed cell death (10). To clarify the biological role of vaccinia virus with respect to apoptosis, we tested whether vaccinia virus infection or transient expression of the SPI genes will interfere with apoptosis induction as does *crmA*.

Semiconfluent HeLa cells (American Type Culture Collection) were infected with the purified Western Reserve (WR) strain of vaccinia virus (kindly provided by Bernard Moss and Carl J. Baldick) at 100 PFU (assayed on BSC-1 cells as previously described [19]) or mock infected in Dulbecco modified Eagle medium containing 10% fetal bovine serum. After 14 h, the cells were challenged with a monoclonal IgM antibody against the fas receptor (CH-11; Medical and Biological Laboratories; 50 ng/ml) in the same medium with cycloheximide (40 μg/ml), or TNFα (Boehringer-Mannheim; 10 ng/ml) and cycloheximide, or cycloheximide alone. Eight hours later, most noninfected cells treated with anti-fas antibody (Fig. 1c) or TNFα (Fig. 1e) had undergone morphological changes typical for apoptosis, whereas the cells treated with cycloheximide alone did not exhibit any visible changes (Fig. 1a). In contrast, the vast majority of the vaccinia virus-infected cells were not affected by the treatment with anti-fas antibody (Fig. 1d) or TNFα (Fig. 1f) and displayed a morphology virtually undistinguishable from that of cells treated with cycloheximide alone (Fig. 1b). A terminal desoxyribonucleotidyltransferase-mediated dUTP-biotin nick-end-labeling (TUNEL) assay for DNA fragmentation (37) was performed and revealed that the noninfected (Fig. 2a to c) but not the infected (Fig. 2d to f) cells had undergone apoptosis when treated with anti-fas antibody or TNFα and cycloheximide as described above. We conclude that infection with vaccinia virus efficiently protects HeLa cells against apoptosis mediated by fas or TNFα.

Vaccinia virus rapidly shuts off host cell protein synthesis. To address whether this alone might influence the response of the infected cells to an inducer of apoptosis, we pretreated cells with an inhibitor of protein synthesis (cycloheximide at 40 μg/ml) or RNA synthesis (actinomycin D at 0.5 μg/ml) 14 h before induction of apoptosis. As shown in Fig. 2m through r, this pretreatment did not prevent apoptosis. Fas-mediated apoptosis was even accelerated in these cells, whereas TNF-mediated apoptosis was slightly delayed by both cycloheximide and actinomycin D (not shown). Interestingly, pretreatment with actinomycin D, but not with cycloheximide, increased the frequency of spontaneous apoptosis in the absence of any specific inducer (Fig. 2p), as has been reported for insect cells (6). On the basis of these controls, we conclude that vaccinia virus inhibits apoptosis not merely by a shutdown of protein synthesis but rather by a mechanism that is more specifically directed against programmed cell death.

Our next goal was to identify specific genes within vaccinia virus that mediate the protective effect of the viral infection against apoptosis. The inhibitory effect of vaccinia virus infection on apoptosis was not affected by the addition of an inhibitor of viral DNA replication, cytosine-arabinoside (14), to the culture media at a concentration of 40 μg/ml at the time of viral infection (Fig. 2s to x). Further, the inhibition of apoptosis can already be observed at 2 h (but not yet at 1 h) after

* Corresponding author. Phone: (609) 258-5993. Fax: (609) 258-1704. Electronic mail address: mdobbelstein@watson.princeton.edu.

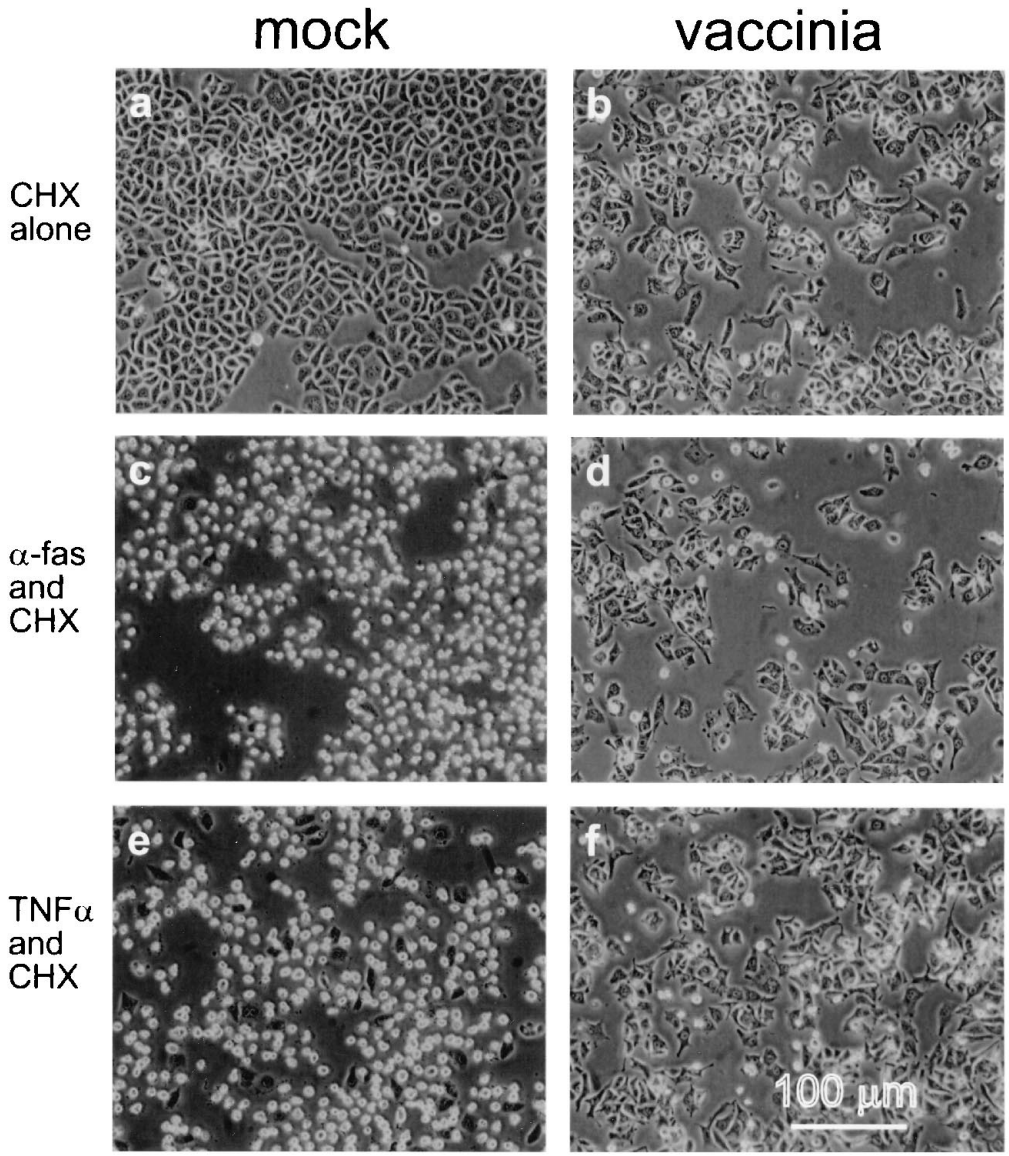

FIG. 1. Protection against apoptosis by vaccinia virus infection. HeLa cells were infected with vaccinia virus strain WR (b, d, and f) or were mock infected (a, c, and e). Fourteen hours later, apoptosis was induced with anti-fas antibody and cycloheximide (c and d) or TNFα and cycloheximide (e and f). As a control, cells were treated with cycloheximide alone (a and b). After 8 h, the cells were photographed using phase-contrast microscopy. Morphologic changes indicative of apoptosis could be observed in the vast majority of the mock-infected cells (c and e) but only rarely in the infected cells (d and f).

infection, and the effect reaches its maximum at 4 h after infection (not shown). This suggests that the effect is mediated by one or several early gene products. Because the cowpox *crmA* has been shown to have an antiapoptotic effect, the best candidate genes within vaccinia virus were SPI-2 and SPI-1: SPI-2 is 93% identical and 97% similar to *crmA* at the amino acid level; SPI-1 is about 40% identical to both *crmA* and SPI-2 (based on the amino acid sequence of the gene products). Both SPI-1 and SPI-2 are expressed early after infection (15, 28). To test whether SPI-2 contributes to apoptosis inhibition, a recombinant virus lacking SPI-2 and its revertant (generous gift from G. L. Smith [15]) were tested for the ability to prevent apoptosis. Most cells infected with the knockout mutant virus were induced to undergo apoptosis (Fig. 2g to i), and their light microscopic appearance was indistinguishable from that of noninfected apoptotic cells (not shown), whereas the cells infected by the revertant virus were protected (Fig. 2j to l). This indicates that SPI-2 is responsible for much of the antiapoptotic effect that is confered by vaccinia virus. However, even with the virus lacking SPI-2, some cells were protected from apoptosis, and this number clearly exceeded the surviving cells after mock infection (not shown). We therefore suggest that additional portions of the vaccinia virus genome can also contribute to apoptosis inhibition. In any case, our data show that SPI-2 can protect cells from undergoing apoptosis in the context of a vaccinia virus infection.

To determine whether the SPI-1 and SPI-2 gene products, like that of *crmA*, could protect against apoptosis in the absence of virus, the coding regions of all three genes were cloned into the eukaryotic expression vector pCI (Promega) to create the plasmids pCISPI-1, pCISPI-2, and pCIcrmA, respectively. The plasmids were constructed as follows. The cod-

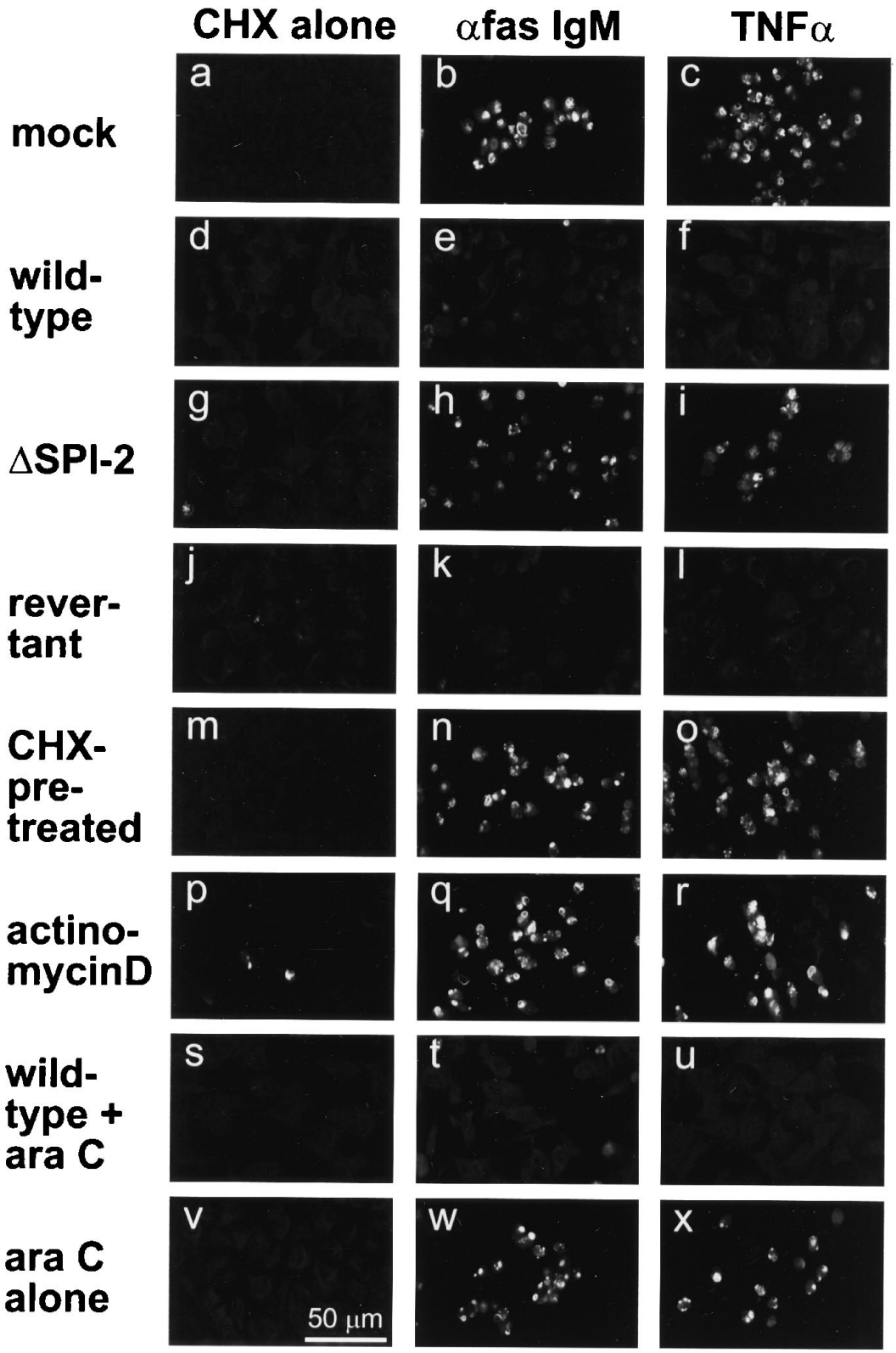

FIG. 2. DNA fragmentation after treatment with agents inducing apoptosis. HeLa cells were treated as described in the legend to Fig. 1 and subsequently assayed for DNA fragmentation using a TUNEL procedure. After infection, the cells were either treated with cycloheximide (CHX) alone, or with anti-fas antibody (αfas IgM), or with TNFα, each combined with cycloheximide. At 14 h before induction of apoptosis, the cells were treated in the following way: mock infection (a to c); infection with wild-type vaccinia virus, strain WR (d to f); infection with a recombinant vaccinia virus lacking the SPI-2 gene (g to i); infection with the revertant construct expressing SPI-2 (j to l); incubation with cycloheximide at 40 μg/ml (m to o); incubation with actinomycin D at 0.5 μg/ml (p to r); infection with wild-type vaccinia virus and simultaneous incubation with cytosine-arabinoside (ara C) at 40 μg/ml (s to u); mock infection and incubation with ara C (v to x).

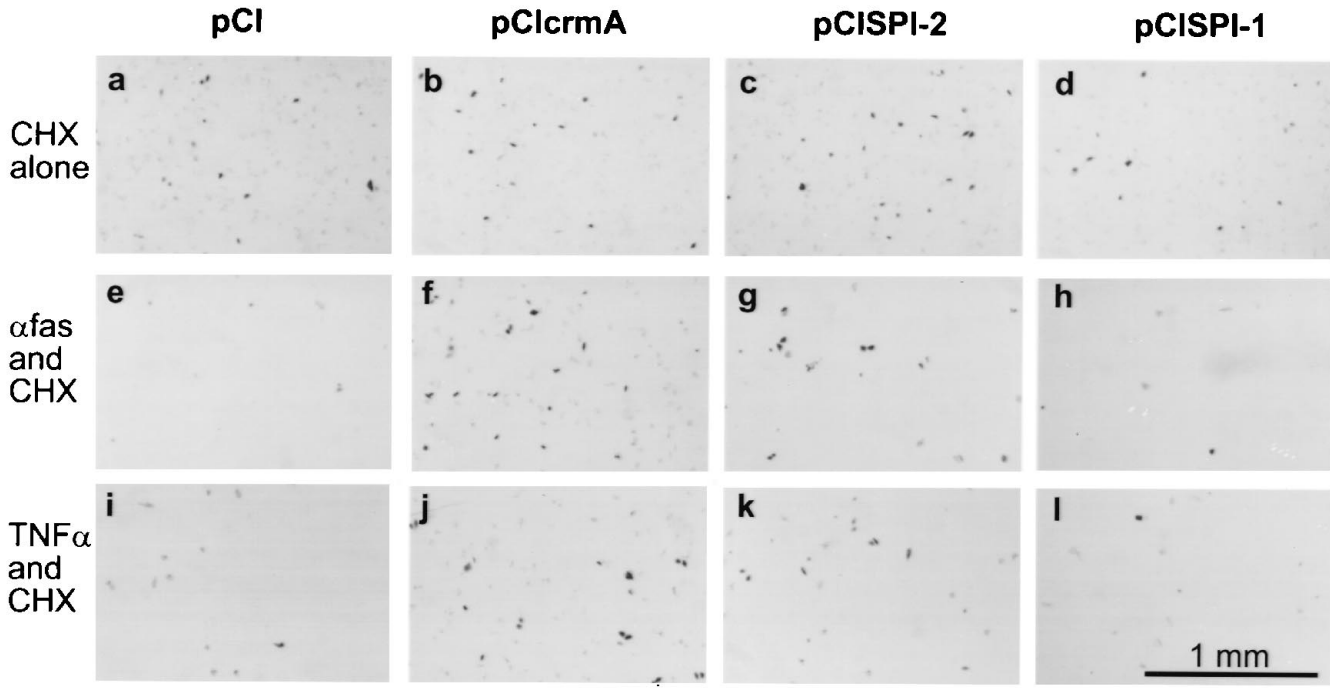

FIG. 3. Transfection of cells with plasmids expressing *crmA* or SPI-2 protects them from the induction of apoptosis with anti-fas antibody or TNFα. HeLa cells were cotransfected with a β-galactosidase expression construct and plasmids expressing *crmA* (pCIcrmA; b, f, and j), vaccinia virus SPI-2 (pCISPI-2; c, g, and k), vaccinia virus SPI-1 (pCISPI-1; d, h, and l), or the expression vector alone (pCI; a, e, and i). Each plasmid was cotransfected with an equal amount of a β-galactosidase expression construct (pCMVβ-gal; Stratagene). A suspension of 2×10^6 cells in 0.4 ml of Dulbecco modified Eagle medium–10% fetal bovine serum containing 10 μg of both plasmids in a cuvette of 0.4 cm gap width was subjected to a voltage pulse (230 V, 960 μF) using a Gene Pulser (Bio-Rad). The cells from one cuvette were allowed to attach to a dish of 3.5 cm in diameter. Fourteen hours after electroporation, the cells were treated with cycloheximide alone (a, b, c, and d) or with cycloheximide in combination with anti-fas antibody (e, f, g, and h) or TNFα (i, j, k, and l). Eight hours later, the dishes were washed four times. Cells remaining on the plate were stained for β-galactosidase expression using an X-Gal substrate.

ing regions for SPI-1 and SPI-2 were PCR amplified from viral genomic DNA isolated from purified vaccinia virus and the coding region for *crmA* from a plasmid containing this sequence (pHD1.2 [9], gift from J. Yuan). The primer sequences were: SPI-1, 5′ CGGAAATTCGTTATTGGTTTTGGTTGT ATACC; SPI-1, 3′ GCTCTAGATTTATTGCGGATAGCAG TATTTCC; SPI-2, 5′ CGGAATTCGCGCTACACGACCA ATATCG; SPI-2, 3′ GCTCTAAGATGGTTAACAATTAGT TGTCGGA; *crmA*, 5′ CGGAATTCGACTATCTCTATCG TCACAC; *crmA*, 3′ GCTCTAGATTTAATTAGTTGTTGG AGAGC. The PCRs were carried out with Expand high-fidelity DNA polymerase (Boehringer-Mannheim) according to the manufacturer's suggestions, with an annealing temperature of 62°C and a total of 30 cycles. The PCR products and the vector were treated with *Eco*RI and *Xba*I and ligated to create the plasmids pCISPI-1, pCISPI-2, and pCIcrmA, respectively. Using T7 RNA polymerase and a T7 promoter contained within the vector, the plasmids were used as templates in a coupled in vitro transcription and translation reaction (TNT system; Promega), yielding a single protein of the expected size in each case (data not shown).

Each plasmid was then cotransfected with an equal amount of a β-galactosidase expression construct (pCMVβ-gal; Stratagene) into HeLa cells. After 14 h of incubation, the cells were challenged with anti-fas antibody or with TNFα and cycloheximide as described above. Eight hours later, the dishes were washed four times with phosphate-buffered saline, removing most of the apoptotic cells. The cells that still attached to the plate were fixed and stained in situ for β-galactosidase activity with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (1). The number of blue survivors remaining on the dish thus indicated how efficiently a cotransfected plasmid can provide protection against apoptosis. After transfection of *crmA* or SPI-2 along with the β-galactosidase expression construct, a considerable number of cells remained adherent after treatment with either fas antibody (Fig. 3f and g) or TNFα (Fig. 3j and k). In contrast, the expression plasmid for SPI-1 did not protect transfected cells from apoptosis mediated by either fas antibody (Fig. 3h) or TNFα (Fig. 3l), nor did the vector pCI alone (Fig. 3e and i). Treatment with cycloheximide alone did not lead to apparent cell death, and the expression of β-galactosidase did not show substantial differences between the four constructs cotransfected with the β-galactosidase expression plasmid (Fig. 3a to d).

To address this phenomenon more quantitatively, the cells were first transfected and exposed to the apoptosis-inducing agents or cycloheximide alone as before. Once the nonadherent cells were removed, the remaining cells were harvested, lysed, and assayed in triplicate for β-galactosidase activity using a 1,2 dioxetane chemiluminescent substrate (Tropix) and a luminometer (Analytical Luminescence Laboratories) according to the manufacturers' recommendations. For all four plasmid combinations, the β-galactosidase activity that was retained after treatment with apoptosis inducers was calculated relative to the activity measured after treatment with cycloheximide alone. The cells cotransfected with the *crmA* expression plasmid retained 54% of the β-galactosidase activity after treatment with fas antibody (Fig. 4, column 2) and 33% after exposure to TNFα (Fig. 4, column 6). Expression of SPI-2 resulted in 31 and 15% retained activity, respectively (Fig. 4, columns 3 and 7). Expression of SPI-1 or cotransfection with

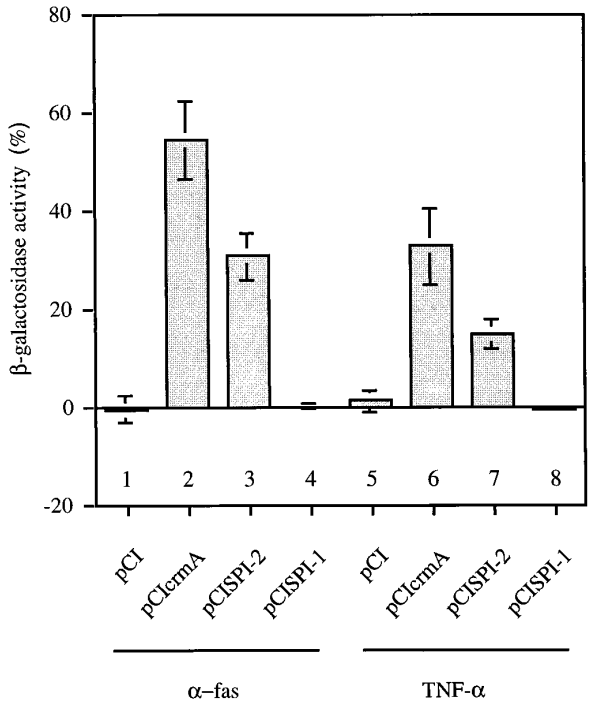

FIG. 4. Quantitative assessment of the ability of *crmA* and SPI-2 to block apoptosis. HeLa cells were cotransfected with a β-galactosidase expression construct and plasmids expressing *crmA* (pCIcrmA; columns 2 and 6), vaccinia virus SPI-2 (pCISPI-2; columns 3 and 7), vaccinia virus SPI-1 (pCISPI-1; columns 4 and 8), or the expression vector alone (pCI; columns 1 and 5). The cells were then treated with cycloheximide alone or in combination with anti-fas antibody (columns 1 to 4) or TNFα (columns 5 to 8). Nonadherent cells were removed by washing. Cells remaining on the plate were lysed, and the lysates were assayed quantitatively in triplicate for β-galactosidase activity, using a chemiluminescent substrate. For each plasmid, the ratio of β-galactosidase activity was calculated between the cells treated with anti-fas-cycloheximide or TNFα-cycloheximide and the cells treated with cycloheximide alone. The ratios are shown as columns representing percentages. The triple standard error is indicated by the bars.

the pCI vector did not allow any significant β-galactosidase activity to be retained (Fig. 4, columns 1, 4, 5, and 8).

These results were confirmed by a TUNEL assay that was performed along with simultaneous immunostaining using a monoclonal antibody against β-galactosidase (Promega) and a secondary antibody coupled to Texas red (Amersham), using methods described previously (7). When pCI (Fig. 5a and e) or pCISPI-1 (Fig. 5d and h) were cotransfected with the β-galactosidase expression plasmid and treated with anti-fas antibody (Fig. 5a to d) or TNFα (Fig. 5e to h), the transfected cells staining for β-galactosidase (labelled ".1") also showed DNA fragmentation (labelled ".2"). In contrast, after transfection with pCIcrmA (Fig. 5b and f) or pCISPI-2 (Fig. 5c and g), the signals indicating β-galactosidase and DNA fragmentation were mutually exclusive. Thus, both *crmA* and SPI-2 expression confer protection against apoptosis mediated by fas or TNFα. These data confirm that expression of either *crmA* or SPI-2 is protective against apoptosis, whereas SPI-1 or the expression

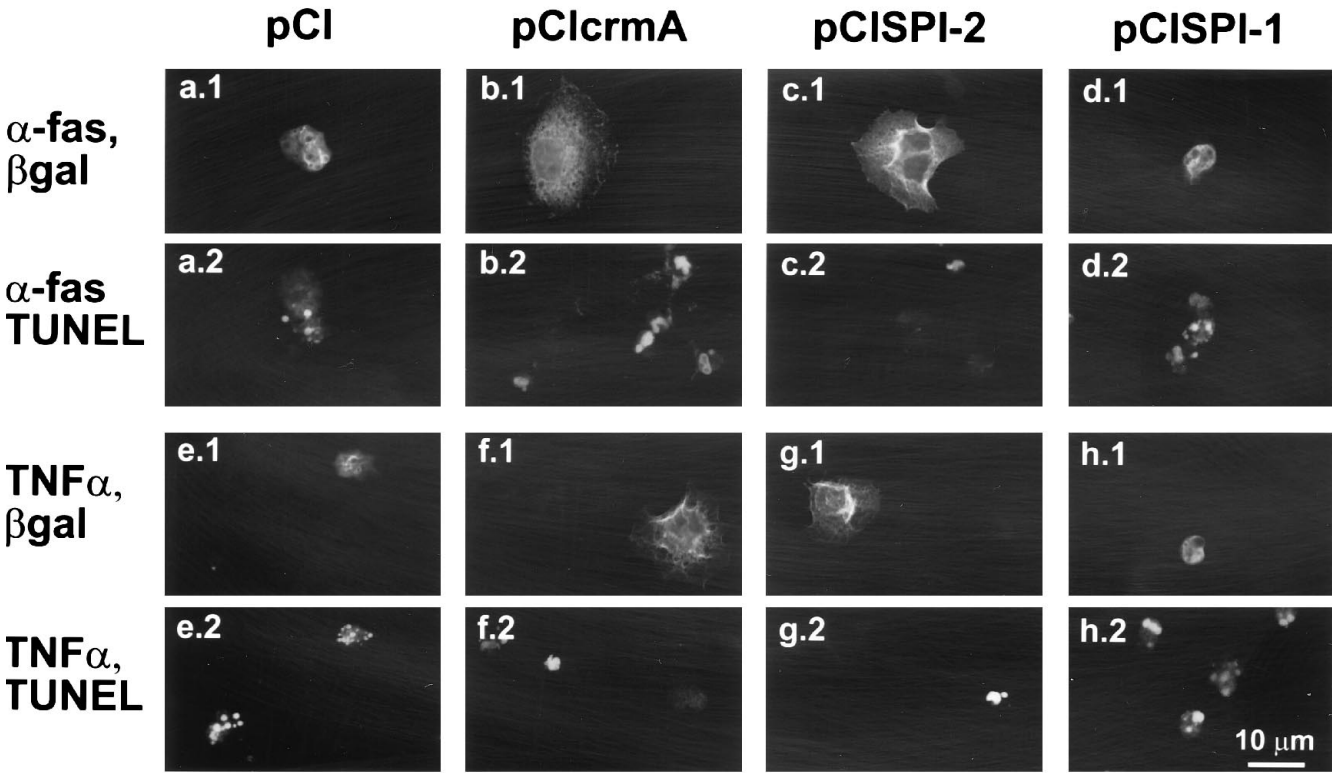

FIG. 5. SPI-2 or *crmA* blocks DNA fragmentation after treatment of cells with anti-fas antibody or TNFα. HeLa cells were cotransfected with a β-galactosidase expression construct and plasmids expressing *crmA* (pCIcrmA; b and f), vaccinia virus SPI-2 (pCISPI-2; c and g), vaccinia virus SPI-1 (pCISPI-1; d and h), or the expression vector alone (pCI; a and e). The cells were subsequently treated with cycloheximide in combination with anti-fas antibody (a, b, c, and d) or TNFα (e, f, g, and h). Subsequently, the cells were fixed and simultaneously stained for galactosidase expression (labelled ".1") and for DNA fragmentation (TUNEL; labelled ".2").

vector alone is without a detectable effect in this assay. SPI-2 may be somewhat less efficient than *crmA* but still can clearly be identified as an inhibitor of programmed cell death.

We have shown that vaccinia virus infection and SPI-2 alone are both capable of preventing fas- or TNFα-mediated apoptosis. Thus, the expression of SPI-2 is likely one way by which vaccinia virus can prevent apoptosis. However, it remains possible that the virus may employ more than just one mechanism to counteract this or other types of apoptosis. In fact, it is likely that poxviruses can influence cell death, immunologic response, and inflammatory reaction at many different levels, as exemplified by the secretion of an interleukin-1β binding protein (29) and as reviewed elsewhere (5, 23).

SPI-2 and *crmA* are highly homologous, including the region that is commonly believed to be active as a protease inhibitor (17). *crmA* inhibits a variety of proteases that are likely to be involved in the regulation of apoptosis, including the interleukin-1β-converting enzyme (ICE) (16, 18, 21, 26), Yama/CPP32β (32), and granzyme B (25). It will be of interest to determine if SPI-2 is capable of inhibiting the same subset of proteases or if there are differences in the repertoire of targets between SPI-2 and *crmA*.

SPI-1 did not confer protection against apoptosis mediated by fas or TNFα. This gene may be involved in the regulation of apoptosis mediated by other types of inducers. Alternatively, it may regulate cell fusion, as has been shown for the weakly homologous SPI-3 gene (34). The SPI-1 gene from rabbitpox virus has been demonstrated to inhibit cell death that otherwise can be induced by the viral infection itself (4). Further studies might show if vaccinia virus SPI-1 can substitute for the rabbitpox gene's function and what pathway, if any, leading to apoptosis might be modified by SPI-1.

During cowpox virus infection, *crmA* is responsible for the development of hemorrhage in the virus-induced lesions, and viruses lacking the *crmA* gene do not produce hemorrhage in infected animals (24). In contrast, no change in phenotype could be observed in a murine intranasal model when the SPI-2 gene was deleted from vaccinia virus (15). However, it remains possible that the choice of animal model affects the outcome of experiments aimed at finding phenotypical consequences of an SPI-2 deletion.

The activation of *fas*, along with the perforin-granzyme pathway, is believed to be a major mechanism for T-cell-mediated cytotoxicity (3). The finding that vaccinia virus can inhibit fas-mediated apoptosis makes it likely that it can suppress the immune response against an infected cell. This assumption is supported by the apparent functional parallelism between SPI-2 and *crmA*, since *crmA* has already been directly shown to prevent the killing of infected cells by cytotoxic T cells (33). This is of practical relevance, since vaccinia virus has been widely used as a vaccine against smallpox and shows promise as a vector for recombinant vaccines against different targets (22). The deletion of both SPI-1 and SPI-2 has been reported to result in a suppressed antibody response in certain systems (36). It is conceivable that by inhibiting apoptosis of infected cells, the effectiveness of a viral vaccine can be modified.

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REFERENCES

1. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1987. Current protocols in molecular biology, vol. 1. John Wiley & Sons, Inc., New York.
2. **Beidler, D. R., M. Tewari, P. D. Friesen, G. Poirier, and V. M. Dixit.** 1995. The baculovirus p35 protein inhibits Fas- and tumor necrosis factor-induced apoptosis. J. Biol. Chem. **270:**16526–16528.
3. **Berke, G.** 1995. The CTL's kiss of death. Cell **81:**9–12.
4. **Brooks, M. A., A. N. Ali, P. C. Turner, and R. W. Moyer.** 1995. A rabbitpox virus serpin gene controls host range by inhibiting apoptosis in restrictive cells. J. Virol. **69:**7688–7698.
5. **Buller, R. M., and G. J. Palumbo.** 1991. Poxvirus pathogenesis. Microbiol. Rev. **55:**80–122.
6. **Crook, N. E., R. J. Clem, and L. K. Miller.** 1993. An apoptosis-inhibiting baculovirus gene with a zinc finger-like motif. J. Virol. **67:**2168–2174.
7. **Dobbelstein, M., A. K. Arthur, S. Dehde, K. van Zee, A. Dickmanns, and E. Fanning.** 1992. Intracistronic complementation reveals a new function of SV40 T antigen that co-operates with Rb and p53 binding to stimulate DNA synthesis in quiescent cells. Oncogene **7:**837–847.
8. **Enari, M., H. Hug, and S. Nagata.** 1995. Involvement of an ICE-like protease in Fas-mediated apoptosis. Nature (London) **375:**78–81.
9. **Gagliardini, V., P. A. Fernandez, R. K. Lee, H. C. Drexler, R. J. Rotello, M. C. Fishman, and J. Yuan.** 1994. Prevention of vertebrate neuronal death by the crmA gene. Science **263:**826–828. (See comments.)
10. **Gonzalo, J. A., A. Gonzalez-Garcia, T. Kalland, G. Hedlung, C. Martinez, and G. Kroemer.** 1994. Linomide inhibits programmed cell death of peripheral T cells in vivo. Eur. J. Immunol. **24:**48–52.
11. **Gregory, C. D., C. Dive, S. Henderson, C. A. Smith, G. T. Williams, J. Gordon, and A. B. Rickinson.** 1991. Activation of Epstein-Barr virus latent genes protects human B cells from death by apoptosis. Nature (London) **349:**612–614.
12. **Henderson, S., M. Rowe, C. Gregory, D. Croom-Carter, F. Wang, R. Longnecker, E. Kieff, and A. Rickinson.** 1991. Induction of bcl-2 expression by Epstein-Barr virus latent membrane protein 1 protects infected B cells from programmed cell death. Cell **65:**1107–1115.
13. **Ink, B. S., C. S. Gilbert, and G. I. Evan.** 1995. Delay of vaccinia virus-induced apoptosis in nonpermissive Chinese hamster ovary cells by the cowpox virus CHOhr and adenovirus E1B 19K genes. J. Virol. **69:**661–668.
14. **Keck, J. G., C. J. Baldick, and B. Moss.** 1990. Role of DNA replication in vaccinia virus gene expression: a naked template is required for transcription of three late trans-activator genes. Cell **61:**801–809.
15. **Kettle, S., N. W. Blake, K. M. Law, and G. L. Smith.** 1995. Vaccinia virus serpins B13R (SPI-2) and B22R (SPI-1) encode M(r) 38.5 and 40K, intracellular polypeptides that do not affect virus virulence in a murine intranasal model. Virology **206:**136–147.
16. **Komiyama, T., C. A. Ray, D. J. Pickup, A. D. Howard, N. A. Thornberry, E. P. Peterson, and G. Salvesen.** 1994. Inhibition of interleukin-1 beta converting enzyme by the cowpox virus serpin CrmA. An example of cross-class inhibition. J. Biol. Chem. **269:**19331–19337.
17. **Kotwal, G. J., and B. Moss.** 1989. Vaccinia virus encodes two proteins that are structurally related to members of the plasma serine protease inhibitor superfamily. J. Virol. **63:**600–606. (Erratum, **64:**966, 1990.)
18. **Los, M., M. Van de Craen, L. C. Penning, H. Schenk, M. Westendorp, P. A. Baeuerle, W. Droge, P. H. Krammer, W. Fiers, and K. Schulze-Osthoff.** 1995. Requirement of an ICE/CED-3 protease for Fas/APO-1-mediated apoptosis. Nature (London) **375:**81–83.
19. **Mackett, M., G. L. Smith, and B. Moss.** 1985. The construction and characterization of vaccinia virus recombinants expressing foreign genes, p. 191–211. *In* D. M. Glover (ed.), DNA cloning: a practical approach. IRL Press, Oxford.
20. **Miura, M., R. M. Friedlander, and J. Yuan.** 1995. Tumor necrosis factor-induced apoptosis is mediated by a CrmA-sensitive cell death pathway. Proc. Natl. Acad. Sci. USA **92:**8318–8322.
21. **Miura, M., H. Zhu, R. Rotello, E. A. Hartwieg, and J. Yuan.** 1993. Induction of apoptosis in fibroblasts by IL-1 beta-converting enzyme, a mammalian homolog of the C. elegans cell death gene ced-3. Cell **75:**653–660.
22. **Moss, B.** 1991. Vaccinia virus: a tool for research and vaccine development. Science **252:**1662–1667.
23. **Palumbo, G. J., R. M. Buller, and W. C. Glasgow.** 1994. Multigenic evasion of inflammation by poxviruses. J. Virol. **68:**1737–1749.
24. **Pickup, D. J., B. S. Ink, W. Hu, C. A. Ray, and W. K. Joklik.** 1986. Hemorrhage in lesions caused by cowpox virus is induced by a viral protein that is related to plasma protein inhibitors of serine proteases. Proc. Natl. Acad. Sci. USA **83:**7698–7702.
25. **Quan, L. T., A. Caputo, R. C. Bleackley, D. J. Pickup, and G. S. Salvesen.** 1995. Granzyme B is inhibited by the cowpox virus serpin cytokine response modifier A. J. Biol. Chem. **270:**10377–10379.
26. **Ray, C. A., R. A. Black, S. R. Kronheim, T. A. Greenstreet, P. R. Sleath, G. S. Salvesen, and D. J. Pickup.** 1992. Viral inhibition of inflammation: cowpox

virus encodes an inhibitor of the interleukin-1 beta converting enzyme. Cell **69:**597–604.
27. **Shen, Y., and T. E. Shenk.** 1995. Viruses and apoptosis. Curr. Opin. Genet. Dev. **5:**105–111.
28. **Smith, G. L., S. T. Howard, and Y. S. Chan.** 1989. Vaccinia virus encodes a family of genes with homology to serine proteinase inhibitors. J. Gen. Virol. **70:**2333–2343.
29. **Spriggs, M. K., D. E. Hruby, C. R. Maliszewski, D. J. Pickup, J. E. Sims, R. M. Buller, and J. VanSlyke.** 1992. Vaccinia and cowpox viruses encode a novel secreted interleukin-1-binding protein. Cell **71:**145–152.
30. **Talley, A. K., S. Dewhurst, S. W. Perry, S. C. Dollard, S. Gummuluru, S. M. Fine, D. New, L. G. Epstein, H. E. Gendelman, and H. A. Gelbard.** 1995. Tumor necrosis factor alpha-induced apoptosis in human neuronal cells: protection by the antioxidant *N*-acetylcysteine and the genes *bcl-2* and *crmA*. Mol. Cell. Biol. **15:**2359–2366.
31. **Tewari, M., and V. M. Dixit.** 1995. Fas- and tumor necrosis factor-induced apoptosis is inhibited by the poxvirus crmA gene product. J. Biol. Chem. **270:**3255–3260.
32. **Tewari, M., L. T. Quan, K. O'Rourke, S. Desnoyers, Z. Zeng, D. R. Beidler, G. G. Poirier, G. S. Salvesen, and V. M. Dixit.** 1995. Yama/CPP32 beta, a mammalian homolog of CED-3, is a CrmA-inhibitable protease that cleaves the death substrate poly(ADP-ribose) polymerase. Cell **81:**801–809.
33. **Tewari, M., W. G. Telford, R. A. Miller, and V. M. Dixit.** 1995. CrmA, a poxvirus-encoded serpin, inhibits cytotoxic T-lymphocyte-mediated apoptosis. J. Biol. Chem. **270:**22705–22708.
34. **Turner, P. C., and R. W. Moyer.** 1992. An orthopoxvirus serpinlike gene controls the ability of infected cells to fuse. J. Virol. **66:**2076–2085.
35. **White, E., P. Sabbatini, M. Debbas, W. S. Wold, D. I. Kusher, and L. R. Gooding.** 1992. The 19-kilodalton adenovirus E1B transforming protein inhibits programmed cell death and prevents cytolysis by tumor necrosis factor alpha. Mol. Cell. Biol. **12:**2570–2580.
36. **Zhou, J., L. Crawford, L. McLean, X. Y. Sun, M. Stanley, N. Almond, and G. L. Smith.** 1990. Increased antibody responses to human papillomavirus type 16 L1 protein expressed by recombinant vaccinia virus lacking serine protease inhibitor genes. J. Gen. Virol. **71:**2185–2190.
37. **Zhu, H., Y. Shen, and T. Shenk.** 1995. Human cytomegalovirus IE1 and IE2 proteins block apoptosis. J. Virol. **69:**7960–7970.