Characterization of the ectromelia virus serpin, SPI-2

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Ectromelia virus (EV) is a member of the Orthopoxviridae family of viruses and is a natural pathogen of mice. In resistant mice, EV causes an acute infection, requiring inflammatory and CD8+ T cell responses for effective resolution of the virus (Blanden, 1970; Karupiah et al., 1993a, b, 1996; Mullbacher et al., 1992; Ramshaw et al., 1997). Poxviruses have evolved numerous strategies to subvert these antiviral responses, including a family of proteins homologous to mammalian serine protease inhibitors (serpins) (Buller & Palumbo, 1991; Turner & Moyer, 1998). The first viral serpin described was a 38 kDa protein encoded by cowpox virus (CPV) called cytokine response modifier A (crmA), also known as SPI-2 (Palumbo et al., 1989; Pickup et al., 1986). Modulation of the host response by crmA was demonstrated in chorioallantoic membranes of embryonated eggs infected with CPV (Pickup et al., 1986). Infection with crmA-mutant CPV resulted in the conversion of the usually observed red pox into white lesions due to the recruitment of leukocytes, suggesting that crmA down-regulated the inflammatory response. This effect on inflammation is likely to result from the inhibition of interleukin-1 (IL-1) processing, since crmA inhibits the activation of the cysteine protease IL-1-β-converting enzyme (ICE; caspase-1) (Ray et al., 1992) which converts pro-IL-1 into the active inflammatory mediator IL-1β (Cerretti et al., 1992; Thornberry et al., 1992).

Highly conserved homologues of crmA/SPI-2 have been identified in rabbitpox virus (RPV), variola virus (Var) and vaccinia virus (VV; Western Reserve strain) (Kettle et al., 1997), suggesting their importance for virus propagation. To determine whether EV encodes a functional SPI-2 gene, DNA was amplified from EV [Moscow strain; originally cloned from Mos-3-P1 and kindly provided by R. M. L. Buller (Chen et al., 1992)] by PCR using oligonucleotides 5′ CGGATATCATGG-GACCTGATAC 3′, containing an EcoRV site (underlined), and 5′ GCCGAGAATTAGTGGAGAGCAATATC 3′, containing an XhoI site (underlined). The primers were based on EV SPI-2 sequences submitted to GenBank (accession nos AJ007935 and AJ244012). The PCR product was digested with EcoRV and XhoI and ligated into the expression vector pEF FLAG (Huang et al., 1997; Newton et al., 1998), digested with the same restriction enzymes. This enabled the in-frame fusion of the FLAG epitope to the N terminus of SPI-2 to form pEF SPI-2. The pEF SPI-2 construct was sequenced using ABI PRISM BigDye terminator chemistry (PE Biosystems) and oligonucleotides 5′ CAAGCTTCGAGGACATCTTCAGGGAG 3′ and 5′ ACATCTTCAGGGAGACCTGATAC 3′, containing an EcoRV site (underlined), and 5′ GCCGAGAATTAGTGGAGAGCAATATC 3′, containing an XhoI site (underlined). The primers were based on EV SPI-2 sequences submitted to GenBank (accession nos AJ007935 and AJ244012). The PCR product was digested with EcoRV and XhoI and ligated into the expression vector pEF FLAG (Huang et al., 1997; Newton et al., 1998), digested with the same restriction enzymes. This enabled the in-frame fusion of the FLAG epitope to the N terminus of SPI-2 to form pEF SPI-2. The pEF SPI-2 construct was sequenced using ABI PRISM BigDye terminator chemistry (PE Biosystems) and oligonucleotides 5′ CAAGCTTCGAGGACATCTTCAGGGAG 3′ and 5′ ACATCTTCAGGGAGACCTGATAC 3′. At least three different PCR clones were sequenced. Sequencing revealed that EV SPI-2 contains an open reading frame of 344 amino acids. BLAST comparisons of EV SPI-2 showed approximately 95% identity at the DNA and protein level to SPI-2 homologues from VV and RPV and about 92% identity with Var and CPV versions. Sequence variation between EV SPI-2 and other poxviruses occurs within the region spanning amino acids 45–64 (Fig. 1a). CPV crmA differs most from EV SPI-2 within the 10 amino acids from positions 50–60 including three deletions at positions 51, 52 and 55. There are four amino

Poxviruses encode multiple proteins that enable them to evade host responses. Among these are serine protease inhibitors (serpins). One of the earliest serpins described, cowpox virus crmA, acts to inhibit inflammation and apoptosis. crmA homologues, known as SPI-2, are conserved in rabbitpox, vaccinia and variola viruses. Here, we describe the characterization of ectromelia virus (EV) SPI-2. EV SPI-2 encodes a protein of approximately 38 kDa showing >94% identity with other poxviral homologues. Conservative changes in amino acid sequence were found within the reactive site loop and the serpin backbone. Like crmA, transient expression of SPI-2 protected cells from tumour necrosis factor-mediated apoptosis and inhibited the activity of caspases-1 and -8 but not caspases-3, -6 or granzyme B. Overall, this study demonstrates that EV SPI-2 is functionally similar to crmA, based on in vitro assays.
The inhibitory activity of crmA for other caspases also suggests that crmA may block apoptosis of infected cells in vivo. Indeed, crmA blocks apoptosis mediated by the tumor necrosis factor (TNF) receptor family members TNF receptor type 1 (TNFR1) and Fas in vitro (Turner & Moyer, 1998). Apoptosis via these receptors depends on the recruitment and activation of caspase-8 (Boldin et al., 1996; Chinnaiyan et al., 1995, 1996; Hsu et al., 1995, 1996; Muzio et al., 1996; Tartaglia et al., 1993). The resulting aggregation of caspase-8 results in autoactivation and the initiation of a caspase cascade that ultimately leads to apoptosis. crmA has been shown to inhibit caspase-8 as well as caspase-1 (Ekert et al., 1999; Garcia-Calvo et al., 1998), and to inhibit TNF- and Fas-dependent apoptosis (Muzio et al., 1996; Zhou et al., 1997). Thus, crmA may target both the inflammatory and apoptotic responses of infected hosts.

To determine whether EV SPI-2 is functionally equivalent to crmA, the antiapoptotic activity and caspase specificities of EV SPI-2 were investigated. The vectors used for FLAG epitope-tagged protein expression, pEF FLAG, pEF-FLAG-Bcl-2/puro and pEF-FLAG-crmA/puro, were a kind gift from David Huang and Andreas Strasser and have been described previously (Huang et al., 1997; Newton et al., 1998). Expression of EV SPI-2 was demonstrated by transfection of murine L929 cells (CSL, Melbourne, Australia; cultured in modified Eagle’s medium supplemented with 5% FCS, 2 mM L-glutamine, 10 mM HEPES, 10 µg/ml penicillin and streptomycin) with pEF-SPI-2 using Lipofectamine Plus reagent (Life Technologies) according to the manufacturer’s instructions. The cells were harvested 48 h after transfection for SDS–PAGE analysis. Protein blots were probed with anti-FLAG antibody (M2, 1:2000; Sigma) followed by biotinylated anti-mouse IgG (1:400; Amersham). The secondary antibody was detected using conjugated strepavidin–horseradish peroxidase (1:500; Sigma). The resulting bands were visualized using ECL (Amerham) according to the manufacturer’s instructions. Transfection of pEF SPI-2 resulted in the expression of a protein of approximately 38 kDa (Fig. 2a).

To examine the effect of EV SPI-2 on apoptosis, adherent L929 cells were co-transfected with the appropriate pEF FLAG expression vector and pCH110 for β-galactosidase expression (Pharmacia) at a ratio of 5:1. After 24 h, cells were incubated with 5 ng/ml of murine recombinant TNF (PeproTech) and 4 µg/ml actinomycin D (Sigma) for 16 h. Cells were then washed and fixed in 0.5% glutaraldehyde, 0.01% sodium deoxycholate and 0.02% NP40 in PBS for 20 min at 4 °C. β-Galactosidase activity was assayed by incubating cells in a solution containing 2 mM MgCl2, 0.01% sodium deoxycholate, 0.02% NP40. 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide and 1 mg/ml X-Gal. The number of cells undergoing TNF-mediated apoptosis was determined by...
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Fig. 2. EV SPI-2 encodes a product of approximately 38 kDa which inhibits TNF-dependent apoptosis. (a) EV SPI-2 was expressed as a FLAG-tagged protein in L929 cells and detected using SDS–PAGE electrophoresis and Western blotting as described. The migration of the SPI-2-FLAG product (lane 3) is compared to extracts from cells transfected with pEF FLAG (lane 1), pEF-bcl-2 (lane 2) or EV SPI-2 derived from yeast extracts (lane 4). (b) L929 cells were co-transfected with pCH110 (for β-galactosidase expression) and either pEF, pEF SPI-2 or pEF crmA in molar excess as described. After 24 h duplicate wells were either untreated or incubated with TNF and actinomycin D. Protection was measured as the percentage of the number of blue cells in the untreated wells as the number of blue cells in the TNF-treated wells as the number of blue cells in the untreated wells. Two representative experiments are shown.

In order to investigate the caspase-targeting specificity of EV-SPI-2, the ability of SPI-2 to protect yeast (Schizosaccharomyces pombe) expressing a range of autoactivating caspases or granzyme B was assessed. While yeast do not express any known caspase homologues, expression of various mammalian caspases in yeast is toxic and has been a productive approach to investigating caspase inhibitors (Ekert et al., 1999; Wang et al., 1999). Due to the lack of a signalling mechanism in yeast, most caspases require artificial stimuli in order to aggregate and hence autoactivate. Whereas caspase-8 naturally contains a pro-domain that facilitates auto-aggregation, caspases-1, -3 or -6 can alternately be auto-activated by either C-terminal fusion of the lacZ gene (Ekert et al., 1999), or other manipulations (Colussi et al., 1998; Srinivasula et al., 1998) which do not appear to alter the characteristics of any of the caspases tested (J. Silke, unpublished). A yeast protection assay which has been described previously (Ekert et al., 1999) was used to assess the target specificity of SPI-2. Briefly, S. pombe cells were transformed with a vector, pURAS-SPI-2, for constitutive SPI-2 expression and a second expression vector, pNEU KA, encoding various caspases under the control of a thiamin-repressible promoter. Yeast transformations were performed using a standard lithium acetate protocol, and clones were maintained on selective medium. Transformed yeast were plated in serial 10-fold dilutions onto either thiamin-containing or normal agar. Protection was measured by comparing growth on the two plates. Expression of EV SPI-2 in yeast was not toxic (Fig. 3b), and the SPI-2 product expressed by yeast was indistinguishable from that of mammalian cells by Western blotting (Fig. 2a). When expression of caspases was induced, SPI-2 expression inhibited caspase-1 and caspase-8 equally well (Fig. 3a), and yeast replicated to the same extent as when the caspases were not induced (Fig. 3b). This is consistent with reports showing that crmA has high affinity for both these caspases, implicating them as the in vivo targets of crmA (Garcia-Calvo et al., 1998; Zhou et al., 1997). In contrast, EV SPI-2 did not protect yeast expressing caspases-3 or -6 or granzyme B (Fig. 3a). When the effects of crmA and EV SPI-2 were compared in the same assays, both serpins equally and potently protected yeast against the toxicity of caspases-1 and -8 (Fig. 3c), and neither inhibited granzyme B activity. Comparison of the inhibitor kinetics for several recombinantly produced caspases showed that crmA inhibits caspases-1 and -5 most potently, followed by caspase-8, with K_i values in the pM range (Garcia-Calvo et al., 1998; Zhou et al., 1997). CrmA also inhibits caspases-4, -5, -9 and -10. However, based on binding kinetics and affinity, caspases-3, -6 or -7 do not appear likely targets of crmA. Thus, the profile of caspase inhibitory activity for EV SPI-2 and crmA, as measured by the yeast survival assay, is the same and matches, in a qualitative fashion, the K_i values previously obtained for crmA. As EV SPI-2 did not inhibit yeast toxicity induced by the activation of caspase-6 (Fig. 3a), it is unlikely to be a physiological target despite an affinity between caspase-6 and crmA (Zhou et al., 1997).

Cytotoxic T lymphocytes (CTL) are critical for the resolution of poxvirus infection (Blanden, 1970) and granule-mediated apoptosis is the major apoptotic pathway used by CTL and NK cells to kill virus-infected cells (Mullbacher et al., 1999a). The granule protein, granzyme B, mediates apoptosis via direct activation of caspases, especially caspases-7 and -10. Granzyme B is a serine protease (Smyth & Trapani, 1995) which has been shown to be inhibited by crmA (Quan et al., 1995; Martin et al., 1996), leading to the hypothesis that poxviruses may also target CTL-mediated apoptosis of infected cells. Inhibition of granzyme B-induced activation of CPP32 and apoptosis in a cell-free apoptotic nuclei assay was also observed (Martin et al., 1996). However, crmA is a much more potent inhibitor of caspase-1 than either granzyme B or CPP32 (Quan et al., 1995; Martin et al., 1996).
Fig. 3. Inhibition of activity of various caspases by EV SPI-2 and crmA. Yeast cells constitutively expressing EV SPI-2 and autoactivating caspases-1, -8, -3, -6 or granzyme B under the control of a thiamin-repressible promoter were serially diluted and plated onto (a) normal or (b) thiamin-containing agar as described. (c) Comparison of the caspase inhibitory activity of SPI-2 and crmA. Yeast cells were transformed with vectors to enable constitutive expression of EV SPI-2, crmA or the empty vector (Vect) andcotransformed with autoactivating caspases-1, -8 or granzyme B. Yeast cells transformed with empty vector or granzyme B were not protected. No toxicity was seen when yeast expressed a catalytic pentapeptide mutant caspase (caspase-mut) in which QACRG has been converted to QAARG, or when yeast were plated on thiamin-containing media (non-inducing; lower panels).

Expression of EV-SPI-2 or crmA did not inhibit the toxic effects of granzyme B in yeast (Fig. 3b) suggesting that, despite the documented interaction of these molecules in vitro, the physiological significance of these interactions is less clear. Together, these results indicate that EV SPI-2 is more likely to inhibit CTL activity by targeting Fas-mediated apoptosis. In this regard, crmA was shown to block CTL-induced apoptosis by inhibiting a Ca\textsuperscript{2+}-independent pathway and not Ca\textsuperscript{2+}-dependent granule exocytosis (Tewari et al., 1995). Furthermore, a recent study showed that CPV crmA blocked CTL-mediated killing of alloreactive target cells by potently inhibiting Fas-dependent mechanisms (Mullbacher et al., 1999b). To a lesser extent, crmA also inhibited granzyme B-dependent apoptosis, although this was independent of granzyme B. Interestingly, no effect of crmA on virus-specific MHC class I-restricted CTL was observed.

In conclusion, the data presented show that EV encodes a full-length SPI-2 protein which appears functionally identical to CPV crmA. Data from this study and others show that SPI-2/crmA proteins selectively inhibit inflammatory and apoptotic responses, due to their inhibitory activity for specific caspases.

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