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Cowpox virus CrmA, *Myxoma virus* SERP2 and baculovirus P35 are not functionally interchangeable caspase inhibitors in poxvirus infections

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Cowpox virus (CPV) expresses the serpin (serine proteinase inhibitor) CrmA, an anti-inflammatory, anti-apoptotic protein required for production of red pocks on chicken chorioallantoic membranes (CAMs). *In vitro*, CrmA inhibits several caspases and granzyme B. Altering the critical P1-aspartate in the CrmA reactive centre loop to alanine resulted in a virus (CPV-CrmA-D303A) that resembled CPV deleted for CrmA (CPVΔCrmA::lacZ); on CAMs it produced white, inflammatory pocks with activated caspase-3 and reduced virus yields, suggesting that CrmA activities are mediated via proteinase inhibition. CrmA in CPV was replaced with SERP2 from *Myxoma virus* (MYX) or baculovirus P35, which inhibit similar proteinases *in vitro*. SERP2 and P35 each blocked caspase-3-mediated apoptosis but were unable to control inflammation of CAMs. However, SERP2 and P35 restored virus yields, indicating that the decreased virus titres seen with CPVΔCrmA::lacZ resulted from apoptosis rather than inflammation. To compare the activities of CrmA and SERP2 further, rabbits were infected with MYX recombinant viruses. Intradermal infection of rabbits with MYX was uniformly lethal, generating raised primary lesions and many secondary lesions. In contrast, deletion of SERP2 from MYX (MYXΔSERP2::lacZ) caused little mortality and produced flat primary lesions with few secondary lesions. Replacement of SERP2 with CrmA (MYXΔSERP2::CrmA) resulted in partial complementation with flat primary lesions, many secondary lesions and death in 70 % of the rabbits. Therefore, CrmA and SERP2 were not functionally interchangeable during infection of CAMs or rabbits, implying that these serpins have activities that are not evident from biochemical studies with human caspases.

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INTRODUCTION

Poxviruses are large, double-stranded DNA viruses that replicate within the cytoplasm of infected cells (Moss, 1996) and encode many proteins that deflect host immune responses (Moss & Shisler, 2001). Serpins (serine protease inhibitors) are included within a group of poxvirus proteins that are non-essential for growth in tissue culture. Serpins are found in plants, animals and bacteria (Silverman *et al.*, 2001; Irving *et al.*, 2002), but poxviruses are the only viruses known to encode functional serpins (Silverman *et al.*, 2001). The cytokine response modifier (CrmA) encoded by *Cowpox virus* (CPV) was the first poxvirus serpin described (Pickup *et al.*, 1986). Homologues of CrmA (SPI-2, B13R) exist in other orthopoxviruses.

Most serpins function as proteinase inhibitors, although some have functions (including hormone transport and regulation of blood pressure) that do not involve proteinase inhibition (Silverman *et al.*, 2001). Inhibitory serpins form a stable 1:1 complex with target enzymes. The interaction is mediated by the reactive centre loop (RCL) of the serpin (Silverman *et al.*, 2001). The specificity of a serpin is determined primarily by the P1 residue within the RCL. CrmA, with a P1 of aspartate (P1-Asp), is a cross-class inhibitor that targets the serine proteinase, granzyme-B (Quan *et al.*, 1995) and caspases, which are thiol-proteases (Komiyama *et al.*, 1994). CrmA is a potent inhibitor of caspase-1 (Komiyama *et al.*, 1994; Garcia-Calvo *et al.*, 1998), which processes pro-interleukin (IL)-1α, proIL-1β and proIL-18 to mature, proinflammatory cytokines (Thornberry *et al.*, 1992; Ghayur

et al., 1997; Gu *et al.*, 1997). The importance of IL-1β in controlling poxvirus infections is supported by the orthopoxvirus-encoded IL-1β receptor (Spriggs *et al.*, 1992; Alcami & Smith, 1992), which controls fever during vaccinia virus (VV) infection (Alcami & Smith, 1996; Kettle *et al.*, 1997). IL-18 is a factor that induces synthesis of IFN-γ. The significance of IL-18 and IFN-γ in poxvirus infections can be inferred because poxviruses encode binding proteins for IL-18 (Born *et al.*, 2000; Smith *et al.*, 2000; Calderara *et al.*, 2001; Xiang & Moss, 2001a, b; Symons *et al.*, 2002a; Reading & Smith, 2003) and IFN-γ (Upton *et al.*, 1992; Alcami & Smith, 1995; Mossman *et al.*, 1995; Verardi *et al.*, 2001; Smith & Alcami, 2002; Symons *et al.*, 2002b).

Infection of embryonated chicken egg chorioallantoic membranes (CAMs) has been used to follow innate immune responses to CPV infection. CAMs at this stage of development lack mature lymphocytes (Chen *et al.*, 1994; Masteller *et al.*, 1997). Wild-type (wt) CPV produces red, haemorrhagic, non-inflammatory lesions (pocks) on CAMs, whereas CPV deleted for CrmA produces white, inflammatory pocks (Palumbo *et al.*, 1989). White pocks produce less virus, contain heterophils and reduce nitro blue tetrazolium (NBT), indicating the presence of activated heterophils. Control of inflammation by CrmA may be due to the inhibition of caspase-1-mediated production of IL-1β (Palumbo *et al.*, 1994).

In addition, CrmA blocks apoptosis in CPV-infected swine cells (Ray & Pickup, 1996). Consistent with these results, CrmA inhibits caspase-8 and -10, which initiate apoptosis (Zhou *et al.*, 1997; Garcia-Calvo *et al.*, 1998). *In vitro* studies have shown that CrmA also inhibits apoptosis induced by allogeneic cytotoxic T lymphocytes (CTLs) (Tewari *et al.*, 1995); the level of protection differs depending on target-cell and effector-CTL populations (Mullbacher *et al.*, 1999). The role of CrmA during virus infections *in vivo* is less well understood.

Deletion of CrmA from CPV caused attenuation in intranasally infected (Thompson *et al.*, 1993) and intracranially infected (Palumbo *et al.*, 1994) Balb/c mice. In contrast, deletion of SPI-2 from VV had no effect on virulence in intranasally infected Balb/c mice (Kettle *et al.*, 1995), although intradermal infection of mouse ear pinnae produced larger lesions than wt VV (Tscharke *et al.*, 2002). An early report (Thompson *et al.*, 1993) that rabbitpox virus RPVΔSPI-2 was attenuated in mice infected intranasally was flawed; the virus contained a second mutation in the K1L gene (R. W. Moyer, unpublished data).

We used CPV-infected CAMs to address the following questions: (i) Does CrmA prevent white pock formation by proteinase inhibition? (ii) Does CrmA function solely as a caspase inhibitor? The first question was addressed by mutating P1-Asp in the CrmA RCL to alanine (D303A), which should abolish serpin function and induce white pocks. The second question was addressed by replacing CrmA in CPV with other caspase inhibitors such as P35 of the baculovirus *Autographa californica* nuclear polyhedrosis virus or SERP2 from *Myxoma virus* (MYX).

The baculovirus P35 protein is not a serpin, but it prevents apoptosis in several systems including insect cells, *Caenorhabditis elegans*, *Drosophila melanogaster* and mammalian cells (Hay *et al.*, 1994; Xue & Horvitz, 1995; Izquierdo *et al.*, 1999). P35 directly inhibits human caspase-1, -3, -6, -7, -8 and -10 (Zhou *et al.*, 1998) and *Spodoptera frugiperda* caspase-1 (Ahmad *et al.*, 1997) but not granzyme B (Zhou *et al.*, 1998). If the role of CrmA during CPV infection is solely to inhibit caspases, then the virus formed by replacing CrmA in CPV with P35 should resemble wt CPV.

The MYX protein SERP2 is a serpin and contains P1-Asp within the RCL (Petit *et al.*, 1996), but only shares 35 % amino acid identity with CrmA. MYX derivatives lacking SERP2 are attenuated during myxoma infection of European rabbits (Messud-Petit *et al.*, 1998). SERP2, like CrmA, inhibits caspase-1 and granzyme-B *in vitro* (Turner *et al.*, 1999). We set out to ask whether SERP2 could substitute for CrmA in CPV infection of the chicken CAM and whether CrmA could substitute for SERP2 in myxoma infection of the European rabbit.

We found that despite the similarities between CrmA, P35 and SERP2 *in vitro*, these proteins were not interchangeable *in vivo*, indicating important differences in biological activity.

METHODS

Cells and viruses. CV-1 and RK-13 cells were maintained in minimum essential medium with Earle's salts (Life Technologies) (Thompson *et al.*, 1993) and LLC-PK1 cells in Medium 199. CPV strain Brighton Red was grown in CV-1 cells and MYX strain Lausanne in RK-13 cells.

CrmA site-directed mutagenesis. The P1-Asp residue at position 303 of CrmA was mutated to alanine using the Altered Sites system (Promega). The CrmA coding region was cloned into pAlterEx1. Primer FS275 (5′-pTGTGCGCTGGTGGCAGCATGCGCATCAACAGTTACA-3′) was used to create the mutations (underlined). The resulting plasmid, pAlterEx1CrmA-D303A, was verified by sequencing.

Construction of recombinant cowpox viruses. The following plasmids were constructed to create CPV recombinant viruses. Underlining in primers indicates the restriction sites *Xba*I, *Eco*RI, *Hin*dIII, *Sma*I and *Nco*I.

pBS-CLF. The upstream flanking sequence of the CrmA coding region (322 nt) was PCR amplified with Vent polymerase (New England Biolabs) from CPV genomic DNA using primers GM17 (5′-GATCTCTAGAGCGGCCGCGGTTCGGTGGCAAACTTACATGGAA-3′) and GM19 (5′-TCGATCGAATTCCATGGCAATCGATTTTGTTGT-3′) and then cloned into pBluescriptII KS(+) (Stratagene) using the *Xba*I and *Eco*RI sites.

pBS-CCF. The downstream flanking sequence of the CrmA coding region (347 nt) was PCR amplified from CPV genomic DNA using primers GM21 (5′-ATCGTACGAATTCCCGGGCATATGATCACATTCTTAATATTAGAATATTAG-3′) and GM22 (5′-TGCTACAAGCTTGATGAACACTGATTCCGCATC-3′). The right flank PCR product was cloned into the *Eco*RI and *Hin*dIII sites of pBS-CLF.

pBS-CgS/A. The $P_{7\cdot5}$-*gpt* cassette was derived from pBS-gptA (Turner & Moyer, 1992) and cloned into the *Sal*I and *Apa*I sites of pBS-CCF.

pCglacZ. The P_{11}-*lacZ* cassette was PCR amplified from pSC11 (Chakrabarti *et al.*, 1985) with Vent polymerase using primers FS129 (5′-GTCAGATCCATGGTTGAATTCCGAGCTTGGCTG-3′) and FS130 (5′-AGTCAACGCCCGGGTACGCTCACAGAATTCCCG-3′) and then cloned into pBS-CgS/A using the *Nco*I and *Sma*I sites.

pBS-D303ACgS/A. The CrmA-D303A coding region was PCR amplified from pAlterEx1CrmA-D303A using FS307 (5′-TACGT-CCATGGATATCTTCAGGGAAA-3′) and FS308 (5′-CAGCTA-CCCGGGTTATAATTAGTTGTTGGAGAGC-3′) and cloned into the *Nco*I and *Sma*I sites of pBS-CgS/A.

pC35. A clone of P35 was provided by Lois Miller (University of Georgia). pC35 was constructed by inserting P35 between the *Nco*I and *Sma*I sites of pBS-CgS/A by recombinant PCR (Turner & Moyer, 1992); the start codon of P35 replaced the natural ATG at the *Nco*I site. The primer pair used to generate the left flank was FS1 (5′-GATCTCTAGAGCGGCCGCGGTTCGGTGGCAAACTTACATGGAA-3′) and FS91 (5′-GGCAATCGATTTTGTTG-3′). The primer pair used to generate P35 was FS89 (5′-CAACAAAATCGATTGCCATG-TGTGTAATTTTTCCGG-3′) with the underlined portion complementary to FS91 and FS74 (5′-TACGTCACCCGGGTTATTTAATTG-TGTTTAATATTAC-3′). Finally the left flank was linked to P35 using primers FS1 and FS74. The full-length PCR product was cloned into the *Xba*I and *Sma*I sites of pBS-CgS/A.

pCSERP2. The SERP2 gene was subcloned from pTM1-SERP2 into the *Nco*I and *Sma*I sites of pBS-CgS/A.

The CrmA open reading frame (ORF) was replaced with *lacZ*, CrmA-D303A, P35 or SERP2. CrmA regulatory elements were retained. First, CPVΔCrmA::lacZ was generated using pCglacZ, which contained a *gpt* cassette conferring resistance to mycophenolic acid (MPA), enabling transient dominant selection (TDS) for virus recombinants (Falkner & Moss, 1990). Wild-type CPV-infected CV-1 cells were transfected with pCglacZ, MPA^R plaques selected and MPA^S segregants screened for blue staining with X-Gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside) to isolate CPVΔCrmA::lacZ. Subsequently, recombinant viruses were obtained from CPVΔCrmA::lacZ by transfection with plasmids containing the appropriate ORF, TDS and loss of X-Gal staining. All viral constructs were verified by sequencing.

Immunoblotting. CV-1 or LLC-PK1 cells (2×10^6) were infected at an m.o.i. of 10. Eighteen hours post-infection (p.i.), infected cells were resuspended in buffer (100 mM Tris/HCl, pH 8·0, 100 mM NaCl, 0·5 % Triton X-100) and underwent three freeze–thaw cycles. Debris was pelleted and 100 μg supernatant protein was electrophoresed on 10 % acrylamide/SDS gels and transferred to nitrocellulose membranes (Micron Separations) in 25 mM Tris/HCl, pH 8·3, 192 mM glycine, 20 % methanol using a semi-dry transfer apparatus (Fisher Scientific). Membranes were blocked in 5 % non-fat milk in PBS, pH 7·4, 0·1 % Tween 20 for 1 h at room temperature and probed with primary antibody for 1 h. Polyclonal rabbit antiserum to P35 was diluted 1 : 10 000 (provided by Paul Friesen, University of Wisconsin). Polyclonal rabbit antiserum to SERP2 diluted 1 : 500 (Turner *et al.*, 1999) and mouse monoclonal antibody to CrmA diluted 1 : 2000 (Macen *et al.*, 1998) have been described. Membranes were washed three times (5 min each) in wash buffer (PBS, pH 7·4, 0·1 % Tween 20) and incubated with secondary antibodies (diluted 1 : 2500) of either goat anti-mouse IgG conjugated to horseradish peroxidase or goat anti-rabbit IgG conjugated to horseradish peroxidase (Southern Biotechnology Associates) for 1 h at room temperature. After five washes (10 min each) in wash buffer, immunoreactive proteins were visualized by enhanced chemiluminescence (Amersham ECL kit).

CAM infections. Embryonated chicken eggs from SPAFAS, Inc. were incubated at 38·5 °C with 50 % humidity for 11 days. The dropped CAMs were inoculated with 10 p.f.u. and the eggs incubated for 72 h. Infected CAMs were harvested, washed twice in PBS (pH 7·2) and scanned on a Microtek ScanmakerIII scanner at 500 d.p.i. Individual pocks were excised and stored at −80 °C. Alternatively, membranes were inoculated with 500 p.f.u. and CAMs were harvested 48 h later yielding confluently infected membranes.

Staining reactive oxygen intermediates. CAMs from eggs were harvested at 72 h p.i. and washed twice with PBS. The membranes were incubated in 0·1 % NBT (Sigma) in PBS at 37 °C for 1 h, the NBT was removed and the membranes were washed once with PBS and scanned.

MTT reduction assay. Individual pocks were harvested at 72 h p.i. and pooled (each pool weighed 15–30 mg), then submerged in 200 μl PBS containing 5 mg 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) ml^{-1} for 30 min at 37 °C. The MTT solution was removed and the reduced formazan extracted by grinding the membranes with a microfuge pestle in 200 μl DMSO (Sigma). The dissolved formazan was clarified by centrifugation at 12 000 ***g*** for 2 min. The absorbance of the solution was read at 550 nm. Absorbance values were expressed as A_{550} $(\text{mg tissue})^{-1}$. The standard error of the mean was calculated from four experiments.

Terminal caspase activity assay. Individual pocks isolated from CAMs at 72 h p.i. were ground in 100 μl 10 mM HEPES, pH 7·5, 2 mM EDTA, 0·1 % CHAPS and 10 mM DTT, freeze-thawed three times and clarified by centrifugation at 12 000 ***g*** for 5 min. Terminal caspase activity in 25 μg of pock extract was measured as increase in fluorescence s^{-1} of aminomethylcoumarin (AMC) from the substrate Ac-DEVD-AMC (Bachem) used at 10 μM in 200 μl of buffer (100 mM HEPES, pH 7·5, 10 % sucrose, 0·1 % CHAPS, 10 mM DTT). Substrate cleavage was monitored using a Tecan SpectraFluor microplate reader with excitation at 380 nm and emission at 460 nm.

Construction of recombinant myxoma viruses. MYXΔSERP2::lacZ was constructed via a plasmid containing *lacZ* under the CPV-ATI promoter flanked by SERP2 flanks. Nt 1–310 of SERP2 were PCR amplified with primers NP1 (5′-GCGGGTACCATGGAGCT-TTTCAAGCATTTCCT-3′) and NP2 (5′-GCGAAGCTTTGGCCG-CCACCCATCGGTTGA-3′) and cloned using *Kpn*I (underlined) and a natural *Eco*RV site near NP2. Nt 745–1002 of SERP2 were amplified with primers NP3 (5′-GCGTCTAGAATCTTCGCACATCC-TAACTTCGAGGA-3′) and NP4 (5′-GCGGAGCTCTTAGTAATTGG-GAGAAGTGACT-3′). The SERP2 flanks were cloned to either side of an *Msc*I–*Xba*I fragment containing P_{ATI}-*lacZ*. The resulting plasmid was transfected into wt MYX-infected RK-13 cells. Blue plaques were obtained and verified by Western blot analysis and sequencing to confirm that *lacZ* had been inserted into the SERP2 locus.

MYXΔSERP2::CrmA contained the CrmA ORF inserted in place of the SERP2 ORF under the control of the SERP2 promoter. The region upstream from SERP2 including the SERP2 promoter was PCR amplified with primer F1 (5′-GCGGGTACCTCTTGTTTAACAACG-CGATACA-3′) and M1 (5′-TCCCTGAAGATATCCATAATCGCAC-TTATACATTATA-3′). The CrmA ORF was amplified with M2 (5′-ATGGATATCTTCAGGGA-3′) and M3 (5′-GCGTCTAGATTAATT-AGTTGTTGGAGA-3′), then joined to the F1/M1-amplified product by recombinant PCR (Turner & Moyer, 1992) to create P_{SERP2}-CrmA. The region downstream of SERP2 was PCR amplified with NP3 and NP4 and joined to the P_{SERP2}-CrmA by an *Xba*I site. The $P_{7\cdot5}$-*gpt* cassette was inserted downstream from CrmA. The final construct was transfected

into MYXΔSERP2::lacZ-infected RK-13 cells. MPA-resistant white plaques were selected. The resulting MYXΔSERP2::CrmA virus containing *gpt* was confirmed by sequencing.

Infection of rabbits with recombinant myxoma viruses. All animal procedures were approved by UF-IACUC. Female, specific-pathogen-free New Zealand White (NZW) rabbits (*Oryctolagus cuniculus*) weighing 2 kg were purchased from Myrtle's Rabbitry (Thompson Station, TN, USA). Areas of 3 cm^2 on each thigh were surgically prepared and 500 p.f.u. virus in 0·1 ml PBS was injected intradermally. Rabbits were killed if signs of respiratory distress were observed or by 10 days p.i. Prior to killing, rabbits were anaesthetized by intramuscular injection of 50 mg Ketaject kg^{-1} and 10 mg Xylaject kg^{-1} (100 mg ketamine.HCl ml^{-1} and 20 mg xylazine ml^{-1}; Phoenix Pharmaceuticals). Blood was collected by cardiac puncture and stored in EDTA. Rabbits were killed by intracardiac injection of 3 ml Beuthanasia-D-Special (Schering-Plough Animal Health).

Tissue sample processing. Tissue samples from necropsies were stored in 10% buffered formalin (Protocol; Fisher Scientific) for 24 h. The thickness of the integument at the centre of the virus inoculation site was measured with calipers three times and the mean value was recorded. Tissues were trimmed, placed in cassettes and stored in 10% buffered formalin before paraffin embedding, sectioning and staining with haematoxylin and eosin (Richard-Allen).

RESULTS

Properties of recombinant cowpox viruses expressing CrmA-D303A, P35 or SERP2

Experiments were designed to ask whether CrmA inhibits inflammation and apoptosis by functioning as a caspase inhibitor. The CrmA ORF was deleted and replaced with *lacZ* in CPVΔCrmA::lacZ. Next CPV-CrmA-D303A was generated in which P1-Asp of CrmA, the key amino acid for proteinase recognition, was mutated to alanine. In addition, the CrmA ORF was replaced either by P35 or SERP2; both proteins are potent caspase inhibitors (Petit *et al.*, 1996; Zhou *et al.*, 1998) and might be expected to substitute fully for CrmA. In each construct, P35 or SERP2 was placed under the control of the native CrmA promoter. Fig. 1A shows the plasmids used to construct these viruses.

Expression of proteins by the recombinant CPVs was analysed by immunoblots of infected cell lysates (Fig. 1B). Lack of CrmA expression by CPVΔCrmA::lacZ (Fig. 1B, lane 3) and expression of CrmA-D303A by CPV-CrmA-D303A (Fig. 1B, lane 4) was confirmed by probing with anti-CrmA antibody. CrmA from wt CPV infections migrated as a 38 kDa protein (Fig. 1B, lane 2), whereas CrmA-D303A had slower mobility resulting in an apparent molecular mass of 40 kDa (Fig. 1B, lane 4). Altered electrophoretic mobilities of serpins as a result of point mutations within the RCL have been observed previously (K. Moon & R. Moyer, unpublished data). P35 was detected following infection with CPVΔCrmA::P35 (Fig. 1B, lane 5) and SERP2 after infection with CPVΔCrmA::SERP2 (Fig. 1B, lane 8). Each of the proteins was stable.

Wild-type CPV infection of CAMs produced red, haemorrhagic pocks, while CPVΔCrmA::lacZ produced white pocks as expected (Palumbo *et al.*, 1989) (Fig. 2). The white pocks produced by CPVΔCrmA::lacZ stained blue with NBT indicating the presence of activated heterophils. CPV-CrmA-D303A also produced white pocks that stained blue

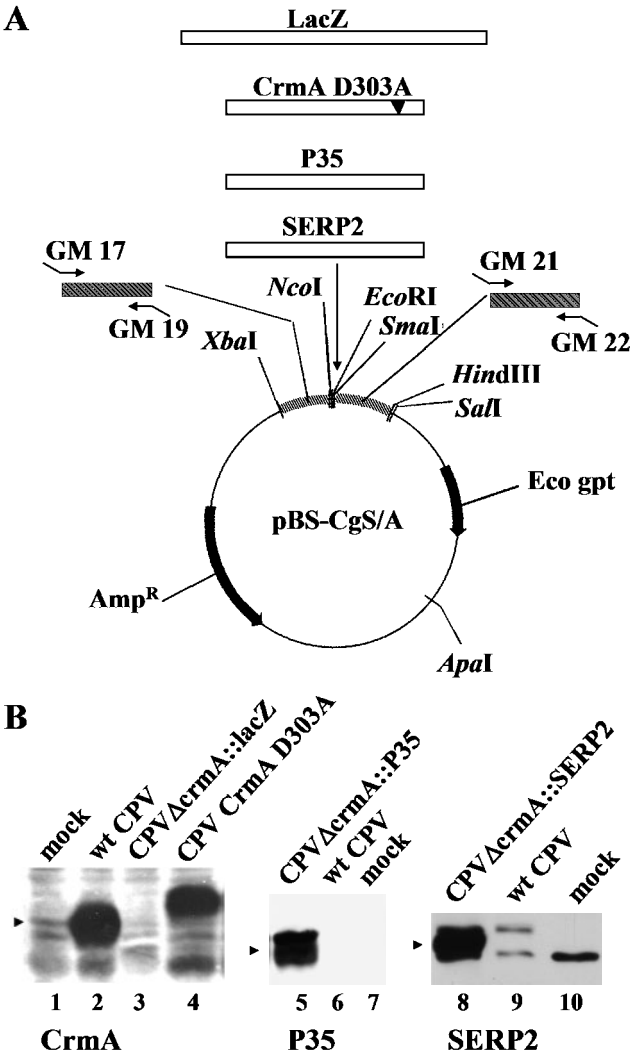

Fig. 1. Construction of recombinant CPV expressing CrmA D303A, P35 and SERP2. (A) Structure of the shuttle vector pBS-CgS/A used to replace CrmA in CPV with mutated or foreign genes. The flanks upstream and downstream of the CrmA coding region indicated by grey boxes were amplified using the primers shown. The genes were cloned in between the CrmA flanks and recombinant CPVs were generated by transient dominant selection (Falkner & Moss, 1990) using the *E. coli gpt* gene (Eco gpt) outside the CrmA flanks as the selective marker. (B) Western blots showing expression of inserted genes. CV-1 cells (lanes 1–4) or LLC-PK1 cells (lanes 5–10) were infected at an m.o.i. of 10 with the viruses indicated above the lanes, harvested at 18 h p.i. and extracts were analysed by immunoblotting with antisera to CrmA (lanes 1–4), P35 (lanes 5–7) or SERP2 (lanes 8–10). Arrowheads indicate the position of proteins: CrmA (38 kDa), P35 (35 kDa) and SERP2 (34 kDa).

with NBT (Fig. 2), suggesting that P1-Asp within the RCL is required for CrmA to inhibit inflammation. Surprisingly, both P35- and SERP2-recombinant viruses produced white pocks with positive NBT staining (Fig. 2). Both P35 and SERP2 failed to inhibit inflammation during CPV infections of CAMs.

All white pocks contain heterophils and high levels of NADPH oxidase

Uninfected CAMs (Fig. 3, mock) had an ectoderm with capillaries and two layers of chorionic epithelial cells, a mesoderm with mesenchymal cells and a single layer of endoderm-derived allantoic epithelial cells. The pocks of all infected CAMs (Fig. 3, remaining panels) had hyperplastic ectoderm, mesoderm and endoderm and moderate ectodermal necrosis localized to regions of pock formation. Wild-type CPV-infected pocks exhibited haemorrhage. In contrast, pocks from CPVΔCrmA::lacZ infection had heterophilic inflammation within the mesoderm, similar to previous reports (Palumbo *et al.*, 1989). The pock histology from CPV-CrmA-D303A infections was nearly identical to CPVΔCrmA::lacZ infections, suggesting that protease inhibition is necessary to block inflammation. The pocks caused by infection with CPVΔCrmA::P35 and CPVΔCrmA::SERP2 were also similar to those of CPVΔCrmA::lacZ, with marked heterophilic inflammation. It was clear that P35 and SERP2 could not block inflammation in the CAM induced by CPV infection but it was not clear whether differences observed in CAM thickness and cellularity were the results of the different viruses or artefacts of sample sectioning. Therefore, the activity of inflammatory cells in pocks was quantified by NADPH oxidase.

Activated heterophils contain NADPH oxidase, which produces superoxide radicals that can be measured by the reduction of tetrazolium salts such as MTT (Merrill *et al.*, 2001). We compared the levels of NADPH oxidase within pooled pocks by measuring MTT reduction over a 30 min period (Fig. 4). Wild-type CPV-infected membranes showed low activity [$0{\cdot}01 \pm 0{\cdot}02$ A_{500} (mg tissue)$^{-1}$]. Significantly higher activity [$>0{\cdot}15$ A_{500} (mg tissue)$^{-1}$] was observed from white pocks produced by CPVΔCrmA::lacZ and CPV-CrmA-D303A. Infection with CPVΔCrmA::P35 and CPVΔCrmA::SERP2 increased NADPH oxidase activity to levels similar to CPVΔCrmA::lacZ infection. This indicated that functionally equivalent numbers of heterophils were activated in all white pocks following infection with recombinant CPVs.

P35 and SERP2 restore virus yields in CPV-infected CAMs

When compared with pocks produced by CPV, the pocks produced by CPV deleted for CrmA are inflammatory and contain less infectious virus (Palumbo *et al.*, 1989). The ability of P35 and SERP2 to restore virus yields was measured following infection of CAMs with CPV derivatives lacking CrmA. Individual pocks were harvested at 3 days p.i., homogenized and virus titre was determined on CV-1 cells (Fig. 5). Wild-type CPV pocks yielded approximately 5×10^6 p.f.u. per pock, while CPVΔCrmA::lacZ yields were at least tenfold lower (4×10^5 p.f.u. per pock), consistent with previous reports (Palumbo *et al.*, 1989).

Virus yields from CPV-CrmA-D303A pocks were similar to those of CPVΔCrmA::lacZ, consistent with the expectation that CrmA promotes higher virus yields during CPV infection by functioning as a proteinase inhibitor. Both P35 and SERP2 recombinant viruses were able to restore virus yields (Fig. 5). Hence, P35 and SERP2 could substitute for CrmA to restore virus titre within the context of a CPV infection of the CAM. Since P35 and SERP2 were unable to block inflammation in this system, these results imply that the restoration of virus yields by P35 and SERP2 may result from prevention of apoptosis in the developing pock.

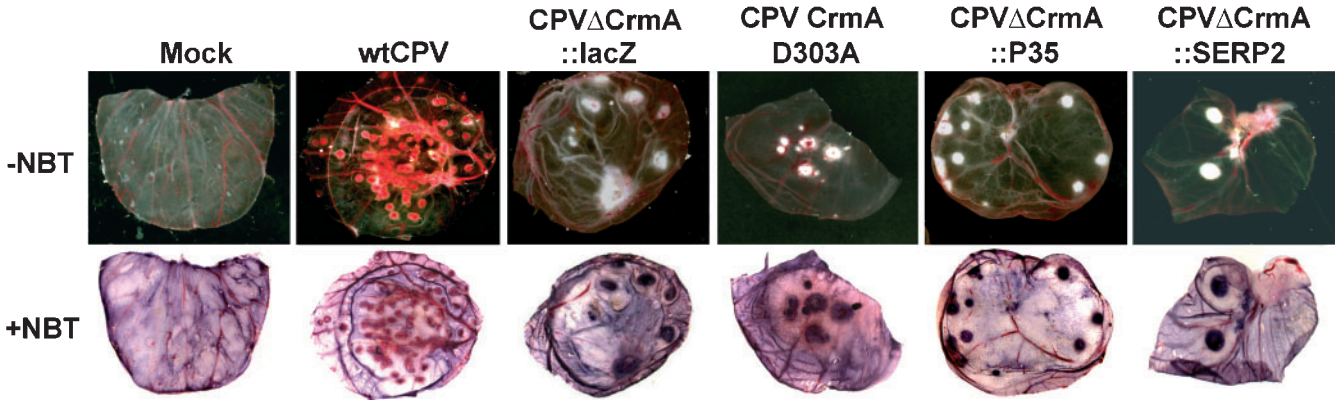

Fig. 2. Appearance of pocks produced by recombinant viruses on the chicken CAM. CAMs of 11-day-old chicken embryos were mock infected or infected with wt CPV, CPVΔCrmA::lacZ, CPV-CrmA-D303A, CPVΔCrmA::P35 or CPVΔCrmA::SERP2. Excised membranes were photographed at 3 days p.i. before (−) and after (+) staining with NBT.

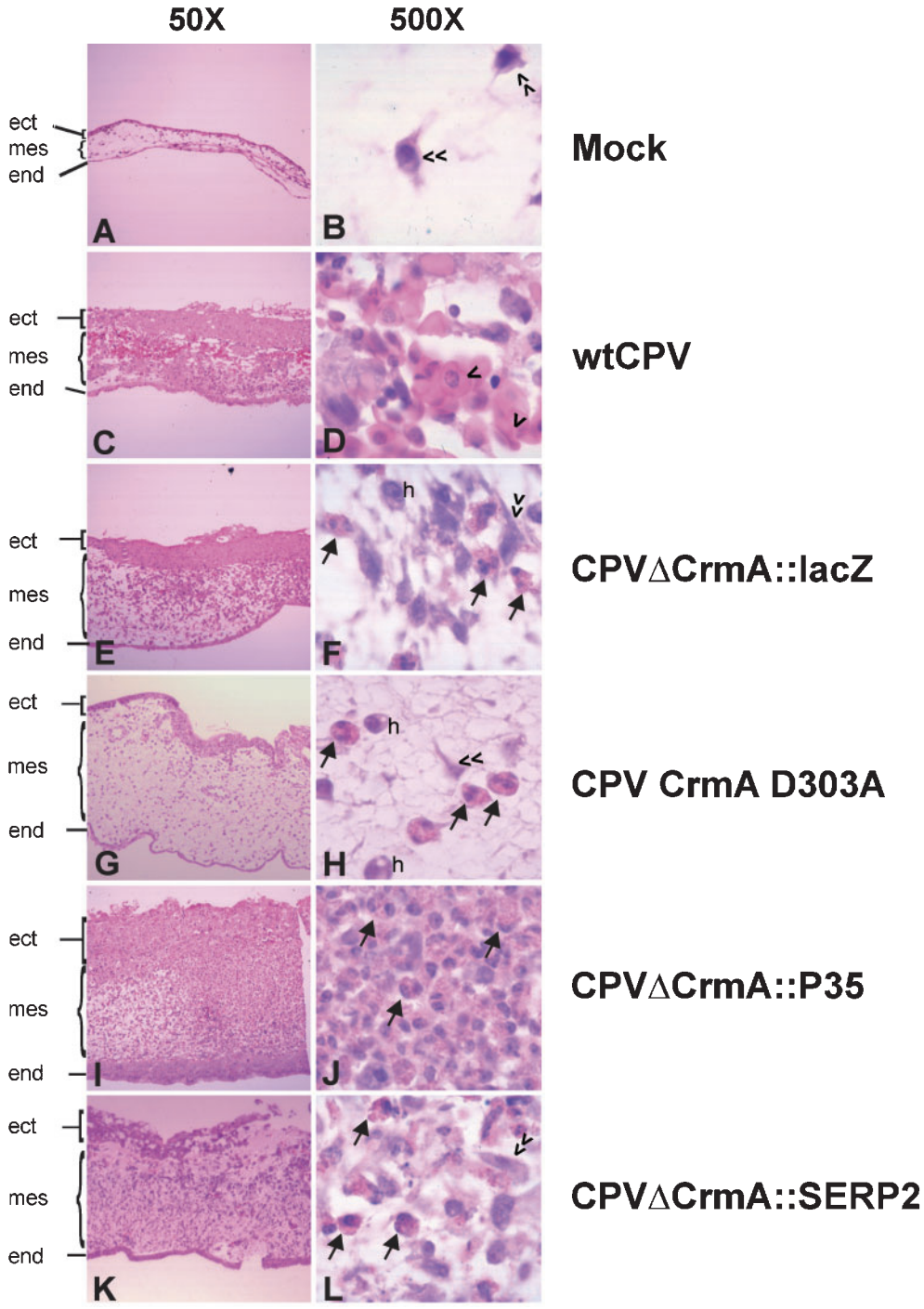

Fig. 3. Histological examination of pocks from infected CAMs. Infected CAMs were harvested at 3 days p.i. and individual pocks were sectioned and stained with haematoxylin and eosin. The panels at 500 × magnification show an area within the mesoderm of each of the CAMs. Cell types are represented as follows: mesenchymal cells (≪), erythrocytes (<), histiocytic cells (h), heterophils (↑). (A) Mock-infected CAMs consisting of an ectoderm (ect) with two layers of chorionic epithelial cells, a mesoderm (mes) with low numbers of stellate mesenchymal cells and a thin endoderm (end) that is one cell layer thick. (B) Individual mesenchymal cells from mock-infected CAMs with a stellate appearance. (C) CPV-infected pocks showing marked ectodermal and mesodermal hyperplasia and mild hyperplasia of the endoderm. Haemorrhage can be seen between the ectodermal and mesodermal layers of the CAM. (D) CPV-infected pocks showing the haemorrhagic ectodermal and mesodermal junction. (E–L) CPVΔCrmA::lacZ (E, F), CPV-CrmA-D303A (G, H), CPVΔCrmA::P35 (I, J) and CPVΔCrmA::SERP2 (K, L) pocks showing hyperplasia of all layers of the CAM and extensive heterophilic inflammation in the mesoderm without evidence of haemorrhage. Increased numbers of histiocytic cells were also present in many high-power fields of CPVΔCrmA::P35 and CPVΔCrmA::SERP2 but are not shown in (J) or (L).

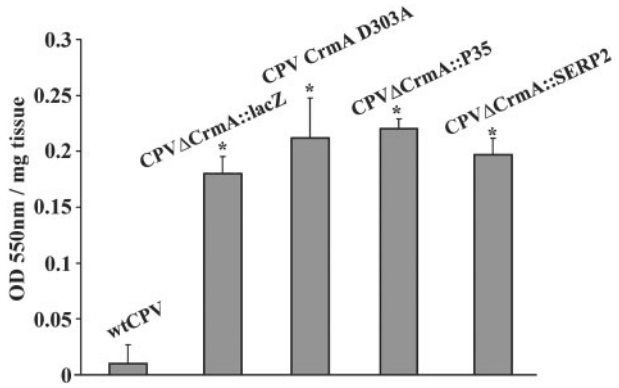

Fig. 4. Levels of oxidative burst in infected CAMs. Confluently infected CAMs were harvested at 48 h p.i. Reactive oxygen intermediates were measured by incubating pooled pocks in the presence of MTT for 30 min. The reduced formazan was dissolved in DMSO and the absorbance read at 550 nm. The results are expressed as A_{550} (mg tissue)$^{-1}$. The low levels observed from uninfected CAMs have been subtracted. Results are the standard error of the mean of four such experiments. *, $P<0{\cdot}05$ by Student's *t*-test compared with CPV.

CrmA, P35 and SERP2 block terminal caspase induction within infected pocks

We evaluated apoptosis within infected pocks by measuring the activity of terminal caspases. Extracts were evaluated for cleavage of Ac-DEVD-AMC, a fluorogenic substrate for caspases-3, -6 and -7 (Fig. 6). Little caspase induction was noted in mock-infected or wt CPV-infected CAMs, but infection with CPVΔCrmA::lacZ resulted in cleavage of Ac-DEVD-AMC. CPVΔCrmA::lacZ and CPV-CrmA-D303A induced terminal caspase activity to similar levels, reaffirming that P1-Asp of the CrmA RCL is essential for CrmA function. Expression of either P35 or SERP2 was able to block induction of terminal caspase activity within the context of CPVΔCrmA-infected pocks.

These results suggested that the restoration of pock titres seen in both P35- and SERP2-recombinant infections is a result of prevention of apoptosis and does not require reduction of inflammation. Thus, while CrmA controls both inflammation and apoptosis within CAMs, P35 and SERP2 can only substitute for CrmA to control apoptosis.

Infection of rabbits with recombinant myxoma viruses lacking SERP2 or expressing CrmA

Poxvirus infection of mammals with fully developed immune systems provides a more complete model for virulence than chicken CAMs. In view of conflicting data about whether deletion of CrmA from CPV (Thompson *et al.*, 1993; Palumbo *et al.*, 1994) or SPI-2 from VV (Kettle *et al.*, 1995; Tscharke *et al.*, 2002) causes virus attenuation in mice, we did not examine CPVΔCrmA::SERP2 for virulence in mice. Instead, we asked whether CrmA could replace the pathogenic function of SERP2 by infecting rabbits with MYXΔSERP2::CrmA, a recombinant that expresses CrmA in place of SERP2.

Infection of NZW rabbits with wt MYX resulted in the formation of primary dermal lesions that were prominent at day 3 and continued to enlarge until day 8. Lesions were raised, erythematous and ulcerated (Fig. 7A). In contrast,

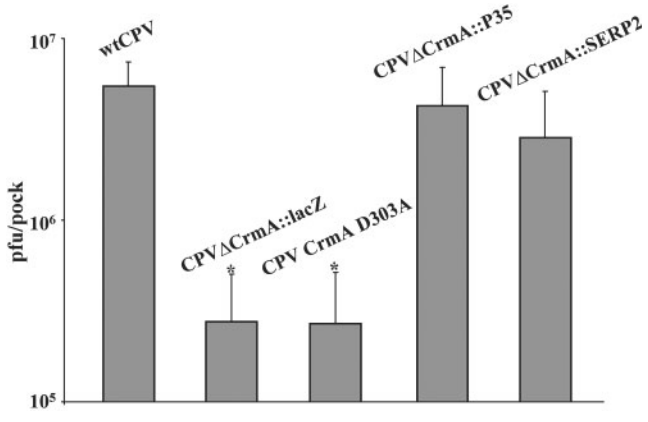

Fig. 5. P35 and SERP2 restore virus yields from infected CAMs. Individual pocks were excised from CAMs at 3 days p.i, homogenized with a microfuge pestle in 500 µl PBS, lysed by three cycles of freeze-thawing and titred on CV-1 cells. The results are expressed as p.f.u. per pock and are means of viral titres from 12 pocks. Error bars are standard deviations of the mean. *, $P<0{\cdot}05$ by Student's *t*-test compared with CPV.

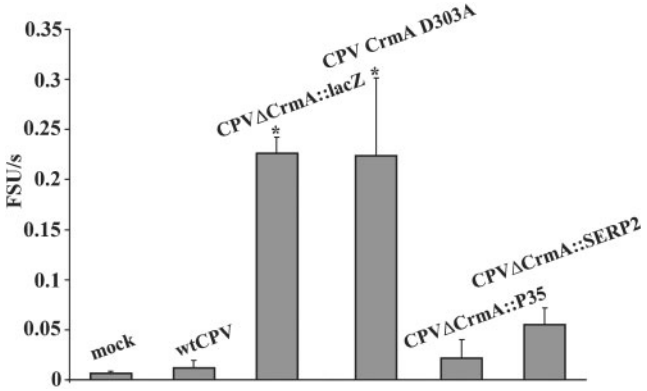

Fig. 6. P35, SERP2 and CrmA block terminal caspase activity within infected pocks. Individual pocks from infected 11-day-old CAMs were isolated at 3 days p.i. and lysates prepared from individual pocks. CAM extract (25 µg) was tested for the ability to cleave the substrate Ac-DEVD-AMC. The rates of cleavage are expressed arbitrarily as fluorescence signal units (FSU) s^{-1} and are means of assays from 20 individual pocks (10 individual pocks tested for CPV-CrmA-D303A). The error bars are standard deviations of the mean. *, $P<0{\cdot}05$ by Student's *t*-test compared with wt CPV.

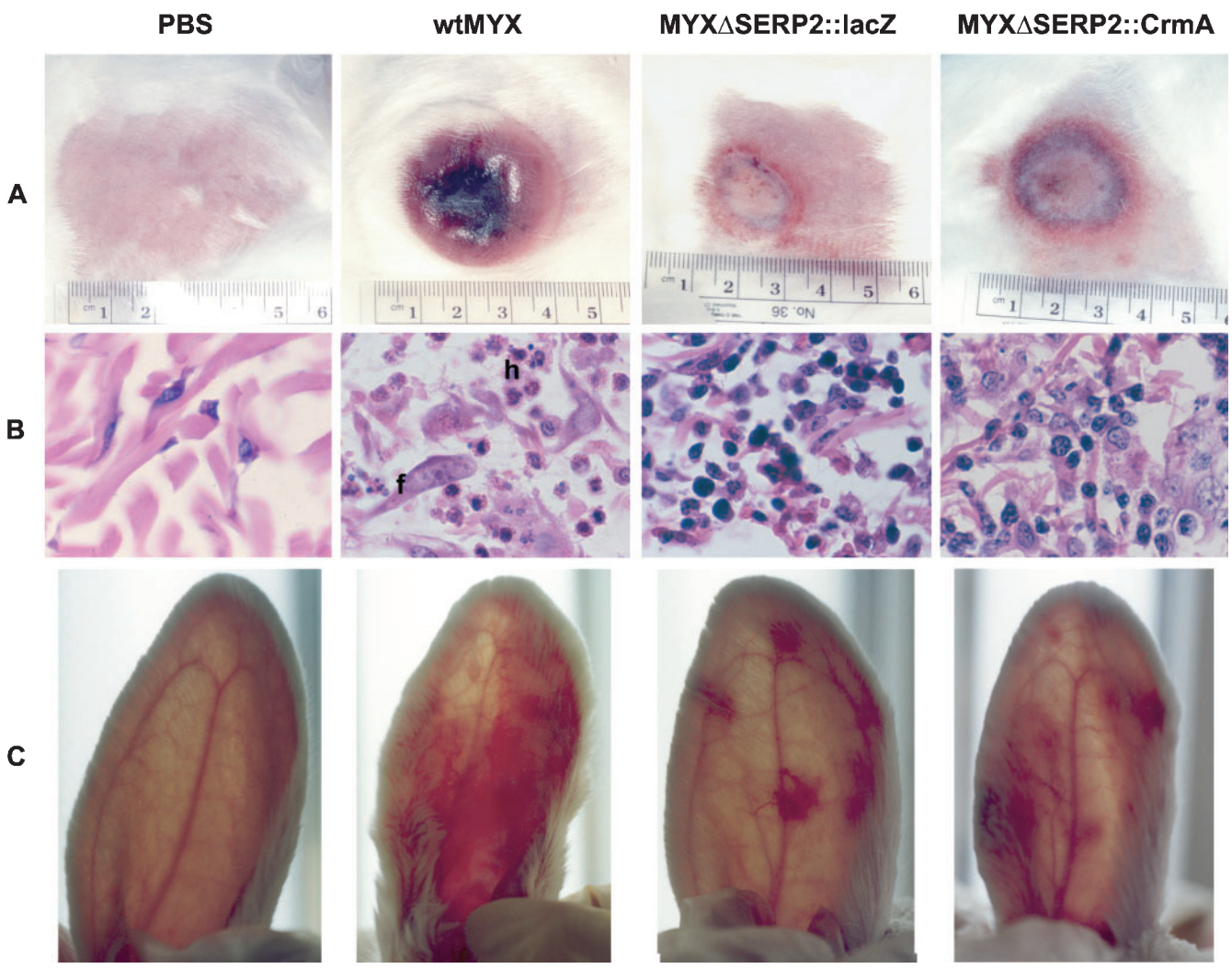

Fig. 7. Lesions caused by recombinant MYX virus infection of NZW rabbits. (A) Photographs of the primary lesions at the site of virus inoculation were obtained prior to necropsy. Lesions infected with wt MYX were visibly thicker and more proliferative than those of MYXΔSERP2::lacZ or MYXΔSERP2::CrmA. (B) Histopathology of the primary dermal lesions showed an area of ischaemic necrosis infiltrated with heterophils and surrounded by mononuclear inflammation in all infected lesions; however, severe fibroblast hypertrophy and myxoedema were only observed with wt MYX infection. The wt MYX section illustrates the reactive fibroblasts (f), heterophils (h) and oedema observed at day 9 after virus inoculation. MYXΔSERP2::lacZ and MYXΔSERP2::CrmA sections show large numbers of mononuclear cells associated with the edges of the lesions 9 days after virus inoculation. The necrotic centres of the lesions are not shown. (C) Photographs of secondary lesions on the ears of rabbits were obtained prior to necropsy. Both wt MYX and MYXΔSERP2::CrmA infections caused large numbers of secondary lesions; fewer secondary lesions were observed during infection with MYXΔSERP2::lacZ virus.

MYXΔSERP2::lacZ and MYXΔSERP2::CrmA caused lesions with a central area of necrosis (0·5–1·5 cm in diameter) surrounded by concentric circles of erythema (1·0–1·5 cm wide) and blanched skin (0·3–0·5 cm wide) (Fig. 7A). Nine days p.i., the mean lesion diameters caused by the mutated viruses were not significantly different from wt MYX lesions.

In all infected rabbits, histopathology of primary dermal lesions collected during necropsy (Fig. 7B) revealed extensive ulceration and necrosis of the epidermis and dermis with severe vascular necrosis. Heterophils were scattered throughout the necrotic tissue and a mononuclear infiltrate was observed at the edges of the ulcer. Vesicular degeneration of the epidermis and viral inclusions were observed in several lesions. The secondary lesions did not have ulceration or necrosis but did have a mixed inflammatory infiltrate. In both the primary and secondary dermal lesions, wt MYX lesions contained large numbers of hypertrophied fibroblasts and a clear extracellular matrix consistent with myxoedema, whereas lesions infected with MYXΔSERP2::lacZ or MYXΔSERP2::CrmA did not.

Wild-type MYX caused secondary lesions on mucocutaneous junctions and ears by 6 days p.i. Like the primary lesions, the secondary lesions were raised and erythematous (Fig. 7C). Infection with MYXΔSERP2::lacZ or MYXΔSERP2::CrmA caused flat secondary lesions that tended to be more numerous in animals that developed more severe signs of respiratory disease (Fig. 7C). Progression of infection with MYXΔSERP2::lacZ and MYXΔSERP2::CrmA followed the same time course observed with wt MYX infection. No lesions were observed on the control rabbit.

Due to production of myxoid extracellular matrix by fibroblasts and severe oedema, the primary lesions caused by wt MYX were markedly thicker than those of MYXΔSERP2::lacZ or MYXΔSERP2::CrmA. On average, wt MYX lesions were 33 mm in diameter and 9·7 mm thick at necropsy (Fig. 8). However, the lesions of MYXΔSERP2::lacZ and MYXΔSERP2::CrmA were flat, averaging 4·0 mm thick with MYXΔSERP2::lacZ and 4·9 mm thick with MYXΔSERP2::CrmA (Fig. 8).

All infected rabbits became febrile at 6 or 7 days p.i. Respiratory rates decreased when rabbits began to show signs of respiratory disease between 8 and 10 days p.i. Severe monocytosis was noted in all infected rabbits. Mean weight gain over a 10-day period was decreased compared with the rabbit inoculated with PBS.

At necropsy, all infected rabbits had mild diffuse splenomegaly, patchy consolidation of lung lobes, pulmonary haemorrhage and slightly enlarged sublumbar lymph nodes. Microscopic examination of the lungs revealed non-suppurative interstitial pneumonia with patchy oedema, haemorrhage and congestion. Rabbits with more severe clinical disease had more severe lymphocytic inflammation. Lung tissue showed mild to moderate growth of *Bordetella bronchiseptica*, a normal inhabitant of the respiratory tract of rabbits that is considered non-pathogenic (Deeb *et al.*, 1990). Sublumbar lymph nodes were examined for apoptosis by TdT-mediated dUTP nick end-labelling (TUNEL) staining, but no appreciable differences between virus infections were observed (data not shown).

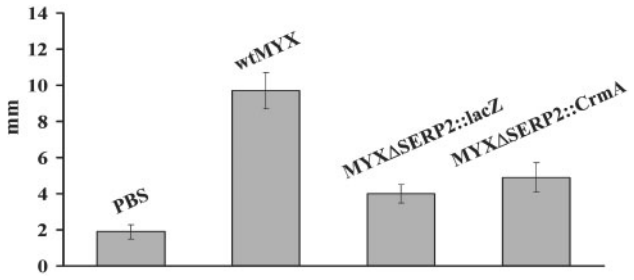

Fig. 8. Integument thickness of the primary lesion caused by recombinant MYX virus infection of NZW rabbits. Primary dermal lesions were excised at necropsy, placed in 10 % buffered formalin for 24 h and integument thickness was measured. The graph reflects the mean lesion thickness of 2 (PBS), 16 (MYXΔSERP2::lacZ), 20 (MYXΔSERP2::CrmA) or 22 (wt MYX) primary lesions. The mean thickness of wt MYX lesions was greater than twice the thickness of those caused by MYXΔSERP2::lacZ- or MYXΔSERP2::CrmA-recombinant viruses.

The mortality rate of NZW rabbits infected with wt MYX was 100 %; all 11 rabbits were killed between 8 and 10 days p.i. These rabbits developed harsh lung sounds with crackles and wheezes indicative of interstitial lung disease and airway narrowing. The mortality rate of rabbits infected with MYXΔSERP2::lacZ was less than 15 %. Only one of eight rabbits infected with MYXΔSERP2::lacZ had harsh lung sounds that may have progressed beyond day 10. In contrast, the mortality rate of MYXΔSERP2::CrmA-infected rabbits was 70 %; seven of ten animals were euthanized between 8 and 10 days p.i. due to respiratory distress.

These results demonstrated that CrmA can partially substitute for SERP2 to restore MYX virulence but cannot replace SERP2 to produce myxomatous lesions.

DISCUSSION

The phenotype of CPV-CrmA-D303A resembled that of CPVΔCrmA::lacZ in all respects, including the production of white, inflammatory pocks (Figs 2–4) with reduced virus yields (Fig. 5) and high levels of terminal caspase activity (Fig. 6). This indicated that all measurable activities of CrmA are mediated via proteinase inhibition.

We were surprised by the fact that P35 and SERP2 were unable to block inflammation in the chicken CAM (Figs 2–4) but did control apoptosis in this system (Fig. 5). Biochemical data indicate that although P35 is an active inhibitor of a broad range of human caspases, the association rate (k_a) of P35 for human caspase-1 is $8{\cdot}2 \pm 1{\cdot}1 \times 10^3$ M^{-1} s^{-1} (Zhou *et al.*, 1998) compared with $1{\cdot}7 \times 10^7$ M^{-1} s^{-1} for CrmA and caspase-1 (Komiyama *et al.*, 1994). P35 may therefore be less effective as an anti-inflammatory agent than CrmA. In contrast, P35 and CrmA have relatively similar inhibition constants against human caspase-8 with values of $6{\cdot}4 \pm 0{\cdot}1 \times 10^4$ and $9{\cdot}1 \times 10^4$ M^{-1} s^{-1}, respectively, and against caspase-10 of $1{\cdot}2 \pm 0{\cdot}4 \times 10^3$ and $1{\cdot}9 \pm 0{\cdot}4 \times 10^3$ M^{-1} s^{-1}, respectively (Zhou *et al.*, 1998). SERP2 is a weaker inhibitor of human caspase-1 ($K_i = 420$ nM) than CrmA ($K_i = 4$ pM) (Turner *et al.*, 1999), a difference that may account for the lack of SERP2 activity against inflammation in the CAM. At present, chicken caspase-1 has not been expressed and there is no information regarding inhibition by CrmA, P35 or SERP2.

The mechanism of inflammation in the CAM is not well understood. Mammalian pro-IL-1β is activated by cleavage at Asp-27 followed by Asp-116, with both sites conserved (Swaan *et al.*, 2001). However, both critical Asp residues are absent from chicken IL-1β (Weining *et al.*, 1998). We expressed chicken pro-IL-1β using the T7 Quick Coupled Transcription/Translation System (Promega

Corporation), but were unable to detect processing by purified caspase-1 or extracts from CAMs infected with wt CPV or CPVΔCrmA::lacZ (data not shown). Chicken pro-IL-18 does have a potential conserved cleavage site when compared with mammalian pro-IL-18. Chicken pro-IL-18, expressed *in vitro* by transcription/translation, was appropriately processed by 1 U human caspase-1 (data not shown). Extracts from CAMs infected with CPVΔCrmA::lacZ but not wt CPV cleaved chicken pro-IL-18. However experiments with peptide inhibitors showed that the activity was blocked by Ac-DEVD-CHO, an inhibitor of terminal caspases, but not by the caspase-1-specific inhibitor Ac-WEHD-CHO (data not shown). The results indicated the observed cleavage was a by-product of high levels of terminal caspases.

CrmA has been suggested to inhibit generation of pro-inflammatory molecules other than IL-1 or IL-18 (Palumbo *et al.*, 1989). Endothelial injury, which leads to haemorrhage and oedema in CPV and MYX infections, is known to activate factor XII, which activates four cascades that involve pro-inflammatory proteases: the coagulation, fibrinolysis, complement and kinin systems. Vascular injury is also associated with increased arachidonic acid metabolism causing production of pro-inflammatory eicosanoids. Any of these mediators are candidate targets for inhibition by CrmA.

Despite the fact that neither P35 nor SERP2 had anti-inflammatory activity in the CAM, both proteins were functional as apoptosis inhibitors within the context of a CPV infection. Restoration of virus yields under these conditions indicated that control of apoptosis rather than inflammation is required for full virus replication.

CrmA did not substitute fully for SERP2 in MYX-infected rabbits. Although the degree of inflammation in dermal lesions of rabbits infected with wt MYX, MYXΔSERP2::lacZ and MYXΔSERP2::CrmA was similar, fibroblast reactivity was markedly increased with wt MYX compared with the recombinant viruses (Fig. 7). This result indicates that CrmA could not function as SERP2 to produce myxomatous lesions. However, MYXΔSERP2::CrmA caused severe morbidity and mortality, whereas MYXΔSERP2::lacZ was severely attenuated. This suggested that CrmA can function in place of SERP2 to restore virus virulence. The dichotomy of these results parallels that seen in the CPV-infected CAM and supports the notion that SERP2 has functions both in promoting primary lesion development and in lethality.

We conclude that CrmA can restore partial virulence in MYX deleted for SERP2 but cannot restore lesion morphology. Likewise, replacement of CrmA with known caspase inhibitors prevents apoptosis and allows virus replication during CPV infection of CAMs but does not prevent inflammation. In the CPV/CAM system, the targets for CrmA in blocking apoptosis and inflammation are probably different caspases. At present, we can only speculate that the roles of SERP2 in lesion morphology and virulence may reflect different target proteases or that the same protease is present at different levels in different cells or tissues.

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