Characterization and functional analysis of Serp3: a novel myxoma virus-encoded serpin involved in virulence

Jean-Luc Guerin,1 Jacqueline Gelfi,1 Christelle Camus,1 Maxence Delverdier,2 James C. Whisstock,3 Marie-France Amardeih1, Robert Py,1 Stéphane Bertagnoli1 and Frédérique Messud-Petit1

1,2 UMR de Microbiologie Moléculaire and Laboratoire d’Anatomie-Pathologique, Institut National de la Recherche Agronomique and Ecole Nationale Vétérinaire, Toulouse, France
3 Department of Biochemistry and Molecular Biology, Monash University, Melbourne, Victoria, Australia

Myxoma virus (MV), a member of the family Poxviridae, is the causative agent of myxomatosis, a fatal disease of the European rabbit. The MV genome is a linear, double-stranded DNA molecule that encodes several factors important for evasion of the host immune system. Sequencing the right-end region of the MV genome identified an 801 bp open reading frame (ORF) encoding a polypeptide that belongs to the serpin superfamily. To date, two MV-encoded serpins have been characterized: SERP-1 binds to several targets and is an anti-inflammatory molecule, whereas Serp2 is essential for virus virulence and has both anti-inflammatory and anti-apoptotic effects. Thus, Serp3 is the third MV-encoded serpin. DNA sequence analysis of Serp3 indicated a similarity to poxvirus late promoters, which was confirmed by mRNA expression analysis. Serp3 has an atypical serpin motif and has significant sequence deletions as compared to most cellular and viral serpins. However, molecular modelling studies suggested that Serp3 can retain the overall serpin fold. Insertional inactivation of the serp3 ORF led to a significant attenuation of virulence in vivo (as measured by the increase in survival of infected rabbits) and limited dissemination of the virus to secondary sites of infection. In rabbits infected with a Serp3 deletion mutant (MV-Serp3N), the main histopathological feature is the absence of secondary myxomas. Both wild-type MV and MV-Serp3 replicate at comparable levels in vivo. Serp3 may represent a significant virulence factor of MV and probably acts in synergy with other viral proteins.

Introduction

Myxoma virus (MV), a member of the family Poxviridae, is the agent responsible for myxomatosis, a highly lethal disease in the European rabbit (Oryctolagus cuniculus) (Fenner & Ratcliff, 1965). The main anatomical and clinical features of myxomatosis are a fulminant lesion at the injection site followed by the occurrence of secondary lesions at the cutaneous and visceral levels, named myxomas. These lesions are particularly obvious on the face and ears of the rabbit. MV infection is also accompanied by general dysfunction of cellular immunity, resulting in Gram-negative bacterial superinfections of the respiratory tract culminating in death within 2 weeks.

MV has a double-stranded DNA of 160 kb with a central region containing highly conserved enzymatic and structural genes that are required for maintenance of normal virus replication. Peripheral regions of DNA, within and near the inverted terminal repeats (ITR) at both sides of the genome, encode non-essential factors that contribute to the modulation of host response to infection (Drillien et al., 1987; Upton et al., 1987; Upton & McFadden, 1986).

Several MV proteins have been described previously and shown to be associated with virulence (reviewed by Nash et al., 1999). Serpins (serine proteinase inhibitors; Carrell & Travis, 1985) have been described in poxviruses, such as cowpox virus (CPV) (Pickup et al., 1986), rabbitpox virus (Brooks et al., 1995; Macen et al., 1998) and vaccinia virus (VV) (Boursnell et al., 1988; Kotwal & Moss, 1988; Smith et al., 1989; Turner & Moyer, 1992). These serpins are named SPI-1, SPI-2 (or CrmA in CPV) and SPI-3 (Turner et al., 1995). To date, we and others have identified two MV-encoded serpins, SERP-1 and Serp2.
Two copies of serp-1 (Upton et al., 1990) are located in the ITR of MV and encode a secreted factor that is implicated in modulation of host inflammatory responses (Macen et al., 1993; Nash et al., 1997, 1998). The serp2 gene has been identified near the right ITR (Petit et al., 1996) and is closely related to CPV-encoded CrmA. Serp2 has been shown to be an intracellular inhibitor of caspase-1 and Granzyme B and to interfere with both inflammation and apoptosis (Messud-Petit et al., 1998; Turner et al., 1999). Another gene, previously designated M152R, had been discovered by systematic sequencing of the MV genome and its product was found to be homologous to leuipin, an intracellular serpin (Cameron et al., 1999).

In this paper, we report the characterization of a third MV protein, Serp3, related to the serpin superfamily. Sequence alignment of this protein with other members of the serpin superfamily and molecular modelling studies revealed the presence of characteristic serpin-like motifs and unusual deletions within the viral serpin named Serp3. We measured the time-course of expression of Serp3 mRNA in cell cultures. The functionality of Serp3 was assessed by comparing the clinical course of disease in rabbits infected with either wild-type MV or MV-Serp3−. In each case, we measured the virus load in tissues, the apoptotic response in the lymph nodes and the presence of secondary myxomas. Our findings strongly indicate that Serp3 is a novel virulence factor of MV, the functional mechanism of which is currently under investigation.

**Methods**

**Cells and viruses.** Wild-type MV strain T1 and MV-Serp3− were grown on rabbit kidney cells (RK13) in Dulbecco’s minimum essential medium supplemented with 10% foetal calf serum (FCS). Rabbit lymphocytes (RL5) were grown in RPMI 1640 (Gibco BRL) supplemented with 5% FCS. Wild-type AcNPV and recombinant BakSerp3 baculoviruses were propagated in the insect cell line Sf9 (Spodoptera frugiperda). These cells were maintained in SF 900 II medium (Gibco BRL) supplemented with 10% FCS.

**Cloning and sequencing of DNA sequences.** A set of primers, namely serp1−5′ (5′ TCGACACGGCAGCAGCTTA 3′) and serp2−3′ (5′ GGCCTACGGATGTTCTAACG 3′) was designed to amplify the sequence between the serp-1 and serp2 genes. The PCR product was cloned into a pGEM-T plasmid vector (Promega) and the recombinant plasmid was purified and sequenced using synthetic oligonucleotide primers with an automated sequencer.

**Computer analysis of DNA sequences.** DNA sequences were analysed using DNA Strider 1.3 software (March, 1988) and the BLAST program (GenBank). Edited sequences were analysed with the GAP program package (SWISS–PROT, release 33, March 1996).

**Sequence comparisons and modelling of Serp3.** PSI-BLAST searches (Altschul et al., 1997) of the non-redundant protein database obtained from NCBI (http://www.ncbi.nlm.nih.gov) were performed to convergence using Serp3 as a probe and the BLOSUM62 matrix (h = 0.001). A molecular model of Serp3 was built using the programs MODELLER (Sali & Blundell, 1993) and Quanta (MSI). The alignment between Serp3 and antitrypsin was generated using PSI-BLAST and manually refined to account for the extensive deletions in Serp3. The X-ray crystal structure of native antitrypsin (pdb identifier 1QLP; Elliott et al., 1996) was obtained from a protein database (http://www.rcsb.org) and used as a template for the model. The protein illustrations in Fig. 5 were generated using Molscript (Kraulis, 1991).

**Construction of Serp3− recombinant and revertant viruses.** To characterize Serp3 and to evaluate its role in MV pathogenicity, inactivation of the serp3 gene was performed by disruption with the lacZ gene. Serp3 was amplified by PCR with primers serp3−5′, 5′ CAGCCAAGTTCAATCTGCTGGTACGCTAGCACCTTCCC 3′, and serp3−3′, 5′ CAGGCACTCAGTCTAGGTTTCCCATCAAAAGAAGAGCTC 3′, corresponding to the 5′ and 3′ ends of its ORF (Fig. 1). The PCR product was cloned into the pGEM-T expression vector (Promega). Serp3-pGEM-T plasmid was digested with Clal after which lacZ was inserted at the Clal site. Clones expressing LacZ were screened and amplified. Disruption of the serp3 ORF was assessed by sequencing the recombinant plasmid, namely pserp3::lacZ. This plasmid was used for transfection of MV-infected cells. MV-Serp3− mutant viruses were screened by LacZ phenotype selection. PCR and Southern blot analyses of recombinant virus DNA were performed to confirm the absence of wild-type virus in preparations of MV-Serp3−. Finally, the absence of Serp3 in MV-Serp3− infected cells was assessed by RT–PCR analysis. An MV-Serp3rev revertant virus containing a wild-type serp3 ORF was constructed by transfecting plasmid DNA containing the complete serp3 gene into MV-Serp3−infected RK13 cells and by using reverse white–blue screening.

**Single-step growth analysis of viruses.** RK13 and RL5 cells were infected for 2 h with either MV strain T1 or MV-Serp3− at an m.o.i. of 5. Free virus was then removed and replaced with growth medium. Cells were incubated at 37 °C and harvested at multiple time-points post-infection (p.i.). Virus was released by freeze–thawing and brief sonication. Virus titres in the lysates were determined by standard plaque titration on RK13 cell monolayers.

**RNA extraction and RT–PCR analysis.** RK13 cells (5 × 10⁶) were infected with either wild-type MV strain T1 or mutant MV-Serp3− at an m.o.i. of 5. Total RNA was isolated at 2, 4, 8, 12 and 16 h.p.i. with TRizol Reagent (Gibco BRL), according to the manufacturer’s instructions. In addition, total RNA was also extracted from infected cells treated with 40 μg/ml cytotoxic arabinoside (AraC) at the time of infection. For RT–PCR analysis, the serp3−3′ primer was used for first-strand cDNA

---

**Fig. 1.** The MV serpin genes. (A) Schematic map of the right-end region of the MV genome (strain T1) showing the location of serp3. EcoRI (E) and BamHI (B) sites and the terminal inverted repeat (TIR), indicated in bold, are shown. (B) Insertion of the β-gal cassette in serp3. The cassette was inserted in the opposite orientation to serp3. The positions of primers serp3−5′ and serp3−3′ used for RT–PCR are indicated.
Production of Serp3 in baculovirus. The serp3 gene was cloned, as described previously (O’Reilly et al., 1994), into a pMBac baculovirus expression vector (Stratagene) allowing release of the protein in the supernatant. Recombinant BakSerp3 viruses were screened for the LacZ phenotype. Sf9 cells were infected with BakSerp3 virus and the supernatant was harvested at day 4 p.i.

Infection of rabbits with MV-Serp3-. Male 8-week-old New Zealand White rabbits were obtained from a local supplier and housed in biocontainment facilities under the guidelines of the European Community Council on animal care. Animals were raised in accordance with the European Council directive 86/609/ECC (24 November, 1986). All procedures on the animals were performed by workers accredited to the French Ministry of Agriculture and were aimed at limiting animal pain and distress. Animals were infected by intradermal inoculation to the right ear with 5 × 10^3 p.f.u. of wild-type MV, MV-Serp3− or MV-Serp3+. Rabbits were monitored daily for clinical signs of myxomatosis (Fenner & Ratcliff, 1965). Rabbits that became moribund were sacrificed with TO1 (Distrivent) administered intravenously. For histological studies, six rabbits each were inoculated with either MV strain T1 or MV-Serp3− as described above. At 4, 8 and 11 days p.i., two animals from each group were sacrificed. Two mock-infected rabbits were used as controls.

Histological examination. All animals were subjected to a complete post-mortem examination. Tissue material from the injection site (primary site, ear) and parotid lymph node (secondary site) was taken and stored in 10% neutral formalin for further analysis. After fixation, tissues were routinely processed in paraffin blocks, sectioned at 4 µm and stained with haematoxylin and eosin (H&E) for microscopic examination. Lesions were assessed histologically and graded as minimal (+), light (++), moderate (+++), marked (++++) or severe (+++++). The TUNEL method was used to assess apoptosis of lymphocytes in parotid lymph nodes, as described previously (Messud-Petit et al., 1998). Grading based on the number of apoptotic bodies in each microscope field at a magnification of ×400 was used as follows: minimal apoptosis (+, 25–50 apoptotic bodies); light apoptosis (++, 50–75 apoptotic bodies); moderate apoptosis (+++, 75–100 apoptotic bodies); marked apoptosis (++++, 100–125 apoptotic bodies); or severe apoptosis (+++++ > 125 apoptotic bodies).

Immunostaining of viral antigens. Viral antigens in paraffin-embedded sections of ear (primary site) and parotid lymph node were reactivated using 0.1% trypsin in PBS (pH 7.6) for 30 min at 37 °C. After neutralization of endogenous peroxidases and before incubation with the specific primary antibody (rabbit hyperimmune serum anti-MV), samples were incubated in goat pre-immune serum. This step was performed to minimize the background generated by the secondary antibody (biotinylated goat anti-rabbit immunoglobulin G) provided by Dako (kit K492). Horseradish peroxidase–streptavidin complex was added and revealed by DAB (3,3′-diaminobenzidine tetrahydrochloride), which formed a brown precipitate. Nuclei were counterstained with Mayer’s haematoxylin. Negative controls comprised sections incubated either without specific primary antibody or with a pre-immune serum in place of the specific primary antibody.

Results and Discussion

The MV genome encodes a third serpin protein

We have reported previously the identification of Serp2, an MV-encoded serpin (Petit et al., 1996). Serp2 is located near the right ITR of the genome and is present as a single copy 2 kb away from the serp-1 gene at the right-end region of the genome (Upton et al., 1990). To determine the nucleotide sequence between serp-1 and serp2, we amplified and sequenced the DNA in this region (Fig. 1). Nucleotide analysis of the region adjacent to serp2 revealed an ORF of 801 bp corresponding to a 266 aa polypeptide with three putative N-glycosylation sites (positions 102, 189 and 202) and four potential myristoylation sites (positions 33, 62, 195 and 225). The deduced molecular mass of the polypeptide is about 30-1 kDa. The presence of a TAAAT element immediately upstream of the start codon is strongly suggestive of poxvirus late promoters (Davison & Moss, 1989). A PSI-BLAST search revealed that this protein is a member of the serpin superfamily and, therefore, it was named Serp3. The complete sequence of MV DNA has been recently made available (Cameron et al., 1999) and the published region between serp-1 and serp2 matches exactly our deposited sequence of serp3.

After two iterations, over 200 members of the serpin superfamily were identified with EXPECT scores ranging from 1 × 10−67 to 5 × 10−8. These are highly significant scores that are well below the significance threshold of 1 × 10−6 (Park et al., 1998). Serp3 contains several substantial deletions (discussed in detail below) compared with other members of the serpin superfamily. The overall sequence identity of Serp3 to other members of the serpin superfamily is quite low (e.g. ~19% identity to SPI-1). This is, however, partly a result of the deletions. Serp3 exhibits a high degree of identity (31% over a 73 aa stretch) to the MV serpin Serp2 and, in addition, contains several of the conserved motifs characteristic of serpins (Fig. 2).

Expression of Serp3 in a baculovirus-infected insect cell system

Serp3 was cloned into a pMBac vector and expressed in a baculovirus/Sf9 cell system. pMBac contains a secretory N-terminal signal sequence allowing secretion of the fusion protein into the medium. On day 4 p.i., the supernatant was collected and clarified by low-speed centrifugation. The proteins were then pelleted by ultracentrifugation and resuspended in PBS (pH 8.0). Comparative patterns of the proteins expressed in the recombinant versus the non-recombinant baculovirus allowed identification of a main band with a molecular mass of 120 kDa following SDS–PAGE (data not shown). This band probably corresponds to heat-stable multimers of four subunits of Serp3. The concentration of Serp3 was estimated to be 3 µg/ml of supernatant, representing approximately 50% of total supernatant proteins. However, purification of this high molecular mass protein and injection into rabbits did not result in the production of substantial amounts of anti-Serp3 antibodies.

Expression of serp3 in MV-infected cells

Because of the poor quality of anti-Serp3 antibodies, which was probably due to spontaneous polymerization of the
protein in the baculovirus expression system rendering them less immunogenic when injected into rabbits, it was impossible to visualize Serp3. Both Western blot and immunofluorescence analyses of proteins expressed in MV-infected RK13 cells were inconclusive. However, RT–PCR analysis of total RNA isolated from wild-type virus-infected cells demonstrated that a serp3 transcript was present at 8 h p.i. and could still be detected as late as 16 h p.i. (Fig. 3). No transcript could be detected from either mock- or MV-Serp3−-infected cells. In the presence of AraC, an inhibitor of DNA replication and, thus, of late gene expression, no serp3 transcript could be detected 12 h p.i. These results indicate that serp3 is transcribed as a late and relatively stable mRNA and are consistent with sequence analysis of the promoter region.

**Construction and characterization of MV-Serp3−**

Serp3 is present as a single copy on the MV genome. To construct MV-Serp3−, we disrupted the serp3 ORF by insertion of the lacZ marker gene, under the control of the VV p7.5 promoter, by homologous recombination (Fig. 1). Because of the presence of the lacZ gene within the genome of MV-Serp3− mutants, isolation of virus foci from background was facilitated by staining with X-Gal. We isolated an MV-Serp3− mutant showing a LacZ phenotype. As a control, we constructed a revertant virus derived from MV-Serp3− in which the complete serp3 ORF was restored by homologous recombination with a plasmid containing the serp3 gene. The resulting wild-type-like MV, designated MV-Serp3rev, was screened and purified by several rounds of white–blue screening. To assess serp3 disruption, we analysed MV-Serp3− DNA by PCR with the specific primers serp3−5′ and serp3−3′. No amplification of MV-Serp3− DNA could be observed compared with wild-type MV strain T1 and revertant MV-Serp3rev amplified DNAs; moreover, Southern blot analysis of viral DNAs revealed consistent restriction patterns for wild-type MV, MV-Serp3− and MV-Serp3rev genomes (data not shown).

Single-step growth-curve analysis showed no defects in the ability of MV-Serp3− to replicate in RK13 fibroblasts. Similar results were obtained with cultures of the rabbit CD4+ T-cell line RL5 (data not shown). These results suggest that disruption of Serp3 does not alter the in vitro replicative capacity of the virus.

**Serp3 is a virulence factor in the European rabbit**

European rabbits were inoculated intradermally with $5 \times 10^3$ p.f.u. of MV strain T1 (n = 8), MV-Serp3rev (n = 4) or MV-Serp3− (n = 10). There was a marked reduction of virulence in rabbits infected with MV-Serp3− compared with those infected with either wild-type MV or MV-Serp3rev (Table 1). On day 4 p.i., there was a typical red, raised and soft nodule (primary myxoma) at the inoculation site in rabbits infected with wild-type MV, whereas in rabbits infected with MV-Serp3−, the lesion was thinner, smaller and less congested. At day 8 p.i., rabbits infected with MV-Serp3− were normally active and displayed signs of respiratory and conjunctival

---

**Fig. 2.** Comparison of carboxy-terminal regions of Serp3 with other serpins. The PFXF motif is shaded; Serp3 contains the sequence STCT in place of this motif. Well-conserved (70%) residues are boxed. The RCL is numbered using the ‘Pn..P3–P2–P1–P1 «–P2 «–P3 «..Pn’ nomenclature [Schechter, 1967 (#461)] where P1–P1 « represents the scissile bond. The conserved hinge region is labelled and spans from P17 to P8. The predicted P1–P1 « of Serp3 is cystine–threonine.

**Fig. 3.** RT–PCR analysis of serp3. RNAs were isolated from mock- (lane 1), wild-type MV- (lanes 2–6, 8) or MV-Serp3−-infected (lane 7) RK13 cells in the absence (lanes 1–7) or presence (lane 8) of 40 µg/ml AraC. RNAs were harvested at 2 (lane 2), 4 (lane 3), 8 (lane 4), 12 (lanes 1, 5, 7, 8) and 16 (lane 6) h p.i. RT–PCR was performed using serp3–5′ and serp3–3′ primers. A molecular mass ladder (MW) is shown on the right.
Table 1. Pathogenicity of MV-Serp3\(^{-}\) in European rabbits

All rabbits infected with either wild-type MV \((n = 8)\) or MV-Serp3\(^{rev}\) \((n = 4)\) were sacrificed due to severity of symptoms. Four out of ten rabbits infected with the MV-Serp3\(^{-}\) mutant survived infection.

<table>
<thead>
<tr>
<th>Day p.i.</th>
<th>Wild-type MV and MV-Serp3(^{rev})</th>
<th>MV-Serp3(^{-})</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Intradermal inoculation with 5000 p.f.u. of virus</td>
<td>Intradermal inoculation with 5000 p.f.u. of virus</td>
</tr>
<tr>
<td>4</td>
<td>Primary lesions at inoculation sites: raised, soft, red (ca. 2 cm)</td>
<td>Primary lesions at inoculation sites: soft, thin, congested (ca. 1.5 cm)</td>
</tr>
<tr>
<td>8</td>
<td>Primary lesions large and diffused. Gram-negative bacterial superinfections of nasal and conjunctival mucosae. Multiple secondary lesions on face and ears. Rabbits prostrated and emaciated</td>
<td>Bacterial infections of nasal and conjunctival mucosae. No secondary lesions on any rabbit. Normal weight. Regular activity</td>
</tr>
<tr>
<td>12</td>
<td>Multiple secondary lesions turning necrotic. Dyspnoea. Severe Gram-negative bacterial infections in conjunctivas and respiratory tract. All rabbits sacrificed due to increased severity of the symptoms</td>
<td>Moderate to severe superinfection of respiratory and conjunctival mucosae. No secondary lesions in nine out of ten rabbits. One rabbit showed a few minute myxomas on the back and nose. These myxomas were neither ulcerative nor necrotic Moderate to severe respiratory infection. Two rabbits were sacrificed due to increased respiratory distress Four rabbits with moderate respiratory infection. Two rabbits were sacrificed at day 19; one rabbit sacrificed at day 20; one rabbit sacrificed at day 25</td>
</tr>
<tr>
<td>14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td></td>
<td>Full regression of the symptoms for four rabbits</td>
</tr>
</tbody>
</table>

bacterial infection only; in contrast, rabbits infected with wild-type MV were prostrated and emaciated and developing clinical signs typical of myxomatosis, with secondary myxomas on the face, back and legs. At day 12 p.i., all rabbits infected with either wild-type MV or MV-Serp3\(^{rev}\) were humanely culled. Of the ten animals that had received MV-Serp3\(^{-}\), all suffered from moderate to severe respiratory superinfections, but only one animal had a few minute myxomas on the back and nose. Six rabbits had to be sacrificed between days 14 and 25 p.i. and four rabbits completely recovered within 30 days p.i. The survival rate of animals infected with MV-Serp3\(^{-}\) was 40%, which contrasts with the 100% mortality typically associated with wild-type MV infection.

Our genetic studies clearly indicate that Serp3 plays a part in the virulence of MV, as the disruption of the corresponding ORF resulted in a decrease of the intensity of the clinical course of infection and overall mortality.

**Histological analysis of lesions from wild-type MV- and MV-Serp3\(^{-}\)-infected rabbits**

We performed a detailed histological examination of tissue material from both the primary (ear) and secondary (parotid lymph node) infection sites at various times during the course of infection. The results of the complete analysis are summarized in Table 2.

At day 4 p.i., the injection sites of the wild-type and the Serp3\(^{-}\) viruses demonstrated the same microscopic lesions, i.e. a light perivascular dermatitis with focal oedema and scattered heterophils. The intensity of inflammatory infiltration (heterophils) was different: light for wild-type MV and minimal for MV-Serp3\(^{-}\). In the parotid lymph node, both the mutant and the wild-type viruses induced a light lymphohistiocytis with lymphoid hyperplasia and diffuse histiocytosis.

At day 8 p.i., the pathological pattern in primary sites was the same for the two viruses. Lesions observed included marked perivascular dermatitis with oedema, accumulation of heterophils (marked for wild-type MV and moderate for MV-Serp3\(^{-}\)), absence of mononuclear cells (light for wild-type MV and moderate for MV-Serp3\(^{-}\)) and a well-developed myxoma (i.e. activated fibroblasts and interstitial mucinosis). The lesion intensity of the myxoma was marked for wild-type MV as opposed to moderate for MV-Serp3\(^{-}\). With both viruses, the parotid lymph node showed the same reaction pattern, i.e. a moderate lymphohistiocytis with lymphoid hyperplasia, diffuse histiocytosis and focal infiltration by heterophils. The main difference was the presence of secondary myxomas in the lymph node of rabbits inoculated with wild-type MV (Fig. 4).

At day 11 p.i., the site of injection with wild-type virus demonstrated a marked perivascular dermatitis with only a light infiltration by heterophils. With the Serp3\(^{-}\) virus, the histological lesions were qualitatively the same but less intense. In particular, the infiltration by heterophils was minimal and mononuclear cells (histiocytes and lymphocytes) were more abundant. Well-developed myxomas were observed in primary sites for all virus strains but secondary myxomas were only...
Table 2. Histological observations of lesions from rabbits infected with either wild-type MV or MV-Serp3−

Samples were taken from lesions at primary (inoculation site) or secondary (parotid lymph nodes) sites. Lesion intensity was graded as minimal (+), light (++) , moderate (+++), marked (++++) or severe (++++++). Lesion topography was assessed as scattered (s), focal (f), multifocal (m), focal extensive (e) or diffuse (d).

<table>
<thead>
<tr>
<th></th>
<th>Wild-type MV and MV-Serp3rev</th>
<th>Wild-type MV and MV-Serp3rev</th>
<th>Wild-type MV and MV-Serp3rev</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Primary sites</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Perivascular dermatitis with:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>epidermal spongiosis and vesicles</td>
<td>-</td>
<td>++ (e)</td>
<td>++ (e)</td>
</tr>
<tr>
<td>oedema</td>
<td>+ + (e)</td>
<td>++ (d)</td>
<td>++ (d)</td>
</tr>
<tr>
<td>focal interstitial microhaemorrhage</td>
<td>+</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>focal thrombosis</td>
<td>-</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>inflammatory infiltrates of:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>heterophils (perivascular and interstitial)</td>
<td>+ + (s)</td>
<td>++ (f)</td>
<td>++ (f)</td>
</tr>
<tr>
<td>histiocytes and lymphocytes (interstitial)</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>activated fibroblasts</td>
<td>+ +</td>
<td>+ +</td>
<td>+ +</td>
</tr>
<tr>
<td>interstitial mucinosis</td>
<td>+ +</td>
<td>+ +</td>
<td>+ +</td>
</tr>
<tr>
<td><strong>Secondary sites</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymphadenitis with:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>lymphodepletion</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>lymphoid hyperplasia</td>
<td>+ +</td>
<td>+ +</td>
<td>+ +</td>
</tr>
<tr>
<td>focal microhaemorrhage</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>focal histiocytosis</td>
<td>+ + +</td>
<td>+ +</td>
<td>+ +</td>
</tr>
<tr>
<td>infiltration by heterophils</td>
<td>+ + +</td>
<td>+ +</td>
<td>+ +</td>
</tr>
<tr>
<td>activated fibroblasts</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>interstitial mucinosis</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Day 4 p.i. Day 8 p.i. Day 11 p.i.

observed in the lymph nodes of rabbits inoculated with wild-type MV. A light lymphadenitis was seen in the parotid lymph nodes of rabbits inoculated with both viruses.

From these observations, we conclude that the main difference between the two types of virus infection was the presence of secondary myxomas in the parotid lymph nodes of rabbits inoculated with wild-type MV and its absence with mutant MV. Other microscopic lesions induced by the two types of virus were qualitatively the same and only minor differences were observed. Firstly, the inflammatory reaction was more rapidly progressive in lesions induced by MV-Serp3− (at day 12 p.i., mononuclear cells were predominant), and secondly, the myxoma in the primary site (ear) was more developed following wild-type MV infection.

**Virus load in tissues**

To show that the differences found in the clinical course of disease and the histological results between both virus infections were not associated with an impairment of the Serp3− mutant virus to replicate in vivo, we measured the viral antigens in the parotid lymph nodes. Eight days after infection with wild-type MV, some cells in the lymph node (mostly fibroblasts and histiocytes, which are characteristic of secondary myxomas) were positively stained with anti-MV antibodies. With MV-Serp3−, the labelled cell populations were mostly histiocytes and, to a lesser extent, lymphocytes (data not shown). The immunostaining of viral antigens is a semi-quantitative method which revealed that both types of virus could replicate in the lymph nodes.

**Histological assessment of lymphoid apoptosis**

The TUNEL method was used to assess apoptosis of lymphocytes in the parotid lymph node. Uninfected control rabbits showed minimal apoptosis (25–50 apoptotic bodies in each microscope field at a magnification of ×400), which was located mainly in the follicular germinal centres. Apoptosis of lymphocytes from rabbits inoculated with either type of virus
Myxoma virus-encoded Serp3

was comparable and was at the same level and localization as in the controls (data not shown). From these results, we conclude that both viruses induce no significant apoptosis of lymphocytes.

Model building of Serp3

The three-dimensional structure of serpins is well characterized (reviewed by Whisstock et al., 1998). Serpins typically comprise ~350-400 aa arranged into three $\beta$-sheets (termed A, B and C; individual strands within each sheet are denoted s1A–s6A, s1B–s6B, s1C–s4C), nine $\alpha$-helices (denoted hA–hI) and a reactive centre loop (RCL), which is responsible in the inhibitory serpins for interacting with the target proteinase. Fig. 5(A) shows the structure of $\alpha_1$-antitrypsin with the sheets and helices labelled. Fig. 5(B) shows an alignment between Serp3 and $\alpha_1$-antitrypsin. Serp3 contains several substantial deletions. The deletion of secondary structure elements in serpins is not unprecedented; two reports described the deletion of D-helix in the inhibitory poxvirus serpin CrmA (Renatus et al., 2000; Simonovic et al., 2000). Comparison between Serp3 and the template sequence $\alpha_1$-antitrypsin reveals two major deletions (Fig. 5 A, B). The first involves the region flanked by the B-helix (aquamarine; Fig. 5 A, B) and strand s3A of the A $\beta$-sheet. The second involves a deletion of the loop linking the I-helix to strand s5A of the A $\beta$-sheet (mauve; Fig. 5 A, B). In addition, there are several more minor deletions, including a truncation of the A-helix (orange; Fig. 5 A, B) and a deletion of part of the G-helix (orange; Fig. 5 A, B).

The major objective of our modelling studies was to reconcile these four deletions with the serpin fold. Strikingly, three out of the four deletions map to structures that, in full-length serpins, are packed against one another (Fig. 5 A), namely the C-, D-, E- and I-helices (and connecting loop), strand s1A and the N terminus of the A-helix. The clustering of these deletions in the bottom half of the serpin suggests that
Fig. 5. (A) Schematic diagram showing the structure of native α1-antitrypsin. The majority of the A β-sheet is in red, the B β-sheet is in green and the C β-sheet is in yellow. Individual strands within each sheet are labelled. The RCL at the top of the molecule is coloured in magenta. Helices hB, hF and hH are in cyan. The elements of secondary structure that we predict are substantially disrupted in Serp3 by deletions are as follows: hC, hD, hE, s1A and s2A are in aquamarine; hI and the loop connecting it to s5A is in mauve and the N-terminal half of hA is in orange; the loop connecting s3B to hG and half of hG is also in orange. (B) Alignment between Serp3 and α1-antitrypsin. Residues conserved between the two sequences are boxed and in bold. The labelled bars above the alignment show each element and the secondary structure of α1-antitrypsin. Elements of secondary structure that we predict are disrupted are the same as in (A): the truncation of the A-helix in Serp3 is shaded in orange; the major deletion following the B-helix is shaded in aquamarine; the deletion between s3B and the end of the G-helix is in orange and the deletion between hI and s5A is in mauve. The highly conserved motif in the B-helix (PSFV), the conserved asparagine in s3A and the tryptophan at the top of s3A are all shaded in light pink. In addition, the histidine residue in s5A, a highly conserved residue in the shutter region, is shaded in light pink. (C) Model of Serp3 with the same colour scheme as in (A). The disrupted regions are represented by extended loops (in light blue, orange and mauve) and we have not attempted to model their structure. The exception is s2A (aquamarine), which we predict is retained in the Serp3 fold. The PFXF motif is ringed in black.
they may have co-evolved in response to one another. The first major deletion is particularly challenging to explain structurally, since it involves the deletion of several elements of secondary structure. At the N-terminal flank of the deleted region is the B-helix (see Fig. 5 B), which is easily identifiable by the presence of the FSPVS motif (light pink; Fig. 5 B). This highly conserved motif is located at the start of the B-helix in a mobile region termed the shutter (Stein & Carrell, 1995). The C-terminal flank of the deleted s3A region can also be placed with confidence, since it contains the highly conserved asparagine residue located in the shutter region and the tryptophan residue located at the top of s3A, which is present in > 99% of serpin sequences (both residues highlighted in pink; Fig. 5 B). A multiple sequence alignment of 252 different serpins (data not shown) suggests that much of the remaining sequence between the B-helix and s3A can be mapped to strand s2A of the A-β-sheet and the F-helix. Thus, we predict that Serp3 will retain these two structural elements (s2A and the F-helix) and that the C-, D- and E-helices and s1A will be severely shortened, even to the point of being reduced to connecting loops.

The second major deletion is shorter and more straightforward to reconcile with the serpin fold. The deletion comprises the loop that, in full-length serpins, connects the I-helix to s5A of the A-β-sheet (mauve; Fig. 5 A, B). We predict, however, that this deletion will disrupt the I-helix. In serpins of known structure, the I-helix and the loop connecting it to s5A pack against the C-helix (Fig. 5 A), which is deleted in Serp3 (see previous paragraph). The minor deletions in Serp3 include the loop between s3B and the G-helix and part of the G-helix itself. We predict that this deletion will disrupt the G-helix and possibly the first turn of the H-helix. In addition, the A-helix is shortened by three turns in Serp3 (Fig. 5 A, B). Truncation of the A-helix is seen in the X-ray crystal structure of the viral serpin CrmA (Renatus et al., 2000; Simonovic et al., 2000).

Despite these considerable changes, Serp3 retains several characteristic features of inhibitory serpins suggesting that it may retain an inhibitory function or at least the ability to undergo conformational change. Firstly, the shutter region of Serp3 contains three important motifs, the FSPVS motif at the top of the B-helix, the highly conserved asparagine residue in strand s3A and the conserved histidine residue in strand s5A (Fig. 5 B). A structural comparison between the intact and cleaved conformations of x1-antitrypsin suggests that these residues play an important role in controlling conformational changes in serpins (Whisstock et al., 2000). Furthermore, the hinge region (Hopkins et al., 1993) of Serp3 (KTVDATAFS) is similar to the consensus sequence (EXGTEAAAX3T, where X3 is usually a glutamine or a lysine residue and X6 is usually an alanine residue) observed in inhibitory serpins (Fig. 2).

Fig. 5 (C) shows a model of Serp3. The model exhibits good stereochemistry with all residues in allowed conformations. Since the deletions make the structure of the sequences connecting hB to s2A, s2A to hF, s5A to s6A and s3B to hH difficult to accurately model, no attempt was made to predict the structure of these sequences. Hence, these regions are shown as extended loops (aquamarine, mauve and orange; Fig. 5 C). However, it is important to note that these loops are sufficiently long enough to link between the elements of secondary structure that they have to connect. Thus, the model reveals that the serpin fold can be maintained, albeit in a reduced form.

**Formation of myxomas**

A remarkable difference between the wild-type and the mutant viruses was in the complete absence of secondary myxomas in the parotid lymph node draining the MV-Serp3− inoculation site, whereas virus replication and apoptosis were apparently similar to that observed with wild-type virus. Obviously, myxomas do not simply result from the replication of virus, since there can be virus without myxomas. More likely, there is a complex interaction between some cellular components and virus factors, e.g. Serp3. There is a need for a better understanding of the series of events leading to the generation of myxomas. These lesions, characteristic of the disease in European rabbits, consist of activated fibroblasts and interstitial mucinosis. The genesis of myxomas is still a black box to pathologists and virologists and it will be very useful to know more about the different cascading events that trigger their formation.

There are previous reports of the absence of secondary myxomas after inoculation of MV: M-T5−, a mutant virus deficient in the T5 virulence factor, does not induce secondary lesions after infection of European rabbits (Mossman et al., 1996). The same observations were seen in rabbits challenged with M-T4− virus, an MV deficient in RDEL-containing protein (Barry et al., 1997). However, with M-T5− as well as with M-T4−, the phenotype seemed to be correlated with an increased ability of the host to control virus infection at the primary site of inoculation, since infection with either M-T5− or M-T4− resulted in apoptosis of cultured CD4+ T cells (RL5) and primary lymphocytes. In contrast, we have shown that MV-Serp3− could replicate efficiently in vitro in CD4+ T-cells and that its dissemination was not impaired in vivo, as indicated by a similar virus load and an absence of apoptosis in the lymph node as compared to rabbits infected with wild-type MV. This has to be paralleled with observations seen after infection with MV-Serp2−. In these experiments, there were few, if any, secondary myxomas, whereas the virus was fully replication-competent in RL5 cells (Messud-Petit et al., 1998). Although viral antigens could be detected at a level comparable to infection with wild-type virus, the high level of lymphocytic apoptosis in the draining lymph node could have accounted for an impairment of further dissemination of MV-Serp2−.

Three serpins are identified for MV. SERP-1 is a secreted factor that can inhibit plasmin, urokinase-type plasminogen activator, tissue-type plasminogen activator, thrombin and...
factor Xa (Lomas et al., 1993; Macen et al., 1993; Maksymowycz et al., 1996; Nash et al., 1997, 1998). Serp2 remains associated within the cells and has both an anti-inflammatory and an anti-apoptotic activity (Messud-Petit et al., 1998; Petit et al., 1996). As Serp3 has none of the usual characteristics of serpins, it is risky to try to assign it a function at this point. However, it is striking that the three serpin genes are present on the MV genome within 5 kb of each other at the right-end region of the genome (Cameron et al., 1999). Is there any biological significance to such a clustering of genes encoding proteins with similar features? Although possible, it is unlikely that the three MV Serp genes are physically close by accident. Viral serpins might be derived from a common ancestor and Serp3 has retained only the minimal characteristics of the family necessary and sufficient for its function. There might also be a co-operative effect between Serp2 and Serp3. The expression of Serp2 is not impaired in MV-Serp3\(^{-}\) mutants (data not shown), indicating that Serp3 is not involved in the regulation of Serp2 production. A double mutant unable to express both Serp2 and Serp3 is being constructed to help clarify a possible interaction between members of the viral serpin family.

We are grateful to E. Coppens for monitoring the rabbits. This work was supported by a grant from Institut National de la Recherche Agronomique. J.C.W. is a National Health and Medical Research Council of Australia Peter Doherty Research Fellow.

References


---

Received 28 September 2000; Accepted 12 January 2001