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The Cowpox Virus SPI-3 and Myxoma Virus SERP1 Serpins Are Not Functionally Interchangeable despite Their Similar Proteinase Inhibition Profiles *in Vitro*

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The myxoma virus (MYX) serpin SERP1 is a secreted glycoprotein with anti-inflammatory activity that is required for full MYX virulence *in vivo.* The cowpox virus (CPV) serpin SPI-3 (vaccinia virus ORF K2L) is a nonsecreted glycoprotein that blocks cell–cell fusion, independent of serpin activity, and is not required for virulence of vaccinia virus or CPV in mice. Although SPI-3 has only 29% overall identity to SERP1, both serpins have arginine at the P1 position in the reactive center loop, and SPI-3 has a proteinase inhibitory profile strikingly similar to that of SERP1 [Turner, P. C., Baquero, M. T., Yuan, S., Thoennes, S. R., and Moyer, R. W. (2000) *Virology* 272, 267–280]. To determine whether SPI-3 and SERP1 were functionally equivalent, a CPV variant was constructed where the SPI-3 gene was deleted and replaced with the SERP1 gene regulated by the SPI-3 promoter. Cells infected with CPVΔSPI-3::SERP1 secrete SERP1 and show extensive fusion, suggesting that SERP1 is unable to functionally substitute for SPI-3 in fusion inhibition. In the reciprocal experiment, both copies of SERP1 were deleted from MYX and replaced with SPI-3 under the control of the SERP1 promoter. Cells infected with the MYXΔSERP1::SPI-3 recombinant unexpectedly secreted SPI-3, suggesting either that the cellular secretory pathway is enhanced by MYX or that CPV encodes a protein that prevents SPI-3 secretion. MYXΔSERP1::SPI-3 was as attenuated in rabbits as MYXΔSERP1::lacZ, indicating that SPI-3 cannot substitute for SERP1 in MYX pathogenesis.

INTRODUCTION

Collectively, poxviruses share a number of characteristics including replication within the cytoplasm of the host cells and a large double-stranded DNA genome with closed hairpin termini (Moss, 1996). However, the diseases caused by these viruses (Fenner, 1996) vary from restricted and relatively benign (e.g., swinepox and molluscum contagiosum) to systemic and with a high mortality rate (e.g., myxomatosis and smallpox). Early during infection the host defenses are mostly nonspecific and include inflammation, the cytolytic action of natural killer cells, the activation of cytokines and chemokines, and programmed cell death. Poxviruses encode a number of proteins that deflect these host responses (Nash *et al.,* 1999; Pickup, 1994; Smith *et al.,* 1997).

Members of the serpin superfamily of serine proteinase inhibitors are included among these poxvirus immunomodulatory proteins. Serpins regulate a variety of biological processes dependent on proteinases, including the maturation of proinflammatory cytokines, apoptosis, complement activation, fibrinolysis, blood clotting, and tissue remodeling (for a review see Potempa *et al.,* 1994). Despite the widespread use of serpins to control cellular pathways, poxviruses are the only family of viruses known to encode functional serpins.

Serpins inhibit specific proteinases by acting as pseudosubstrates. A key region of the serpin that resembles the substrate normally recognized by the proteinase is the reactive center loop (RCL), located near the C-terminus. The RCL protrudes from the surface of the globular serpin and contains a key residue (P1) responsible for proteinase specificity. Inhibition of the proteinase is initiated when the proteinase attacks the bond between the P1 and adjacent P1′ residues of the serpin, leading to the formation of a stable serpin/proteinase complex (Potempa *et al.,* 1994).

The leporipoxvirus myxoma virus (MYX) causes a rapid and fatal systemic disease (myxomatosis) in the European rabbit (*Oryctolagus cuniculus*) that includes immunosuppression (Fenner, 1996; McFadden, 1994). The MYX genome has been completely sequenced (Cameron *et al.,* 1999) and encodes two intact serpins known as SERP1 (Macen *et al.,* 1993; Upton *et al.,* 1990) and SERP2 (Messud-Petit *et al.,* 1998; Petit *et al.,* 1996), together with a truncated serpin SERP3. The SERP2 protein has some resemblance to the crmA/SPI-2 serpin encoded by the orthopoxviruses in that it has been reported to inhibit the interleukin-1β convertase (ICE) (Petit *et al.,* 1996). SERP2 mutants of MYX are strongly atten-

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uated (Messud-Petit *et al.,* 1998), indicating that the natural target of the SERP2 protein plays an important role in host defense against myxoma virus.

The myxoma SERP1 protein (Macen *et al.,* 1993; Nash *et al.,* 1997; Upton *et al.,* 1990) is also required for full virulence of wild-type myxoma virus. Mutation of both copies of the SERP1 gene within the terminal inverted repeats results in a significant attenuation of myxoma virus, such that more than half of the infected animals are able to recover from an otherwise lethal infection (Macen *et al.,* 1993). A mutant of malignant rabbit fibroma virus in which SERP1 had been inactivated was also attenuated relative to wild type (Upton *et al.,* 1990).

The SERP1 protein has been shown to be involved in the suppression of inflammation following infection with wild-type myxoma virus (Macen *et al.,* 1993). Purified SERP1 protein has proved to have potent anti-inflammatory activity in animal models of arthritis (Maksymowych *et al.,* 1996) and to inhibit restenosis following angioplasty (Lucas *et al.,* 1996). SERP1 is a secreted glycoprotein that is expressed late during infection (Macen *et al.,* 1993) and has been shown to inhibit the serine proteinases plasmin, urokinase, and tissue plasminogen activator (tPA) *in vitro* (Lomas *et al.,* 1993) and also thrombin and factor Xa (Nash *et al.,* 1998), consistent with the P1/P1′ sequence of Arg/Asn. Whether any one of these inhibited proteinases is the true "natural target" of SERP1 is not clear.

The orthopoxviruses (e.g., variola, cowpox, vaccinia, and rabbitpox viruses) encode three serpins. The crmA/SPI-2 protein is a potent inhibitor of ICE (caspase-1) (Ray *et al.,* 1992) and of some other caspases (Zhou *et al.,* 1997), thus conferring both anti-inflammatory and anti-apoptosis activities (Bird, 1998; Cohen, 1997). CrmA is termed a cross-class proteinase inhibitor because it inhibits both cysteine proteinases and the serine proteinase granzyme B (Quan *et al.,* 1995). A second orthopoxvirus serpin, SPI-1, is associated with the maintenance of full host range and inhibiting the chymotrypsin-like proteinase cathepsin G (Moon *et al.,* 1999).

The third orthopoxvirus serpin SPI-3 (ORF K2L in vaccinia virus) is known to inhibit cell–cell fusion following infection (Law and Smith, 1992; Turner and Moyer, 1992a; Zhou *et al.,* 1992). SPI-3, like SERP1, has extensive N-linked glycosylation, but unlike SERP1 is expressed at early times in infection (Turner and Moyer, 1995) and is not secreted. Although SPI-3 and SERP1 share only 29% overall identity, typical of any two unrelated serpins, SPI-3 is predicted to have the same P1 Arg residue. However, unlike SERP1, SPI-3 does not appear to be required for virulence as SPI-3 deletion mutants in vaccinia virus (VV) and cowpox virus (CPV) inoculated intranasally were as lethal in mice as wild-type viruses (Law and Smith, 1992; Thompson *et al.,* 1993). Despite the SPI-3 protein having all the features characteristic of an inhibitory serpin, analysis by site-directed mutagenesis indicated that these motifs were not required for the SPI-3 protein to inhibit cell fusion (Turner and Moyer, 1995).

We have shown that SPI-3 protein, like SERP1, is able to form stable complexes with and inhibit the proteinases plasmin, urokinase, and tPA (Turner *et al.,* 2000). As with SERP1, some complex formation is also noted between SPI-3 and thrombin or factor Xa (Nash *et al.,* 1998). Hence, the overall inhibitory spectrum of SPI-3 appears to be very similar to that of SERP1, suggesting that these two proteins may have a similar function *in vivo.* Therefore, SERP1 was tested for its ability to substitute for SPI-3 in cowpox virus in suppressing cell fusion, and SPI-3 was tested for the ability to function in place of SERP1 in conferring full virulence on myxoma virus. When the ORFs were exchanged between the two viruses the natural promoters were left intact in each case, such that the CPV recombinant expressed SERP1 from the early SPI-3 promoter, and the MYX recombinant expressed SPI-3 from the late SERP1 promoter. Our results indicate that SERP1 could not substitute for SPI-3 and vice versa, despite the similar biochemical activity of the two serpins *in vitro.*

RESULTS

Construction and characterization of a CPV recombinant deleted for SPI-3 and expressing SERP1 as an early protein

The recombinant virus CPVΔSPI-3::SERP1 was created following transfection of wtCPV infected cells with the plasmid $pP_{SPI\text{-}3}$-SERP1-$P_{7.5}$gpt (Materials and Methods), which allows deletion of the SPI-3 ORF and replacement by the SERP1 ORF under the control of the SPI-3 early promoter. This plasmid also introduces the *gpt* gene downstream of the SERP1 gene (Fig. 1), enabling selection of recombinants by mycophenolic acid resistance. Deletion of the SPI-3 gene was confirmed by both Western blot and PCR analysis, and insertion of the SERP1 ORF was verified by PCR (data not shown). Expression of SERP1 by CPVΔSPI-3::SERP1 was initially confirmed by immunoblotting of intracellular and extracellular fractions from infected cells with SERP1 monoclonal antibody (Fig. 2A). No SERP1 was detected in extracts of mock or wtCPV infected cells as expected (Fig. 2A, lanes 1–4). An immunopositive band of approximately 55 kDa was present in the supernatants of cells infected with the CPVΔSPI-3::SERP1 recombinant and from control wtMYX infected cells (Fig. 2A, lanes 6 and 8). Little if any SERP1 was detected in the cellular fraction of these cells (Fig. 2A, lanes 5 and 7), indicating that as expected most of the SERP1 was secreted when expressed from either virus. To investigate the possibility that SERP1 is present in the supernatants of CPVΔSPI-3::SERP1 infected cells as a constituent of extracellular enveloped virus (EEV) rather than as an independently

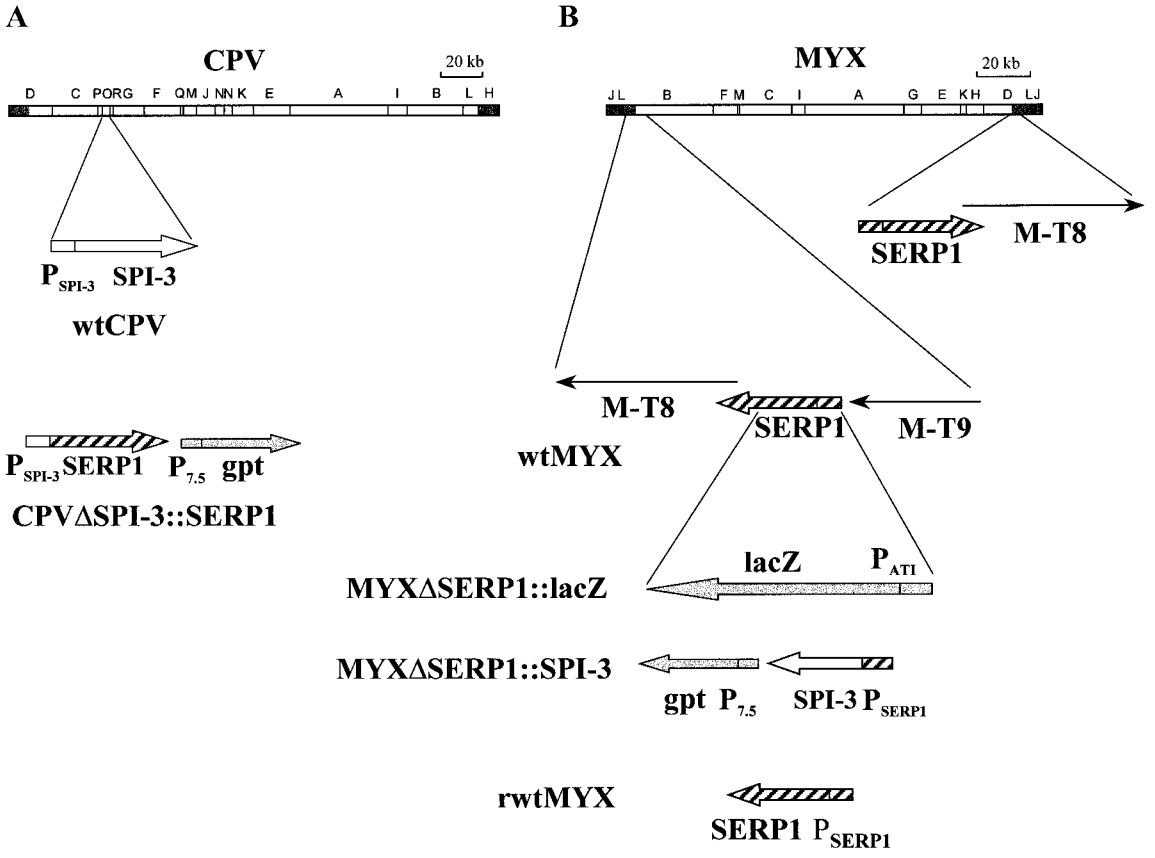

FIG. 1. Construction of the recombinant cowpox and myxoma viruses. (A) The map position of the SPI-3 gene (equivalent to vaccinia virus ORF K2L) is shown within the wild-type CPV *Hin*dIII genomic map (Mackett and Archard, 1979). Gray boxes represent the terminal inverted repeats (TIRs). Below the map, the structure of CPVΔSPI-3::SERP1 is represented with SERP1 under the control of the early SPI-3 promoter. The $P_{7.5}$-gpt cassette was inserted downstream of the SERP1 gene to facilitate selection of recombinant viruses. (B) The structures of myxoma virus derivatives are shown. The location of the region within the TIR encoding the SERP1 gene and neighboring ORFs M-T9 and M-T8 is shown below the MYX *Hin*dIII map (Russell and Robbins, 1989). Note that there is an overlap of 23 bp between the 3′ end of the SERP1 ORF and the 5′ end of M-T8. In MYXΔSERP1::lacZ the bulk of the SERP1 ORF (excluding the region that overlaps M-T8) has been replaced by the lacZ gene driven by the CPV ATI promoter. In MYXΔSERP1::SPI-3, the CPV SPI-3 gene is expressed from the late SERP1 promoter. Recombinants containing the $P_{7.5}$-gpt cassette were selected by resistance to mycophenolic acid. The reconstructed wild-type myxoma recombinant (rwtMYX2) was derived from MYXΔSERP1::SPI-3 as described in the text.

secreted protein, the medium harvested from infected RK-13 cells was further fractionated by high-speed centrifugation under conditions that sediment EEV. SERP1 was found solely in the cleared supernatant of cells infected with CPVΔSPI-3::SERP1, and none was detected in the pellet fraction that contains EEV (data not shown). Hence, expression of SERP1 from within a CPV context does not alter the natural secretion pattern of SERP1 seen in MYX infected cells.

The SPI-3 gene of CPV is normally expressed early prior to DNA synthesis (Smith *et al.,* 1989; Turner and Moyer, 1995), whereas MYX SERP1 is a late gene, expressed only after DNA synthesis (Macen *et al.,* 1993). The recombinant CPVΔSPI-3::SERP1 was designed so that the SERP1 ORF would be regulated by the SPI-3 promoter and hence should be expressed early. SERP1 expression in CPVΔSPI-3::SERP1 infected cells was evaluated in the presence or in the absence of cytosine arabinoside (AraC), an inhibitor of viral DNA synthesis and late gene expression. Supernatants were harvested at 4, 8, 12, and 16 h postinfection (p.i.) and analyzed by immunoblot analysis with SERP1 monoclonal antibody. SERP1 protein was present in the supernatants of infected cells harvested at 8, 12, and 16 p.i., both in the absence (Fig. 2B, lanes 3, 5, and 7) and in the presence (lanes 4, 6, and 8) of AraC. Little or no SERP1 was present in the infected cell pellets (data not shown), indicating that secretion was very efficient. These data indicate that SERP1 is being expressed early by CPVΔSPI-3::SERP1 and show that SERP1 is secreted whether synthesized early in CPV or late in MYX.

SERP1 fails to substitute for SPI-3 in inhibiting cell–cell fusion in CPV infected cells

One of the known functions of SPI-3 protein is to inhibit infected cell–cell fusion (Law and Smith, 1992; Turner and Moyer, 1992a; Zhou *et al.,* 1992). The ability of SPI-3

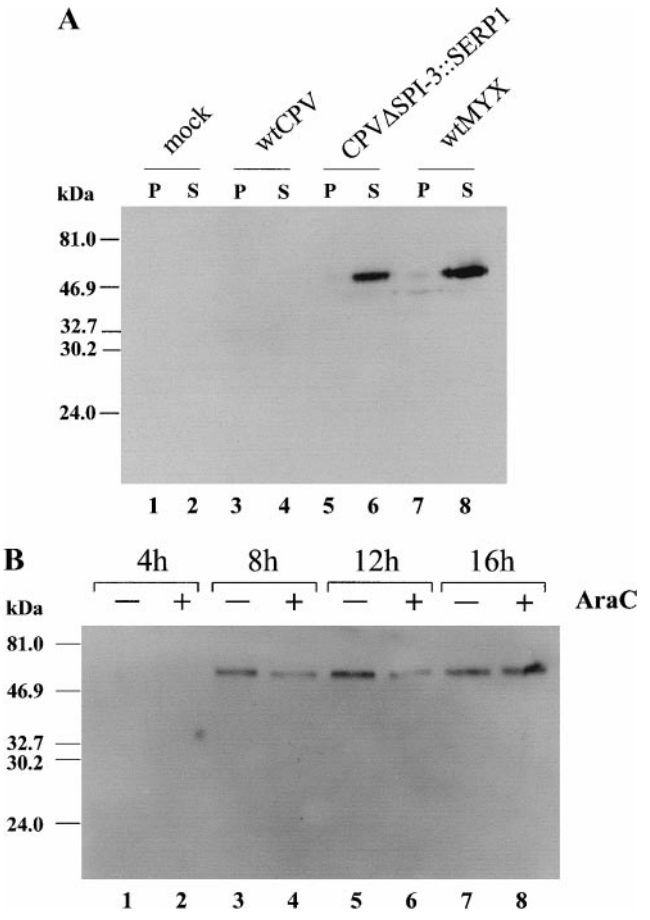

FIG. 2. Expression of SERP1 by CPVΔSPI-3::SERP1 in the presence and in the absence of AraC. (A) RK-13 cells were infected with the indicated viruses at an m.o.i. of 10, harvested at 20 h postinfection, and fractionated into cell-associated pellet (P) and supernatant (S) fractions by low-speed centrifugation. The supernatants were concentrated by ultrafiltration as described under Materials and Methods. Proteins within the pellet and supernatant fractions were subjected to Western blot analysis using anti-SERP1 sera following separation on SDS–polyacrylamide gels. Lane 1, cell-associated pellet from mock-infected cells; lane 2, supernatant from mock-infected cells; lanes 3 and 4, pellet and supernatant, respectively, from wtCPV infected cells; lanes 5 and 6, pellet and supernatant from CPVΔSERP1::SPI-3 infection; lanes 7 and 8, pellet and supernatant from cells infected with wtMYX. (B) RK-13 cells were infected with CPVΔSPI-3::SERP1 at an m.o.i. of 10 in the presence or in the absence of 40 μg/ml AraC. The infected cell supernatants were collected at the times postinfection indicated above each lane and concentrated. Expression of SERP1 was assayed by Western blotting of proteins separated on SDS–polyacrylamide gels. Duplicate samples of infected cells were prepared either in the absence (lanes 1, 3, 5, and 7) or in the presence (lanes 2, 4, 6, and 8) of AraC.

to control cell fusion does not depend on the RCL of the serpin, indicating that serpin activity and control of fusion are two independent functions of the protein (Turner and Moyer, 1995). As the orthopoxvirus hemagglutinin also inhibits fusion (Ichihashi and Dales, 1971) and is glycosylated, we hypothesized that the presence of N-linked glycosylation may be connected with fusion inhibition. We asked whether the glycoprotein SERP1 can substitute for SPI-3 to prevent fusion of CPVΔSPI-3::SERP1 infected cells. Monolayers of CV-1 cells were infected at low multiplicity with wtCPV, CPVΔSPI-3, or CPVΔSPI-3::SERP1 under liquid medium and examined 24 h postinfection. The plaques formed in cells infected with CPVΔSPI-3::SERP1 (Fig. 3C) show extensive fusion as do CPVΔSPI-3 plaques (Fig. 3B) and were clearly differentiated from wild-type CPV plaques, which produced minimal cytopathic effect and no fusion under these conditions (Fig. 3A). Plaques of CPVΔSPI-3::SERP1 were still able to hemadsorb chicken red blood cells (data not shown); i.e., they expressed a functional hemagglutinin as expected. The fact that CPVΔSPI-3::SERP1 is fusogenic is therefore not due to an unintended mutation in the HA gene, but rather indicates that SERP1 is unable to substitute for SPI-3 to inhibit fusion in CPV infected cells.

Replacement of the myxoma virus SERP1 gene with the cowpox virus SPI-3 gene

Although SERP1 has a role in MYX virulence, the gene is dispensible for replication of MYX in tissue culture (Macen *et al.,* 1993; Upton *et al.,* 1990). Since the spectrum of inhibited proteinases appears similar between SPI-3 and SERP1 (Turner *et al.,* 2000), we asked whether SERP1 could be functionally replaced by SPI-3. SERP1 is present in two copies within the inverted repeat regions of MYX. Therefore, we first constructed a MYX deletion mutant (MYXΔSERP1::lacZ; Fig. 1) in which both copies of SERP1 were replaced with the *lacZ* gene under the control of the CPV ATI promoter. This mutant provides a control for the effect of SERP1 on virulence of the virus and was obtained by infecting RK-13 cells with wild-type MYX and transfecting with pSERP1-ATI/lacZ plasmid DNA (Materials and Methods). Several recombinant plaques expressing lacZ, based on X-gal staining, were picked and replaqued several times until all the resulting progeny were blue. This procedure maximizes the likelihood that both copies of SERP1 have been replaced by lacZ, as heterozygotes when replaqued yield a mixture of both white and blue plaques. Potential MYXΔSERP1::lacZ recombinants were tested by both PCR and immunoblotting to verify that both copies of the SERP1 gene had been removed and that there was no expression of SERP1 (data not shown).

Virus in which the lacZ gene was replaced by SPI-3 to generate MYXΔSERP1::SPI-3 (Fig. 1) was generated by transfecting pP_{SERP1}-SPI-3-$P_{7.5}$gpt transfer plasmid DNA into RK13 cells infected with MYXΔSERP1::lacZ followed by selection for mycophenolic acid-resistant plaques. The initial recombinants were still lacZ positive as presumably only one copy of the lacZ gene had been replaced by the SPI-3-gpt cassette. Individual plaques were replaqued three times in the presence of mycophenolic acid, which resulted in isolates yielding only white plaques, consistent with replacement of both copies of the lacZ gene with the SPI-3-gpt cassette. This virus

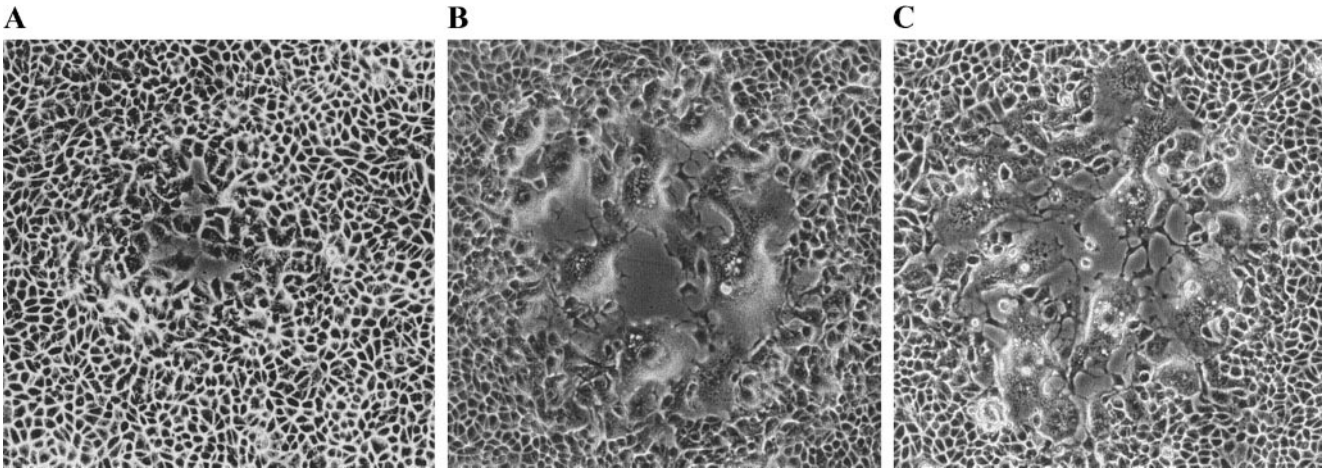

FIG. 3. CPV-mediated fusion of infected cells. Confluent monolayers of CV-1 cells were infected at low multiplicity in liquid medium and individual plaques were photographed using phase-contrast microscopy at 24 h postinfection. A single representative plaque is shown from each infection. (A) wtCPV. (B) CPVΔSPI-3. (C) CPVΔSPI-3::SERP1.

(MYXΔSERP1::SPI-3) was shown to lack the SERP1 and lacZ genes by PCR and to lack SERP1 expression by immunoblot analysis (not shown). In order to confirm that any effects of deleting SERP1 and replacing with SPI-3 were not due to unintended mutations, a reconstructed wild-type virus (rwtMYX2) was obtained by infecting with MYXΔSERP1::SPI-3, transfecting with a plasmid clone containing the wtSERP1 gene and flanks, and selecting for recombinants that had lost the gpt cassette by reverse selection for resistance to 6-thioguanine (Isaacs *et al.,* 1990) using HGPRT$^-$ HeLa cells (Kerr *et al.,* 1991). rwtMYX2 expressed SERP1 protein in the supernatant as anticipated.

Secretion of SPI-3 by MYXΔSERP1::SPI-3 infected cells

The presence of the SPI-3 gene in MYXΔSERP1::SPI-3 was confirmed by PCR (data not shown), and expression of SPI-3 protein was assessed by Western blot analysis of infected cells, examining both cell pellets and low-speed supernatants (Fig. 4). As expected, no synthesis of SPI-3 was noted in mock or wtMYX infected cells (Fig. 4, lanes 1, 2 and 8, 9). No SPI-3 was present in the cell pellet after infection with MYXΔSERP1::SPI-3 (Fig. 4, lane 7), but instead SPI-3 was found in the supernatant as 57- and 55-kDa proteins (Fig. 4, lane 3). To evaluate the possibility that the SPI-3 protein produced by MYXΔSERP1::SPI-3 was not truly secreted, but rather associated with released EEV, medium from infected RK-13 cells was collected at 20 h postinfection and separated into cleared supernatant and extracellular virus fractions by high-speed centrifugation. SPI-3 was found exclusively in the high-speed supernatant, indicating that it was not associated with EEV and was a true secreted protein (data not shown).

SPI-3 in CPV infected cells is normally expressed in small amounts and is not secreted (Turner and Moyer, unpublished data). For the comparisons shown here, the vaccinia/T7 expression system (Fuerst *et al.,* 1986) was used to overexpress SPI-3 to ensure reproducibly detectable levels of SPI-3. As previously reported (Turner and Moyer, 1995), vTF7-3 and VV-P_{T7}–SPI-3 mixed infections produce a large amount of SPI-3 that migrates as 50- and

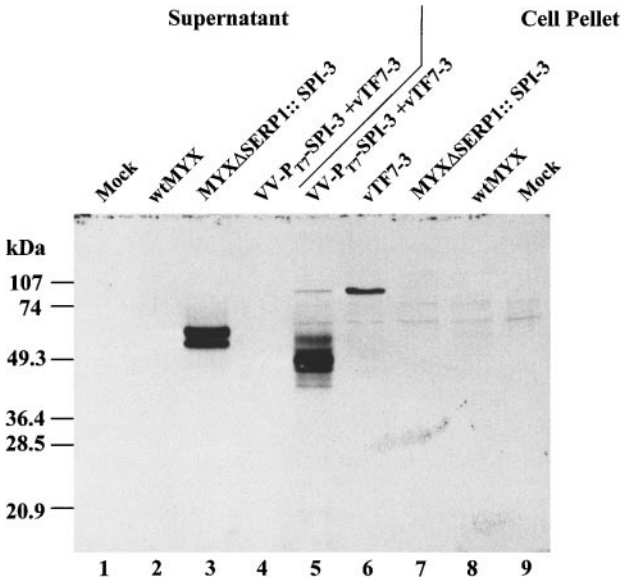

FIG. 4. Comparison of SPI-3 synthesized by vaccinia/T7 expression and by MYXΔSERP1::SPI-3. RK-13 cells were infected either with vTF7-3 and VV-P_{T7}-SPI-3 (for expression in the VV/T7 system) or with wtMYX or MYXΔSERP1::SPI-3. Infected cells were harvested at 20 h postinfection, separated into supernatant and cell-associated pellet fractions, and subjected to Western blot analysis with anti-SPI-3 sera. Lanes 1–4 are from supernatants, and lanes 5–9 are from cell pellets, as indicated above the blot. Lanes 1 and 9, mock-infected RK-13 cells; lanes 2 and 8, wtMYX infected cells; lanes 3 and 7, MYXΔSERP1::SPI-3 infected cells. Lanes 4 and 5 are from cells coinfected with vTF7-3 and VV-P_{T7}-SPI-3. Lane 6 is the cell pellet from infection with vTF7-3 alone.

48-kDa glycosylated proteins. This overexpressed SPI-3, like naturally expressed SPI-3, remains within the cell (Fig. 4, lane 5) and was not found in the cleared supernatant (Fig. 4, lane 4). Hence within the context of vaccinia virus, no overexpressed SPI-3 was secreted.

The extent of SPI-3 glycosylation is different in MYX and VV infected cells

Inspection of the data of Fig. 4 shows that the SPI-3 protein secreted from MYXΔSERP1::SPI-3 infected cells appears somewhat larger than the SPI-3 synthesized from vaccinia virus (Fig. 4, compare lanes 3 and 5). Since RK-13 cells were the host cells in both cases, this difference is dependent on the context (orthopoxvirus or leporipox virus) in which SPI-3 is expressed. The difference in apparent molecular weights between the SPI-3 proteins synthesized by the MYX recombinant and by the vaccinia/T7 system might be due to differences in the extent of N-linked glycosylation of SPI-3, which has four potential sites for modification. N-linked glycosylation of SPI-3 isolated from vTF7-3 and VV-P_{T7}-SPI-3 coinfected cells was confirmed by using the inhibitor tunicamycin. The apparent size of SPI-3 from vaccinia/T7 expression was reduced in the presence of tunicamycin (Fig. 5A, lane 3) compared with expression in the absence of the inhibitor (Fig. 5A, lane 1). If differential glycosylation does account for the apparent size difference for SPI-3 from vaccinia/T7 and from the MYX recombinant, then complete deglycosylation of SPI-3 protein from the two sources should generate polypeptides of the same apparent molecular weight. SPI-3 from VV/T7 and from MYX was compared following treatment with peptide-*N*-glycosidase F (PNGase F), an enzyme that completely removes all types of *N*-glycan chains from glycoproteins. After complete digestion with the enzyme, both proteins migrate with an identical mobility of 42 kDa, consistent with the primary amino acid sequence of SPI-3 (compare lanes 2 and 5 in Fig. 5A). Therefore, differences in glycosylation appear to account for the different electrophoretic mobilities of SPI-3, which in turn are determined by the virus from which the protein is expressed.

We explored the possibility that the different apparent masses of SPI-3 derived from vaccinia and MYX infected cells (Fig. 4) represent differences in how many potential N-glycosylation sites are utilized. SPI-3 protein from the supernatants of RK-13 cells infected with MYXΔSERP1::SPI-3 was partially digested with PNGase F to remove N-linked sugars (Fig. 5B, lanes 2–4). Incomplete digestion produces a total of five protein bands, the smallest of which corresponds in size to the expected size (42 kDa) of the SPI-3 polypeptide predicted from the sequence (Fig. 5B). A similar pattern of five SPI-3 proteins has been seen when the SPI-3 gene is transcribed and translated *in vitro* in the presence of canine microsomal membranes (Turner *et al.,*

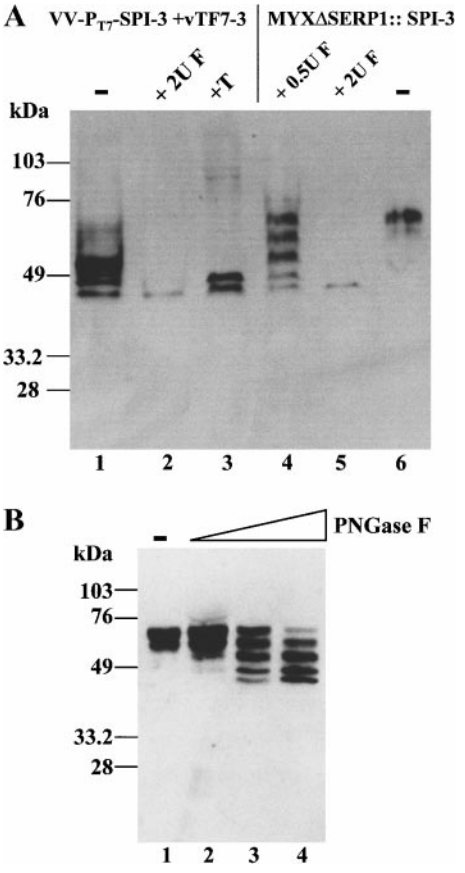

FIG. 5. Effect of endoglycosidase treatment on SPI-3 isolated from vaccinia/T7 expression or from MYXΔSERP1::SPI-3 infection. (A) Digestion with PNGase F of SPI-3 derived from vTF73/VV-P_{T7}-SPI-3 coinfected cells (lanes 1–3, cell pellets) or from MYXΔSERP1::SPI-3 (lanes 4–6, supernatants). Lane 1, extract from RK-13 cells coinfected with VV-P_{T7}-SPI-3 and vTF7-3 (overexpressed SPI-3), untreated with PNGaseF. Lane 2, cellular extract containing overexpressed SPI-3 from VV, digested to completion with 2 units PNGase F. Lane 3, cell extract containing SPI-3 overexpressed in the presence of tunicamycin. Lane 4, partially PNGase F-digested SPI-3 from supernatant of MYXΔSERP1::SPI-3-infected RK-13 cells, treated with 0.5 units PNGaseF. Lane 5, completely digested SPI-3 from MYXΔSERP1::SPI-3 supernatant, treated with 2 units PNGase F. Lane 6, untreated SPI-3 from MYXΔSERP1::SPI-3 supernatant. (B) Partial digestion of secreted SPI-3 from MYXΔSERP1::SPI-3 with peptide N-glycosidase F (PNGase F). Supernatants were either untreated (lane 1) or treated with 0.1, 1, or 2 units PNGase F (lanes 2, 3, and 4, respectively) for 16 h at 37°C before immunoblotting with anti-SPI-3 serum.

2000). Collectively, these results suggest that all four potential N-linked glycosylation sites in the SPI-3 protein are utilized during a MYX infection. The increased size of SPI-3 expressed from the MYX recombinant compared with VV/T7 expression suggests that N-linked glycans of greater molecular mass are being added at the four glycosylation sites in MYX-infected cells; i.e., the ladder of five bands is more widely spaced from MYX than from vaccinia. The different MYX SPI-3 glycosylation pattern may be due in part to the addition of sialic acid residues by the MYX-encoded α2,3-sialyltransferase (Jackson *et al.,* 1999), which is absent from the orthopoxviruses.

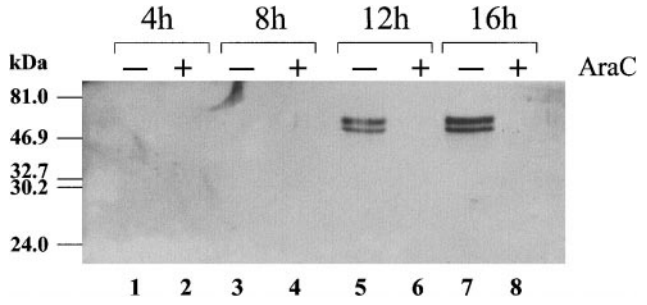

FIG. 6. Time course of SPI-3 secretion by MYXΔSERP1::SPI-3. The supernatants of RK-13 cells infected with MYXΔSERP1::SPI-3 in the absence or in the presence of AraC were harvested at the times postinfection indicated above the lanes, concentrated, and immunoblotted with anti-SPI-3 serum. Lanes 1, 3, 5, and 7 are from infections without AraC, and lanes 2, 4, 6, and 8 are from infections with AraC added at 40 μg/ml. Lanes 1 and 2 are from supernatants removed at 4 h postinfection; lanes 3 and 4, 8 h p.i.; lanes 5 and 6, 12 h p.i.; lanes 7 and 8, 16 h p.i.

MYXΔSERP1::SPI-3 expresses SPI-3 as a late protein

In the MYXΔSERP1::SPI-3 recombinant, the SPI-3 ORF was placed under control of the SERP1 promoter, which should render expression late and susceptible to inhibition by viral DNA synthesis inhibitors such as AraC. Supernatants from RK-13 cells infected with MYXΔSERP1::SPI-3 either in the presence or in the absence of AraC were harvested at 4, 8, 12, and 16 h p.i., concentrated, and analyzed for SPI-3 expression. SPI-3 synthesis was apparent at 12 and 16 h p.i. in the absence of the DNA replication inhibitor AraC (Fig. 6, lanes 5 and 7), but absent when AraC was present (Fig. 6, lanes 6 and 8). These results suggest that SPI-3 is indeed expressed late following infection with MYXΔSERP1::SPI-3 as is SERP1 following a wtMYX infection.

Phenotype in infected rabbits of myxoma virus derivatives expressing SERP1 or SPI-3

To test the ability of SPI-3 to substitute for SERP1 in MYX virulence, male New Zealand White rabbits (*O. cuniculus*) were infected with wtMYX, MYXΔSERP1::lacZ, MYXΔSERP1::SPI-3, or rwtMYX2 virus. The rabbits were injected intradermally with 500 PFU in each flank (a total of 1000 PFU per rabbit) and were monitored on a daily basis for progression of disease symptoms. Primary lesions at the site of inoculation are shown in Fig. 7A, and secondary lesions in the ears following virus spread are shown in Fig. 7B.

All of the myxoma viruses caused a small raised lesion at the inoculation site by day 3 (Fig. 7A). However, after day 5, several differences became apparent between

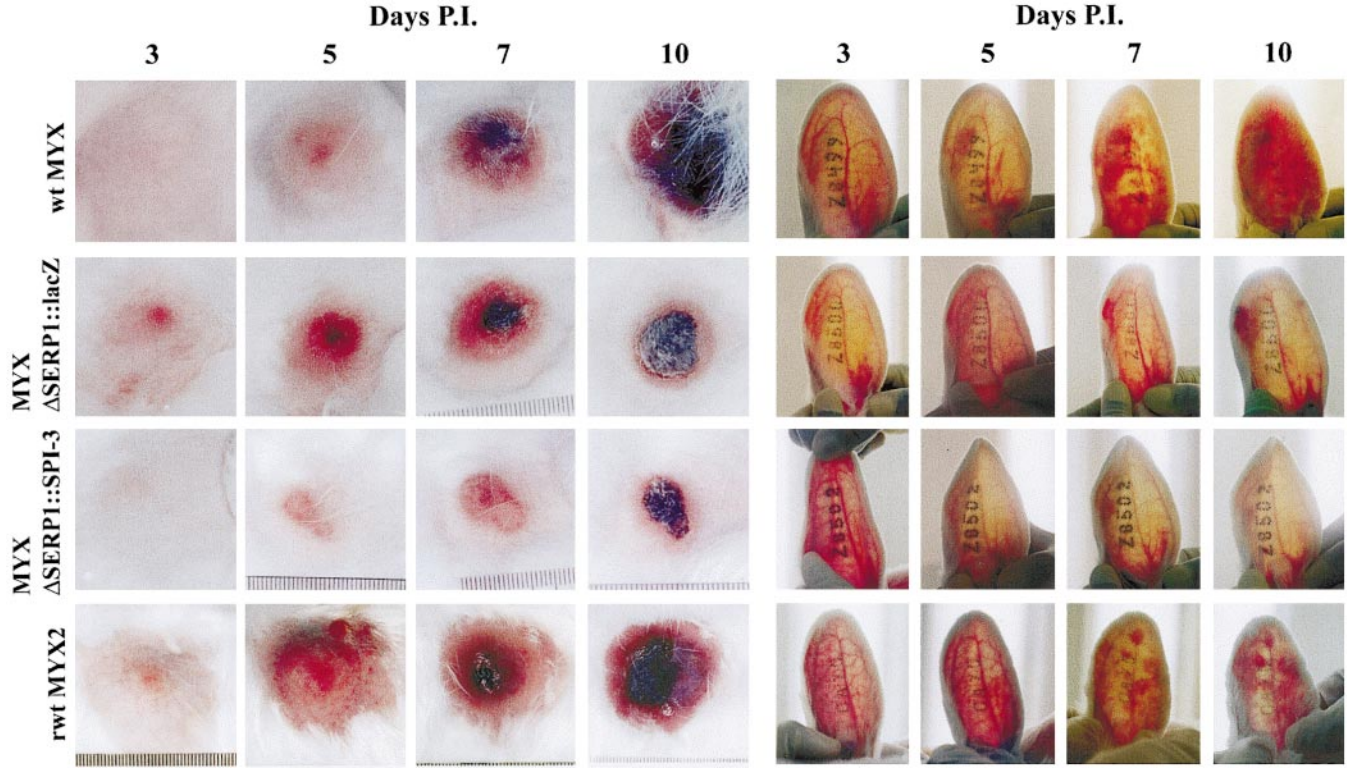

FIG. 7. Primary and secondary lesions produced in rabbits by MYX recombinants. Rabbits were infected intradermally in each flank with 500 PFU of virus and photographed at days 3, 5, 7, and 10 p.i., as shown above the figure. The first row of panels is from wtMYX infected rabbits, the second is from MYXΔSERP1::lacZ, the third is from MYXΔSERP1::SPI-3, and the fourth is from rwtMYX2. Each series is of the same primary lesion or ear followed over time and was representative of other animals in the same experimental group. (A) Primary lesions, photographed with ring flash. (B) Ears showing secondary lesions, photographed with backlighting.

animals infected with the different viruses. For animals infected with wtMYX or with rwtMYX2, the primary lesions developed rapidly, becoming elevated and moist. Blistering and hemorrhaging of the primary lesions of wtMYX2 and rwtMYX2 infected rabbits was evident at day 10. The final sizes of primary lesions for wtMYX and for rwtMYX2 were from 3.0 to 4.0 cm in diameter. In contrast, rabbits infected with either MYXΔSERP1::lacZ or MYXΔSERP1::SPI-3 had primary lesions that developed much more slowly (final size of 1.5–2.5 cm), and by day 7 they were starting to necrose and resolve. The primary lesions from MYXΔSERP1::lacZ or MYXΔSERP1::SPI-3 infections were flat and dry from day 7 onward, and the central areas of the lesions became covered with a dark purple scab (Fig. 7A). Histological analysis of primary lesions harvested at 5 days p.i. showed severe edema and dermal thickening resulting from wtMYX or rwtMYX2 infection and much less pronounced edema and thickening after infection with MYXΔSERP1::lacZ or MYXΔSERP1::SPI-3 (data not shown), consistent with the gross pathology (Fig. 7A).

Secondary lesions, indicative of viremia, were first noted in the ears of wtMYX and rwtMYX2 infected animals by day 5 and continued to develop until at day 10 the ears had near confluent hemorrhagic secondary lesions (Fig. 7B), with pronounced drooping. Lesions were also noted at other sites in wtMYX and rwtMYX2 infected animals including the muzzle and eyelids. In stark contrast the ears of animals infected with MYXΔSERP1::lacZ or MYXΔSERP1::SPI-3 had a much reduced number of small lesions (Fig. 7B) and did not droop. Prominent secondary lesions were not observed at other locations on these animals.

Rabbits infected with wtMYX or with rwtMYX2 developed all the symptoms of myxomatosis and were indistinguishable. At day 7 all wtMYX and rwtMYX2 animals had gram-negative infections as evidenced by conjunctivitis (thickened eyelids) and discharge from the eyes and nose. By day 9, the wtMYX and rwtMYX2 animals had severe gram-negative infections and some breathing difficulties. On day 10, the wtMYX and rwtMYX2 infected rabbits were moribund and were euthanized due to the severity of their symptoms. Infection with MYXΔSERP1::lacZ or with MYXΔSERP1::SPI-3 resulted in only slight to mild gram-negative infections, with no signs of labored breathing. At day 10 the MYXΔSERP1::lacZ and MYXΔSERP1::SPI-3 animals were active and apparently recovering. No significant differences were seen between rabbits infected with MYXΔSERP1::lacZ or with MYXΔSERP1::SPI-3 in primary or secondary lesions or in the development of gram-negative infections.

The results clearly indicate that inactivation of the SERP1 gene in MYXΔSERP1::lacZ results in significant attenuation, confirming that SERP1 is an important virulence factor for MYX (Macen *et al.,* 1993). Replacement of SERP1 with SPI-3 in MYXΔSERP1::SPI-3 generated a recombinant that was as attenuated as MYXΔSERP1::lacZ. Furthermore, reintroduction of the SERP1 gene into MYXΔSERP1::SPI-3 and removal of the SPI-3 gene generated a virus (rwtMYX2) that was as lethal as wtMYX, indicating that an unintended attenuating mutation at a site distinct from the SERP1 locus had not occurred in MYXΔSERP1::SPI-3. These data provide clear evidence that SPI-3 is unable to substitute for SERP1 in MYX pathogenesis, despite the biochemical similarities between these proteins and the fact that both are secreted by MYX.

DISCUSSION

Although SERP1 and SPI-3 have been found to resemble one another in their biochemical activities against proteinases *in vitro* (Turner *et al.,* 2000), the two proteins share only 29% amino acid identity. The results presented here indicate that SERP1 and SPI-3 are unable to substitute for one another within the context of either CPV or MYX. SERP1 clearly does not inhibit cell–cell fusion mediated by a CPV derivative lacking SPI-3 (Fig. 3). SERP1 expression by CPVΔSPI-3::SERP1 was early as judged by resistance to AraC, as expected for transcription from the SPI-3 promoter (Fig. 2). However, SERP1 synthesized by CPVΔSPI-3::SERP1 was secreted into the medium as it is naturally in wtMYX, unlike SPI-3, which is not secreted following CPV infection. In the vaccinia/T7 overexpression system, SPI-3 could be detected in the infected cell pellet but was not present in the cleared supernatant (Fig. 4), suggesting that SPI-3 is cytoplasmic or membrane-bound under these conditions. The SPI-3 protein may be associated naturally with extracellular virus, like the hemagglutinin, which also inhibits cell fusion (Ichihashi and Dales, 1971) and is glycosylated (Payne, 1979). Inhibition of cell–cell fusion by SPI-3 does not require a wild-type sequence within the RCL (Turner and Moyer, 1995), but may depend on N-linked glycosylation. Although SERP1 and SPI-3 are both N-glycosylated (Macen *et al.,* 1993; Turner and Moyer, 1995), the numbers of potential glycosylation sites are different for the two proteins (3 for SERP1 and 4 for SPI-3), and their locations within the two proteins are not conserved (Fig. 1). Secretion of SERP1 or glycosylation differences provide possible explanations as to why SERP1 does not inhibit cell–cell fusion. In addition a fusion phenotype has not been reported for SERP1 knockout mutants of either malignant rabbit virus (Upton *et al.,* 1990) or MYX (Macen *et al.,* 1993) when compared with the corresponding wild-type viruses.

Expression of SPI-3 by MYXΔSERP1::SPI-3 gave rise to two unexpected findings. First, we had not anticipated that SPI-3 would be secreted into the medium by MYXΔSERP1::SPI-3 (Fig. 4). This underscores the importance of viral context in determining localization and

ultimately biological activity of a given gene product. Had the two serpins not been exchanged between the viruses, it is unlikely that the differential secretion of SPI-3 would have been observed. The reason for the phenomenon is of keen interest. The cell association of SPI-3 after infection with orthopoxviruses but not with the MYX recombinant could reflect either retention of the protein in orthopoxviruses through interaction(s) with another viral protein(s) or an effect of MYX on the host cell such that viral proteins are secreted more efficiently. Clearly this hypothetical modification to the secretion apparatus would be predicted not to occur following infection with either CPV or VV. SERP1 contains a cleavable signal peptide from residues 1 to 15, and the cleavage position between Cys-15 and Arg-16 has been confirmed by N-terminal sequencing (P. Nash and G. McFadden, personal communication). SPI-3 contains a sequence that appears by analysis with SignalP (Nielsen *et al.,* 1997) to be similar, with the predicted signal cleavage site between Ala-15 and Tyr-16. The signal peptide is presumably removed following insertion of SPI-3 into the endoplasmic reticulum. We have shown that SPI-3 protein bearing a His-tag at the N-terminus retains the tag following expression in the VV/T7 system when residues 1–15 of the wt SPI-3 have been deleted, but the tag is removed when the putative signal peptide is present (Turner *et al.,* 2000). SPI-3 secreted from MYXΔSERP1::SPI-3 infected cells was not associated with extracellular MYX virions (data not shown) and was functional as an inhibitor of plasmin, uPA, and tPA when concentrated supernatants were tested (data not shown).

Second, the apparent size of SPI-3 synthesized by the MYX recombinant (57 and 55 kDa) was increased compared with that seen for either wtCPV (50 kDa) (Turner and Moyer, 1995) or following overexpression by VV (50 and 48 kDa) (Fig. 4). The larger size of the SPI-3 protein synthesized by MYXΔSERP1::SPI-3 apparently reflects differences in N-linked glycosylation in RK-13 cells infected with MYX or with VV (Fig. 5). The recent discovery of an α2,3-sialyltransferase encoded by MYX is the first description of a natural virus-encoded glycosyltransferase (Jackson *et al.,* 1999) and provides a possible explanation for the observed size discrepancy. It is very possible that SPI-3, after reaction with the GlcNAc-α 1,3/4-fucosyltransferase from the host cell, has been further modified by the α2,3-sialyltransferase such that it contains additional terminal sialic acid residues on the carbohydrate chains, hence increasing the apparent molecular weight of SPI-3 by SDS–PAGE. The MYX MST3N gene encoding the α2,3-sialyltransferase does not have a homolog in VV (Jackson *et al.,* 1999).

SPI-3 synthesis by MYXΔSERP1::SPI-3 was eliminated by treatment with AraC (Fig. 6), as expected for expression from the SERP1 promoter at late times during infection. We do not know how the level of SPI-3 secreted into the medium by MYXΔSERP1::SPI-3 compares with the natural level of SERP1, but we do know that the timing and localization of SPI-3 expression by the recombinant are the same as those seen for SERP1. The finding that SPI-3 does not substitute for SERP1 in MYX pathogenesis (Fig. 7) indicates that the two serpins are fundamentally different, despite their similar inhibition spectrum *in vitro.* The true proteinase target(s) for SERP1 and SPI-3 is almost certainly not plasmin, urokinase, tPA, thrombin, or factor Xa as the apparent second-order association rates between SERP1 or SPI-3 and these proteinases are relatively slow, all at or below 10^5 $M^{-1}s^{-1}$ (Nash *et al.,* 1998; Turner *et al.,* 2000), which is considered the threshold for physiologically significant interactions (Travis and Salvesen, 1983). Also MYX and CPV may not encounter these classical plasma proteinases during natural infections. Our results strongly suggest that the true target proteinases for SERP1 and SPI-3 are different. However, other interpretations are possible. For example, the target might be the same for the two serpins but the species specificity may differ such that SERP1 inhibits the enzyme from rabbits but SPI-3 does not. In this case SPI-3 would be expected to inhibit the homologous proteinase from the natural host for CPV, rodents. Our current efforts are directed toward determining where SPI-3 is localized in natural infections and finding physiologic targets for SPI-3 and SERP1.

MATERIALS AND METHODS

Viruses and cells

The wild-type Brighton Red strain of CPV was obtained from David Pickup (Duke University Medical Center). The wild-type Lausanne strain of MYX was obtained from Grant McFadden (J. P. Robarts Research Institute, London, Ontario, Canada). CV-1 cells (ATCC CCL-70) and RK13 cells (ATCC CCL-37) were grown in Gibco BRL minimum essential medium with 5% fetal bovine serum, 2 mM glutamine, 50 U of penicillin per milliliter, 50 μg of streptomycin per milliliter, 1 mM sodium pyruvate, and 0.1 mM nonessential amino acids.

Plasmid constructions

The *Escherichia coli gpt* gene under control of the VV $P_{7.5}$ promoter [the $P_{7.5}$gpt cassette, originally from pTK61-gpt (Falkner and Moss, 1988)] was excised as a 2-kb *Eco*RI fragment from pBS-gptA (Turner and Moyer, 1992b). pSPH-ATI/lacZ was constructed and the recombinants were selected as described previously (Li *et al.,* 1998).

The scheme for construction of the CPV and MYX derivatives used is shown in Fig. 1. A recombinant PCR method (Turner and Moyer, 1992b) was used to generate a transfer plasmid in which the SPI-3 ORF was replaced by the SERP1 coding region and the SPI-3 promoter was

retained. The 400-bp region downstream of the SPI-3 gene ($SPI\text{-}3_R$) was amplified with the sense primer CD1 (5′-GCGTCTAGATATTGTTTATGGATACGGT-3′) and the antisense primer CD2 (5′-GCGGAGCTCATTCTCTAGAAGATTTTTCA-3′). Primers CU1 (5′-GCGGGTACCTACAACCGGAAAAAGAAATT-3′) and R2 (5′-ACGAGGACCAGATACTTCATGATTTTATGGTATTAATAA-3′) were used to amplify the region upstream from the SPI-3 ORF, including the promoter region ($P_{SPI\text{-}3}$). The underlined portion of R2 is complementary to the 5′ end of the SERP1 coding region, and the remaining part is complementary to the sequence of SPI-3 upstream from the initiating ATG (Fig. 1). The SERP1 coding region acted as the central portion in the recombinant PCR scheme and was amplified with primers R3 (5′-ATGAAGTATCTGGTCCTCGT-3′) and R4 (5′-GCGTCTAGATTATACAGAGGATACGACAT-3′). The $P_{SPI\text{-}3}$ and SERP1 fragments were joined by mixing the purified DNAs and amplifying with the outside primers (CU1 and R4). The first two cycles were denaturation at 94°C for 1 min, followed by slow cooling to 37°C (ramp rate of 10 s/°C). The next 12 cycles were 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min. Extension was completed by a final incubation at 72°C for 10 min. The recombinant and the downstream flank of the SPI-3 gene were inserted serially into pBluescript KS(+) (Stratagene) and then verified by sequencing to produce the plasmid $pP_{SPI\text{-}3}$-SERP1. The $P_{7.5}$gpt cassette was inserted into the unique *Xba*I restriction enzyme site in $pP_{SPI\text{-}3}$-SERP1 in the same orientation as the SERP1 ORF to create the transfer plasmid $pP_{SPI\text{-}3}$-SERP1-$P_{7.5}$gpt.

A plasmid for deleting SERP1 from MYX and inserting a lacZ cassette in its place was constructed as follows. Primers LP1 (5′-GCGGGTACCTCCGTAACAACACGTGTGTC-3′) and LP2 (5′-GCGCTCGAGTTAACCCTCAAAAAAAACGT-3′) were used to amplify the 359-bp region upstream from the SERP1 gene ($SERP1_L$); and RP11 (5′-GCGTCTAGAACGCCCTCACGCCGATCGT-3′) and RP2 (5′-GCGGAGCTCCATAACGAGACTGGGCATAA-3′) were used to amplify the 563-bp region downstream of the SERP1 gene ($SERP1_R$). The left part of $SERP1_R$ includes 150 bp of the SERP1 3′ end coding sequence since this sequence potentially includes the M-T8 promoter (Fig. 1). The two fragments were PCR-amplified, serially cloned into pBluescript KS(+), and sequenced to verify the lack of errors. A 3.4-kb *Msc*I and *Pst*I fragment consisting of the lacZ gene driven by the CPV ATI promoter derived from pSPH-ATI/lacZ (Li *et al.,* 1998) was inserted between the flanking sequences to create pSERP1-ATI/lacZ.

A transfer plasmid was generated in which the SERP1 coding region was replaced by the SPI-3 ORF but the SERP1 promoter was retained. LP1 and C2 (5′-TATCAATAACGCAATCATTTAACCCTAAAAAAAACGT-3′), in which the 18 bases at the 5′ end are complementary to SPI-3 and 20 bases at the 3′ end are complementary to the SERP1 promoter, were used to amplify the left flanking fragment including the SERP1 promoter. C3 (5′-ATGATTGCGTTATTGATA-3′) and C4 (5′-GCGTCTAGATTAAGGAGATTCTACCTTA-3′) were used to amplify the SPI-3 open reading frame. After the amplification, the two fragments were joined by recombinant PCR as described above. After the verification of the sequence, the product and the right flank of the SERP1 gene were serially inserted into pBluescript KS(+) to generate pP_{SERP1}-SPI-3. The $P_{7.5}$gpt cassette was inserted into the unique *Xba*I restriction enzyme site in the same orientation as SERP1 to create the pP_{SERP1}-SPI-3-$p_{7.5}$gpt transfer plasmid.

Plasmid pSERP1 containing the SERP1 ORF and natural flanking sequences was constructed by cloning the PCR product generated from MYX genomic DNA with primers LP1 and RP2. pSERP1 was used to obtain the reconstructed wild-type virus rwtMYX2 from MYXΔSERP1::SPI-3.

Construction of virus recombinants

CV-1 or RK13 cells grown to 80% confluence in 60-mm-diameter dishes were infected at a multiplicity of 0.05, and 5 μg plasmid DNA complexed with 30 μg Lipofectin (Gibco BRL) in a total volume of 100 μl was added at the end of the 2-h adsorption period. The infected and transfected cells were harvested at 48 h postinfection, and virus recombinants were obtained by plaquing. Recombinant viruses containing the *E. coli* xanthine-guanine phosphoribosyltransferase (gpt) gene were selected by resistance to mycophenolic acid (MPA). Gpt selection medium contained 2.5 μg of MPA per milliliter, 250 μg of xanthine per milliliter, and 15 μg of hypoxanthine per milliliter. For the gpt selection, 3 ml of gpt selection medium with 5% fetal bovine serum was added following the infection/transfection procedure. Confluent monolayers of CV-1 or RK13 cells were pretreated for 14 to 24 h with gpt selection medium before infection with virus resulting from the gpt transfections. MPA-resistant (MPA^R) plaques were visualized after 3 days by staining with neutral red (Gibco BRL). To screen for the expression of LacZ, X-Gal was added to a final concentration of 300 μg/ml in the overlaying agarose after plaque formation had occurred. MYX recombinants lacking gpt were selected following infection and transfection of RK-13 cells by plaquing on $HGPRT^-$ HeLa cells (Kerr *et al.,* 1991) in the presence of 1 μg/ml 6-thioguanine (Isaacs *et al.,* 1990). In all cases recombinants were plaque purified at least three times before amplification to produce virus stocks.

Immunoblot analysis

Infected CV-1 or RK13 cells in 35-mm-diameter wells were harvested at 20 h p.i. and the cell pellet was resuspended in 100 μl of cell lysis buffer [100 mM Tris–HCl (pH 8), 100 mM NaCl, 0.5% Nonidet-P40 (NP-40)] containing 1.5 μg of Aprotinin per milliliter. For secreted protein analysis, the supernatants were harvested from

infected cells and centrifuged at 1000*g* for 10 min to pellet residual cells. To remove extracellular virus, the supernatants were centrifuged at 16,000*g* for 20 min. The cleared supernatants were concentrated using Centriprep 10 filters (Amicon). Peptide *N*-glycosidase F (PNGase F, Roche Molecular Biochemicals) digestions were performed according to the manufacturer's instructions. Concentrated serum-free medium from cells infected with MYXΔSERP1::SPI-3 or cell pellets from cells coinfected with vTF7-3 and VV-P_{T7}-SPI-3 were incubated with PNGase F for 16 h at 37°C. The reaction products were analyzed by SDS–PAGE and immunoblotting with anti-SPI-3 serum.

SDS–PAGE protein gels were electroblotted to 0.1-μm-pore-size nitrocellulose. Blots were probed either with anti-MBP-SPI-3 rabbit polyclonal antiserum (Turner and Moyer, 1995) diluted 1:300 or with anti-SERP1 monoclonal antibody at 1:5000 diluted into phosphate-buffered saline with 0.05% NP-40 and 5% (wt/vol) dried milk, followed by horseradish peroxidase (HRP)-conjugated goat anti-rabbit serum (Fisher Biotech) at 1:5000 or HRP-conjugated goat anti-mouse serum at 1:2000. Bound antibodies were detected using enhanced chemiluminescence reagents (Amersham).

Infection of rabbits with myxoma virus derivatives

Male New Zealand White rabbits (*O. cuniculus*) were obtained from Myrtle Rabbitry, Inc. (Thompson Station, Tennessee) and housed in level C containment facilities. Each rabbit was injected intradermally with 500 PFU/hind flank (total of 1000 PFU per rabbit) of wtMYX, MYXΔSERP1::lacZ, MYXΔSERP1::SPI-3, or rwtMYX2 virus. Eight rabbits were infected for each virus group with the exception that only three rabbits were infected with rwtMYX2. Rabbits were monitored on a daily basis for the appearance and progression of disease symptoms. Primary lesions (at the injection site) and secondary lesions in the ears were photographed at regular intervals. On days 1, 3, and 5 postinfection, two rabbits from the wtMYX, MYXΔSERP1::lacZ, and MYXΔSERP1::SPI-3 infection groups were euthanized by intravenous administration of Euthasol and necropsies were performed. The remaining animals, including those infected with rwtMYX2, were sacrificed on day 10 postinfection.

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