

Orthopoxvirus Fusion Inhibitor Glycoprotein SPI-3 (Open Reading Frame K2L) Contains Motifs Characteristic of Serine Proteinase Inhibitors That Are Not Required for Control of Cell Fusion

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The cowpox virus (CPV) SPI-3 gene (open reading frame K2L in vaccinia virus) is one of three orthopoxvirus genes whose products are members of the serpin (serine proteinase inhibitor) superfamily. The CPV SPI-3 gene, when overexpressed by using the vaccinia virus/T7 expression system, synthesized two proteins of 50 and 48 kDa. Treatment with the N glycosylation inhibitor tunicamycin converted the two SPI-3 proteins to a single 40-kDa protein, close to the size of 42 kDa predicted from the DNA sequence, suggesting that the SPI-3 protein, unlike the other two orthopoxvirus serpins, is a glycoprotein. Immunoblotting with an anti-SPI-3 antibody showed that the SPI-3 protein is synthesized early in infection prior to DNA replication. SPI-3 inhibits cell-cell fusion during infections with both CPV and vaccinia virus. A transfection assay was devised to test engineered mutants of SPI-3 for the ability to inhibit fusion. Two mutants with C-terminal deletions of 156 and 70 amino acids were completely inactive in fusion inhibition. Site-directed mutations were constructed near the C terminus of SPI-3, in or near the predicted reactive-site loop which is conserved in inhibitory serpins. Substitutions within the loop at the P1 to P1' positions and P5 to P5' positions, inclusive, did not result in any loss of activity, nor did changes at the P17 to P10 residues in the stalk of the reactive loop. Therefore, SPI-3 does not appear to control cell fusion by acting as a serine proteinase inhibitor.

The orthopoxviruses include the prototype vaccinia virus (VV) and variola virus, the causative agent of smallpox. Poxviruses have DNA genomes of about 200 kbp and replicate in the cytoplasm (25). Their large genomes contain many genes in the terminal regions which counteract host immune defenses (6) by interfering with complement activation (21) or cytokine synthesis or by secreting soluble cytokine receptors (16, 30). Poxviruses are unique in that they contain viral genes whose products are members of the serpin (serine proteinase inhibitor) superfamily (41). Mammalian serpins, many of which are active as serine proteinase inhibitors, regulate a broad range of processes, including inflammation, coagulation, fibrinolysis, complement activation, and prohormone conversion (32). Viral serpin genes were presumably acquired from the host in evolution.

Orthopoxviruses contain three serpin genes, SPI-1, SPI-2, and SPI-3, near the ends of the viral genome (4, 31, 36). The best-characterized orthopoxvirus serpin is SPI-2 (31) (also known as *crmA*, 38K gene, and open reading frame [ORF] B13R in VV). Cowpox virus (CPV) mutants in the SPI-2 gene produce white raised pocks on the chicken chorioallantoic membrane rather than the red, hemorrhagic flat lesions seen with wild-type (wt) CPV (31). The white pocks result from a failure to prevent an influx of inflammatory cells into the developing lesion (13, 27). The SPI-2 protein derived from wt CPV inhibits interleukin-1 β (IL-1 β)-converting enzyme (ICE), a cysteine proteinase which generates the proinflammatory cytokine IL-1 β by cleavage of the precursor pro-IL-1 β (33). The SPI-1 protein (ORF B22R in VV) is 45% identical to

SPI-2 protein. In rabbitpox virus, which contains an active SPI-2 gene, SPI-1 is also required for the production of red pocks, since a SPI-1 mutant gives white pocks on the chorioallantoic membrane (1). However, the biochemical function of SPI-1 has not yet been defined.

The third orthopoxvirus serpin, SPI-3 (ORF K2L in VV), is only distantly related to SPI-1 and SPI-2. SPI-3 mutants of rabbitpox virus and CPV give red pocks on the chorioallantoic membrane (38). The only phenotype associated with mutations in SPI-3 is the occurrence of extensive cell-cell fusion in infected tissue culture cells (22, 39, 44). SPI-3 and the virally encoded hemagglutinin must both be active to prevent fusion (19, 34); however, SPI-3 mutants are not perturbed in the synthesis or localization of hemagglutinin (22, 39, 44), and the mechanism by which the SPI-3 gene product inhibits fusion is not understood. The SPI-3 ORF potentially encodes a 42-kDa protein and is intact in VV (4, 15), rabbitpox virus (3), CPV (11), and variola virus (24, 35).

The first objective of this work was to identify and characterize the protein product of the SPI-3 gene, which appears by Northern (RNA) blot analysis to be transcribed early in infection (36). We then developed an assay for SPI-3 activity to address the relationship between potential proteinase inhibition (serpin activity) and control of cell fusion. Inhibitory serpins, which mimic the natural protein substrates, form very stable 1:1 complexes with their target proteinases via an exposed reactive loop located near the C terminus of the serpin (7, 9, 32). When the complex finally decays, the serpin is ultimately cleaved within the reactive loop between the P1 and P1' residues and released. The amino acid at the P1 position of the serpin is the major determinant of specificity for the target enzyme. The reactive loop consists of about 20 amino acids, with residues around the P1/P1' scissile bond numbered P2, P3, etc., proceeding toward the N terminus and P2', P3', etc., proceeding toward the C terminus of the serpin (32). A con-

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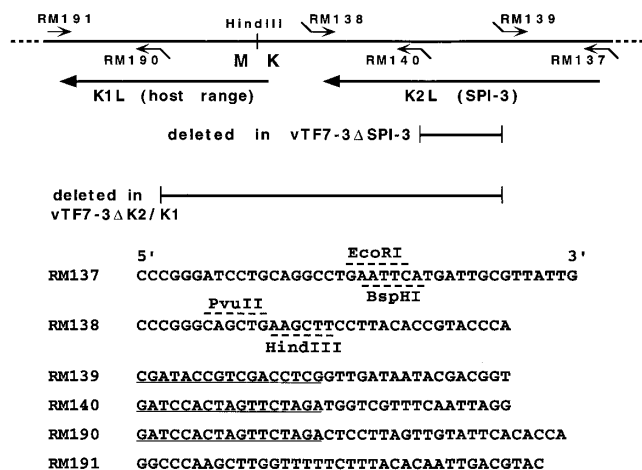


FIG. 1. Primers used for insertion of CPV SPI-3 into expression vectors and for construction of SPI-3 deletions in vTF7-3. The top part shows the locations of primers against SPI-3 (ORF K2L) and the neighboring host range gene K1L. The map spans the junction of *Hind*III fragments M (left) and K (right) in the VV genome; orientation is as in the genome. ORF K1L originates in the *Hind*III K fragment, although it is mostly located in the *Hind*III M fragment. Large arrows represent ORFs; small arrows indicate primers. The bars below show the regions deleted in the VV derivatives vTF7-3ΔSPI-3 and vTF7-3ΔK2/K1. Sequences of primers are indicated at bottom with restriction sites used in cloning into expression vectors pmalCRI, pTM1, and pBluescript. The underlined portion of RM139 is complementary to primer KS; that of RM140 and RM190 is complementary to primer SK. The *gpt* cassette in both pBS-gptA and pBS-gptL is flanked by sites for primers KS and SK (see Materials and Methods).

formational transition involving insertion of the stalk of the reactive loop (residues P17 to P9) into a β sheet located in another part of the serpin is important for the function of inhibitory serpins (8). Site-directed mutations of the CPV SPI-3 protein targeted to these conserved motifs were constructed and tested for the ability to inhibit fusion by means of a novel transient fusion assay developed for this purpose.

MATERIALS AND METHODS

Cells and viruses. CV-1 cells (ATCC CCL70), originally obtained from Ed Stephens, University of Missouri, were grown as monolayers in Gibco-BRL minimum essential medium with 7% fetal bovine serum, 2 mM glutamine, 50 U of penicillin per ml, 50 μ g of streptomycin per ml, 1 mM sodium pyruvate, and 0.1 mM minimal essential medium nonessential amino acids. wt VV strain WR and vTF7-3 (VV/T7) were obtained from Bernard Moss (National Institute of Allergy and Infectious Diseases). wt CPV strain Brighton Red was provided by David Pickup (Duke University). CPVΔSPI-3 and VVΔSPI-3 containing an internal deletion within the SPI-3 gene have been described previously (39).

Expression of an MBP-SPI-3 fusion protein in *Escherichia coli* and preparation of an anti-SPI-3 serum. The SPI-3 ORF was amplified from wt CPV genomic DNA by using primers RM137 and RM138 (Fig. 1), which match the 5' and 3' regions of the SPI-3 coding sequence and contain *Eco*RI and *Hind*III restriction sites, respectively. CPV SPI-3 was cloned into the bacterial maltose-binding protein (MBP) fusion plasmid vector pmalCRI (New England Biolabs) which had been digested with the same enzymes, so that SPI-3 was downstream from and in frame with the MBP sequence. *E. coli* TB1 containing pmalCRI-SPI-3 was induced by adding 0.3 mM isopropyl- β -D-thiogalactopyranoside (IPTG). The resulting MBP-SPI-3 fusion protein was purified by affinity chromatography as recommended by New England Biolabs and migrated with an apparent size of 85 kDa by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), consistent with the components MBP (42 kDa) and CPV SPI-3 (calculated molecular mass of 42.3 kDa). Antiserum was obtained after immunization of two female New Zealand White rabbits with MBP-SPI-3 protein (17).

Overexpression of SPI-3 protein in CV-1 cells. Plasmid pTM1 (2, 26) was used to express high levels of the CPV SPI-3 protein in CV-1 cells by means of the VV/T7 expression system (14). pTM1 contains the encephalomyocarditis virus leader to enhance translation of uncapped transcripts made from the T7 promoter (10). The CPV SPI-3 ORF was amplified by PCR from genomic DNA with primers RM137 and RM138 (Fig. 1), digested with *Bsp*HI and *Pvu*II, and cloned

into pTM1 digested with *Nco*I (end compatible with *Bsp*HI) and *Stu*I to generate pTM1-SPI-3. This construct makes a transcript from the T7 promoter in which the natural AUG codon of the CPV SPI-3 ORF is the first start site encountered during cap-independent translation. The CPV SPI-3 protein was overexpressed following infection of CV-1 cells with vTF7-3 (14), a VV recombinant which synthesizes T7 RNA polymerase, and transfection of pTM1-SPI-3 DNA by LipofectACE (Life Technologies) essentially as described previously (2). Subconfluent (85%) CV-1 cells in six-well plates (35-mm-diameter wells) were infected with vTF7-3 at a multiplicity of 10 and transfected with 5 μ g of plasmid DNA that had been mixed with 12 μ l (16.8 μ g) of LipofectACE in a total volume of 100 μ l of H₂O. Coinfection with vTF7-3 and a VV recombinant containing the CPV SPI-3 gene fused to the T7 promoter was also used to overexpress CPV SPI-3. The VV derivative containing the CPV SPI-3 gene inserted into the thymidine kinase (TK) gene was constructed by infecting with an isatin β -thiosemicarbazone-dependent VV mutant (42), transfecting with pTM1-SPI-3, which contains TK flanking sequences, and selecting for bromodeoxyuridine-resistant (TK⁻) virus.

Metabolic labeling, immunoprecipitation, and immunoblotting. CV-1 cells in 35-mm-diameter wells expressing SPI-3 protein at 6 h postinfection were starved for 20 min at 37°C in 0.3 ml of methionine- and cysteine-free labeling medium (Flow Laboratories); 30 μ Ci of Tran³⁵S-label (ICN Biomedicals, Inc.) was added, and labeling continued for 30 min. Five molar NaCl (4.8 μ l) was added to each well in experiments in which transcripts contained the encephalomyocarditis virus leader to increase cap-independent translation (2). The cells were harvested and resuspended in 100 μ l of cell lysis buffer (100 mM Tris-HCl [pH 8], 100 mM NaCl, 0.5% Nonidet P-40 [NP-40]) containing 1.5 μ g of aprotinin per ml. Immunoprecipitation with anti-SPI-3 serum was in 100 μ l of NP-40 immunoprecipitation buffer (50 mM Tris-HCl [pH 8], 150 mM NaCl, 1% NP-40) for 90 min on ice. Protein A-Sepharose was used to recover antigen-antibody complexes. Following SDS-PAGE analysis on 10% acrylamide gels, radiolabeled proteins were detected by fluorography with PPO (2,5-diphenyloxazole) and autoradiography. Alternatively, proteins resolved by SDS-PAGE were electroblotted to 0.1- μ m-pore-size nitrocellulose and analyzed by Western blotting (immunoblotting) (17) with an anti-MBP-SPI-3 antiserum, using Amersham enhanced chemiluminescence detection reagents. Blots were probed with the anti-MBP-SPI-3 antiserum diluted 1:300 into phosphate-buffered saline with 0.05% NP-40 and 5% (wt/vol) dried milk; the secondary antibody was horseradish peroxidase-conjugated goat anti-rabbit antiserum (Fisher Biotech) at 1:5,000.

Construction of vTF7-3 derivatives containing SPI-3 deletions. The central portion of the SPI-3 gene between primers RM139 and RM140 (Fig. 1) was deleted and replaced with a *P*_{7.5}-*gpt* cassette conferring resistance to mycophenolic acid (5, 12) to make vTF7-3ΔSPI-3. The source of the 2-kb *gpt* cassette was pBS-gptA (40), which contains *gpt* inserted into pBluescript (Stratagene) between the sites for primers KS (5'-CGAGGTCGACGGTATCG-3') and SK (5'-TCTAGAACTAGTGGATC-3'). *gpt* was flanked with the left and right arms of the SPI-3 gene as described previously (39, 40) by recombinant PCR. The locations and sequences of primers RM137, RM138, RM139, and RM140 used to construct vTF7-3ΔSPI-3 are shown in Fig. 1. A second derivative, vTF7-3ΔK2/K1, with a larger deletion of SPI-3 extending into the adjacent K1L host range gene, was made in a similar fashion between primers RM139 and RM190 (Fig. 1). In vTF7-3ΔK2/K1, the C-terminal two-thirds of SPI-3 including the region encoding the reactive loop has been completely deleted. A smaller *gpt* cassette (1 kb) from plasmid pBS-gptL (obtained from Karen Mossman and Grant McFadden) was used in this second construction. *gpt* is still flanked by the KS and SK primer sites in this plasmid. Primer pairs RM137-RM139 and RM190-RM191 were used to flank *gpt* with the left arm of SPI-3 and the right arm of ORF K1L, respectively (Fig. 1). Inactivation of K1L in vTF7-3ΔK2/K1 was confirmed by the inability of this virus to plaque on RK-13 cells, as expected for a mutant in K1L (29). Both vTF7-3ΔSPI-3 and vTF7-3ΔK2/K1 grow well on CV-1 cells, but in each case the infection is characterized by extensive fusion of the cell monolayer due to the inactivation of SPI-3.

Construction of C-terminal deletion and site-directed mutations of the CPV SPI-3 gene. The CPV SPI-3 ORF was PCR amplified from genomic wt CPV DNA with RM137 and RM138 (Fig. 1), digested with *Eco*RI and *Hind*III, and inserted into pBluescript KS(+) (Stratagene) cut with *Eco*RI and *Hind*III. The resulting pBS-SPI-3 construct was confirmed by nucleotide sequencing. Mutant pBS-SPI-3Δ*Acc*I, containing a C-terminal deletion from the *Acc*I site in CPV SPI-3 (at amino acids 216 and 217) through the stop codon to *Acc*I in pBluescript (see Fig. 7A), was made by digestion with *Acc*I and religation. This construct has deleted amino acids 218 to 373 in SPI-3 and added 51 amino acids from the vector. Deletion mutant pBS-SPI-3ΔNH, lacking the region between the *Nde*I site in CPV SPI-3 (at amino acids 303 and 304) through the C terminus to the *Hind*III site in the vector (see Fig. 7A), was obtained by digestion with *Nde*I and *Hind*III, blunting with Klenow enzyme in the presence of all four deoxynucleoside triphosphates, and religating. Accurate end filling and joining was confirmed by the presence of a *Hind*III site (5'-AAGCTT-3') at the junction of filled ends from *Nde*I (... 5'-CATA-3') and *Hind*III (5'-AGCTT-3'...). In pBS-SPI-3ΔNH, the region encoding amino acids 304 to 373 of SPI-3 is deleted, but 56 amino acids are added from the vector.

Site-directed mutants of the CPV SPI-3 gene in the predicted reactive loop region were constructed by recombinant PCR using overlapping mutagenic primers (18). The P1/P1' mutant of pBS-SPI-3 with Gly-Ala replacing Arg-Ser was

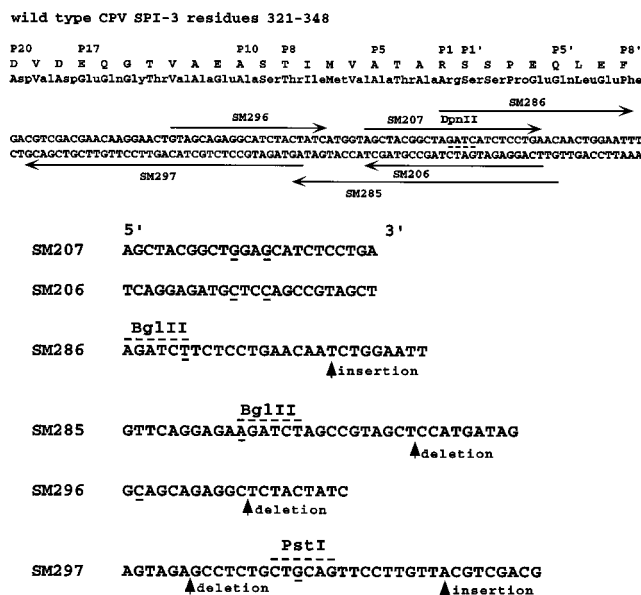


FIG. 2. Primers used for construction of site-directed mutations in CPV SPI-3. At the top is the region of the CPV SPI-3 protein from amino acids 321 to 348 (predicted P20 to P8'). Below are the sequences of both DNA strands, with the locations of mutagenic primers indicated. At the bottom are the sequences of the primers, with introduced restriction sites shown. Bases altered to produce single-nucleotide substitutions are underlined. The positions of single-base insertions and deletions are indicated by arrowheads.

made with the mutagenic primers SM206 and SM207 (Fig. 2). PCR products synthesized from wt SPI-3 DNA with primer pairs RM140-SM206 and SM207-RM138 were joined by recombinant PCR, digested with *NdeI* and *HindIII*, and cloned into pBS-SPI-3 cut with *NdeI* and *HindIII*. The effect of this was to replace the wt *NdeI*-*HindIII* region (see Fig. 7A) with a mutant version with substitutions at the P1 and P1' positions (see Fig. 7B). Screening for recombinant plasmids was facilitated by the loss of a natural *DpnII* site (5'-GATC-3') which overlaps the codons for the P1 and P1' residues in the wt gene (Fig. 2). A similar strategy was used to create the P5/P5' mutant with mutagenic primers SM285 and SM286 (Fig. 2). By introducing a single-base deletion in the P5 codon and a single-base insertion after the P5' codon, the reading frame was shifted after P5 and restored to wt after P5'. The result was replacement of the 10 amino acids from P5 to P5' inclusive (see Fig. 7B). A single-base substitution was also introduced in the center of this region to create a *BglII* restriction site for screening purposes. A P17-to-P10 substitution mutant (see Fig. 7B) was constructed with mutagenic primers SM296 and SM297 (Fig. 2). In this construct, the natural reading frame of codons P17 to P10 (Fig. 2) was displaced by an insertion of one base and then restored by a single-base deletion. Additionally, a *PstI* site was introduced in the P17/P10 mutant by a single-base substitution (Fig. 2). The 300-bp region between the *NdeI* and *HindIII* sites of the P1/P1', P5/P5', and P17/P10 mutants of pBS-SPI-3 was sequenced in each case to confirm the presence of the desired mutations and the absence of unintended changes resulting from misincorporation by *Taq* polymerase.

Transient assay for fusion inhibition. CV-1 cells were grown overnight to confluence in six-well plates (~10⁶ cells per well) and infected with vTF7-3ΔSPI-3 or vTF7-3ΔK2/K1 at a multiplicity of 0.2. Adsorption was for 2 h at 37°C. Thirty minutes before the end of adsorption, 70 μl of H₂O, 12 μl of LipofectACE (16.8 μg) and ~4 μg of plasmid DNA in 20 μl of H₂O were mixed at room temperature to allow complex formation. Infecting virus was removed, cells were washed twice with serum-free medium, and 1 ml of serum-free medium and 100 μl of H₂O-LipofectACE-DNA were added per 35-mm-diameter well. Five hours later, 2 ml of medium containing 7% fetal bovine serum was added to each well (final concentration, 5% fetal bovine serum). Addition of serum at times much later than 5 h resulted in reduced fusion, as cells fuse inefficiently in the absence of serum (22); however, serum was absent during the first 5 h following addition of DNA to allow the LipofectACE reagent to function optimally. Infected and transfected cells were scored for fusion at 24 h postinfection (p.i.).

RESULTS

The CPV SPI-3 protein is N glycosylated. A rabbit polyclonal antiserum raised against an MBP-SPI-3 fusion protein

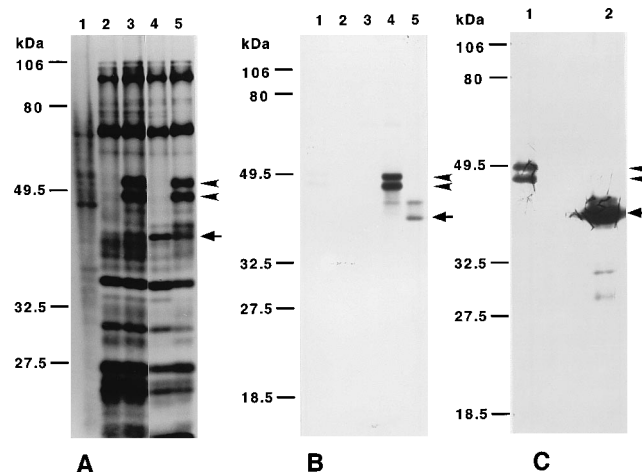


FIG. 3. Overexpression of CPV SPI-3 protein by the VV/T7 system. CV-1 cells were infected with vTF7-3 and transfected with plasmid DNA of pTM1 derivatives. Arrowheads indicate the 50- and 48-kDa forms of glycosylated SPI-3 protein; the arrow shows the 40-kDa unglycosylated protein made when tunicamycin is present. (A) Autoradiograph of total ³⁵S-labeled proteins from cytoplasmic extracts. Lane 1, uninfected CV-1 cells; lanes 2 to 5, cells infected with vTF7-3 and transfected with pTM1 DNA (vector alone) (lane 2), pTM1-SPI-3 DNA (lanes 3, 5; duplicates); and pTM1-SPI-3 DNA in the presence of 5 μg of tunicamycin per ml (lane 4). (B) Immunoprecipitation of ³⁵S-labeled proteins with an anti-SPI-3 serum. Lane 1, SPI-3 sample (from panel A, lane 3) immunoprecipitated with control preimmune serum; lanes 2 to 5, immunoprecipitation with anti-SPI-3 serum of extracts from uninfected CV-1 cells (lane 2) or cells infected with vTF7-3 alone (lane 3) or vTF7-3 plus pTM1-SPI-3 DNA (lane 4) plus pTM1-SPI-3 DNA with 5 μg of tunicamycin per ml (lane 5). (C) Western blot with chemiluminescence detection of SPI-3 protein in the absence (lane 1) or presence (lane 2) of tunicamycin. Infected and transfected cells were harvested at 6 h p.i.

as described in Materials and Methods was used in Western blot analysis to visualize native SPI-3 protein in extracts of CV-1 cells infected with wt CPV. We were unable to detect any SPI-3-specific protein in cells harvested at 6 h postinfection by this method and therefore overexpressed the protein in infected cells to facilitate characterization. The CPV SPI-3 ORF was inserted into the plasmid vector pTM1 (26), as described in Materials and Methods, to overexpress the SPI-3 protein from the transfected plasmid DNA in infected mammalian cells by the VV/T7 system. Novel bands of 50 and 48 kDa were clearly visible in profiles of total radiolabeled proteins from CV-1 cells transfected with pTM1-SPI-3 DNA (Fig. 3A, lanes 3 and 5, arrowheads), which were not seen in a control transfection with pTM1 DNA (Fig. 3A, lane 2). The 50- and 48-kDa proteins were immunoprecipitated by the anti-MBP-SPI-3 antiserum (Fig. 3B, lane 4) but not by preimmune serum from the same rabbit (Fig. 3B, lane 1). The anti-SPI-3 serum recognized proteins of the same sizes in Western blots of extracts containing overexpressed SPI-3 protein (Fig. 3C, lane 1), confirming that the 50- and 48-kDa proteins were derived from SPI-3.

The discrepancy between the observed sizes of 50 and 48 kDa for SPI-3 protein and the expected size of 42.3 kDa for the primary translation product (11) could be explained by post-translational modification. Overexpression in the presence of the N glycosylation inhibitor tunicamycin resulted in a reduction in apparent size of the 50- and 48-kDa forms to a 40-kDa form, which was readily visible in total labeled protein above the background of VV proteins (Fig. 3A, lane 4, arrow). The SPI-3 40-kDa species was detected by the anti-MBP-SPI-3 antiserum both by immunoprecipitation (Fig. 3B, lane 5) and by Western blotting (Fig. 3C, lane 2). The relatively weak band

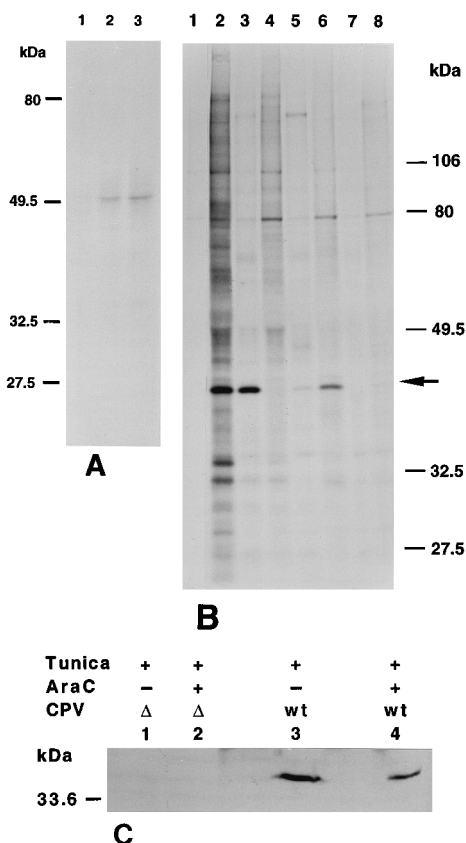


FIG. 4. SPI-3 protein made on infection with wt CPV and VV. (A) Immunoprecipitation of ^{35}S -labeled proteins from CV-1 cells infected with wt CPV at a multiplicity of 10 with preimmune serum (lane 1) and with an anti-SPI-3 serum (lanes 2 and 3). Labeling was from 1 to 6 h p.i. (B) Immunoprecipitation with anti-SPI-3 serum of extracts from CV-1 cells treated with tunicamycin. Labeling with Trans ^{35}S -label was at 4 h p.i. for 30 min unless stated otherwise. Tunicamycin was present throughout at 5 $\mu\text{g}/\text{ml}$. Lane 1, uninfected CV-1 cells; lane 2, cells infected with wt CPV, labeled from 1 to 6 h p.i.; lane 3, cells infected with wt CPV, labeled at 4 to 4.5 h p.i.; lane 4, cells infected with CPVΔSPI-3; lane 5, cells infected with wt CPV, preimmune serum; lane 6, cells infected with wt VV strain WR; lane 7, cells infected with VVΔSPI-3; lane 8, cells infected with wt VV, preimmune serum. (C) Western blot for SPI-3 protein in extracts of cells infected with CPV in the presence of inhibitors of N glycosylation and DNA replication. CV-1 cells were infected with CPVΔSPI-3 (lanes 1 and 2) and with wt CPV (lanes 3 and 4) at a multiplicity of 25 in the presence of 5 μg of tunicamycin (Tunica) per ml (lanes 1 and 3) or tunicamycin plus 100 μg of β -cytosine arabinoside (AraC) per ml (lanes 2 and 4). Cytoplasmic extracts were made from cells harvested at 5.5 h p.i., Western blotted, and probed with an anti-SPI-3 serum by using chemiluminescence detection.

seen by immunoprecipitation may reflect the limited labeling occurring in a 30-min period compared with the strong band seen by Western blotting representing unlabeled protein that has accumulated during the first 6 h of infection. The effect of tunicamycin indicates that the CPV SPI-3 protein is N glycosylated, which is consistent with the presence of four potential N-linked glycosylation sites (see Fig. 6A) in the deduced protein sequence (11). Essentially identical results were obtained by overexpressing SPI-3 protein by coinfection with vTF7-3 and a VV recombinant containing P_{TF7}-SPI-3 inserted into the TK gene (data not shown). Thus, in contrast with SPI-1 and SPI-2 (20, 31), SPI-3 is the only orthopoxvirus serpin protein that is glycosylated.

The native CPV SPI-3 product is an early glycoprotein. SPI-3 protein was barely detectable by immunoprecipitation in CV-1 cells infected with wt CPV (Fig. 4A). We detected a weak

band of 50 kDa (Fig. 4A, lanes 2 and 3) which comigrated with the 50-kDa (uppermost) band immunoprecipitated following overexpression (Fig. 3B, lane 4). However, the 40-kDa SPI-3 protein was readily detectable by immunoprecipitation from extracts of cells infected with wt CPV or wt VV in the presence of tunicamycin (Fig. 4B, lanes 2, 3, and 6) but not from extracts of cells infected with the CPVΔSPI-3 or VVΔSPI-3 deletion mutant (Fig. 4B, lane 4 or 7, respectively) or from extracts of wt CPV- or wt VV-infected cells with preimmune serum (Fig. 4B, lane 5 or 8, respectively). The similar apparent sizes of the CPV and VV SPI-3 proteins would be expected from the nucleotide sequences (4, 11). The 40-kDa species lacking N glycosylation may have been easier to visualize than the native N-glycosylated form (Fig. 4A, lanes 2 and 3) because antibodies were made against bacterial unglycosylated MBP-SPI-3 protein which might not recognize the glycosylated protein as efficiently. Another possibility is that the 40-kDa form accumulated in cells in the presence of tunicamycin.

The CPV SPI-3 protein was detectable by metabolic labeling at between 4 and 4.5 h p.i. (Fig. 4B, lane 3). To test for production of SPI-3 protein prior to DNA replication, the DNA synthesis inhibitor β -cytosine arabinoside was used. CPV SPI-3 protein could not be visualized from wt CPV infections by Western blotting, even with chemiluminescence detection, unless tunicamycin was present (data not shown), and so both inhibitors were used to test for early expression of SPI-3 protein. The 40 kDa SPI-3 band was clearly visible in the presence of tunicamycin (Fig. 4C, lane 3) and was not abolished by additional treatment with β -cytosine arabinoside (Fig. 4C, lane 4). The 40-kDa protein was absent from control infections with CPVΔSPI-3 under the same conditions (Fig. 4C, lanes 1 and 2), as expected, suggesting that it is an underglycosylated SPI-3 translation product. The data show that the CPV SPI-3 protein is made prior to DNA replication and is therefore an early gene product, consistent with results of Northern blotting for SPI-3 transcripts (36).

Transient assay for fusion inhibition by SPI-3. The only function described for the SPI-3 protein to date is the inhibition of infected cell-cell fusion (22, 39, 44). A rapid assay was devised to test for the ability of a given SPI-3 gene to inhibit fusion whereby transfected plasmid DNA containing the SPI-3 gene complements a viral mutant deleted for SPI-3 during infection (Fig. 5A). This scheme allows wt and mutant versions of SPI-3 protein to be tested for activity in inhibiting fusion without the necessity of isolating virus recombinants. The SPI-3 gene on the plasmid was expressed from the T7 promoter (Fig. 5A) rather than the natural SPI-3 promoter to elevate the level of SPI-3, which is normally quite low (Fig. 4). Infection with vTF7-3 would be expected to result in cytopathic effect but no fusion, as this VV derivative contains a functional SPI-3 gene. Therefore, vTF7-3ΔSPI-3, a mutant of vTF7-3 lacking the central portion of the VV SPI-3 gene (Fig. 1), was constructed as described in Materials and Methods and should lead to cell-cell fusion. However, transfection of pTM1-SPI-3 DNA into cells infected with vTF7-3ΔSPI-3 should produce sufficiently high levels of SPI-3 protein to suppress fusion.

The transient assay for fusion inhibition was successful. Compared with uninfected cells (Fig. 5B, panel 1), vTF7-3 infection caused CV-1 cells to round individually and exhibit general cytopathic effect, but no cell fusion was observed (Fig. 5B, panel 2). In contrast, infection with the vTF7-3ΔSPI-3 deletion virus resulted in extensive fusion to generate syncytia, which was unaffected by the introduction of pTM1 vector DNA alone (Fig. 5B, panel 3). This result also suggests that the presence of the LipofectACE reagent did not alter the fusion phenotype of infected CV-1 cells in any detectable manner.

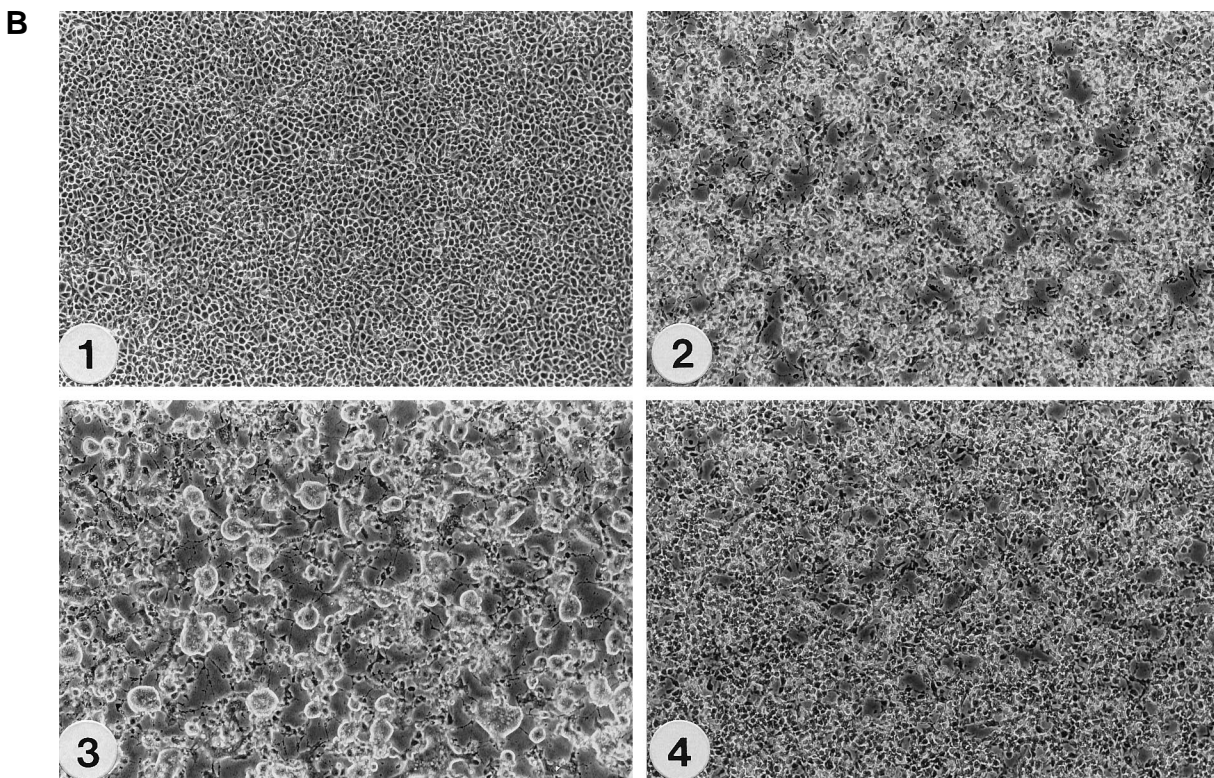
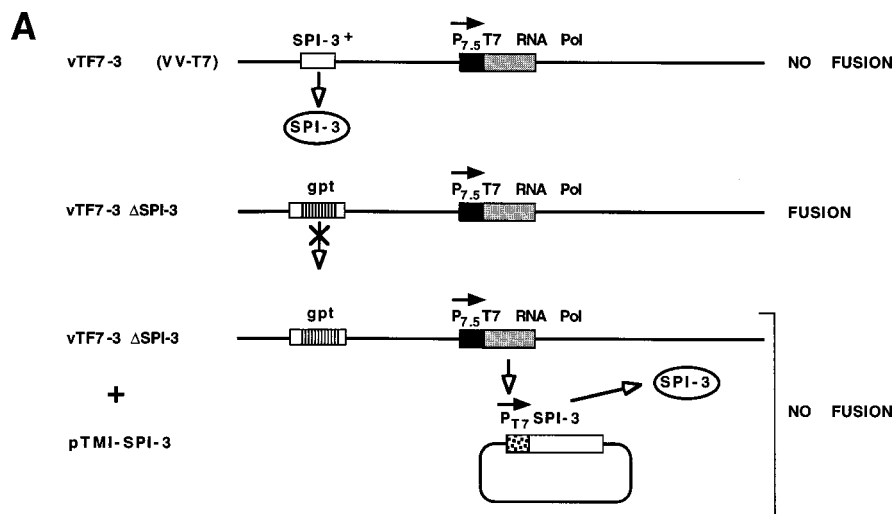


FIG. 5. Transient assay for fusion inhibition by a plasmid-borne SPI-3 gene. (A) Basis of the assay. The thick line represents the genome of vTF7-3, a VV derivative with the T7 RNA polymerase gene fused to the VV promoter $P_{7.5}$ and inserted into the TK gene. On the left is the infecting virus and transfected plasmid DNA; on the right is the expected cell fusion phenotype. The endogenous SPI-3 gene is shown as an open box. In Δ SPI-3 derivatives, part of the SPI-3 gene is deleted and replaced with the *gpt* cassette (vertical hatching) expressed from $P_{7.5}$. Short filled arrows represent promoters; open arrows indicate expression of protein products. The SPI-3 protein is pictured as an oval. (B) Effect on fusion of overexpressing SPI-3 from pTM1-SPI-3. 1, Uninfected CV-1 cells; 2, cells infected with vTF7-3, showing cytopathic effect without fusion; 3, cells infected with vTF7-3 Δ SPI-3 and transfected with pTM1 vector DNA alone, showing the resultant fusion; 4, cells infected with vTF7-3 Δ SPI-3 and transfected with pTM1-SPI-3 DNA, showing no fusion.

Transfection of pTM1-SPI-3 DNA into cells infected with vTF7-3 Δ SPI-3 moreover gave complementation which led to inhibition of fusion (Fig. 5B, panel 4). Expression of wt CPV SPI-3 protein via plasmid transfection can therefore completely inhibit the fusion normally seen with vTF7-3 Δ SPI-3 and provides a convenient assay for SPI-3 activity. These results also indicate that the CPV SPI-3 protein can substitute functionally for the VV SPI-3 protein in this system.

C-terminal deletion mutants of the CPV SPI-3 protein fail to inhibit fusion. SPI-3 protein has been presumed to function as a proteinase inhibitor on the basis of its relatedness to inhibitory serpins (22, 39, 44). We constructed in the SPI-3 protein mutations which were designed to destroy critical structural motifs that are necessary for activity of inhibitory serpins. The object was to determine if SPI-3 protein inhibits cell-cell fusion by acting as a proteinase inhibitor.

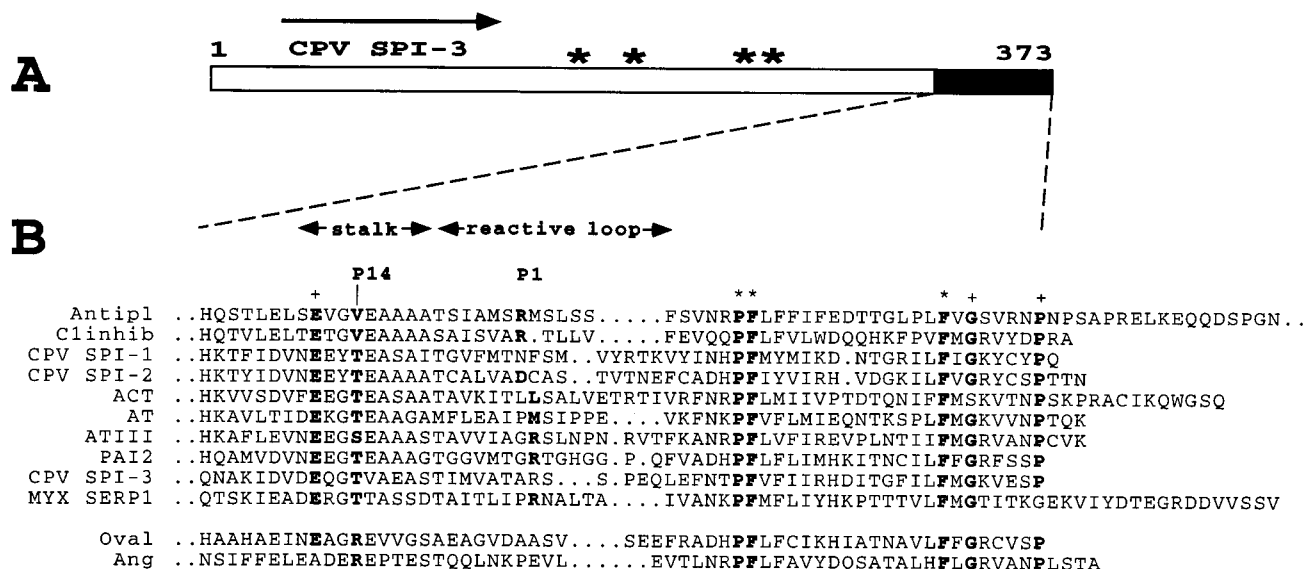


FIG. 6. Reactive loop region of the CPV SPI-3 protein. (A) The box represents the 373-amino-acid CPV SPI-3 protein, with potential N-linked glycosylation sites indicated by asterisks. The black box containing the critical serpin motifs discussed in the text is the region whose sequence is shown below. (B) Alignment of the CPV SPI-3 protein with the CPV SPI-1 and SPI-2 (crmA) proteins, the myxoma (MYX) SERP1 protein, and mammalian inhibitory serpins. The C-terminal portion of the CPV SPI-3 protein from residue 316 to the C terminus at residue 373 is shown, with the corresponding regions of the other serpins. Two noninhibitory serpins, ovalbumin and angiotensinogen, are included for comparison. Note that the 38 C-terminal residues of α_2 -antiplasmin are not shown here. The approximate locations of the reactive loop and the stalk (hinge region) are shown by arrows. The P14 and P1 residues (where known) are indicated by boldface and labeled. Note that for α_2 -antiplasmin, C1 inhibitor, and the CPV SPI-2 (crmA) protein, the P1 residue is displaced one position toward the N terminus compared to the other inhibitory serpins. Residues conserved in all 12 and 11 of 12 serpins are in boldface and marked by * and +, respectively. The PILEUP program of the Genetics Computer Group, Inc., was used to generate the alignment displayed. The order of the lines reflects clustering of similar sequences. Abbreviations: Antipl, α_2 -antiplasmin; C1inhb, C1 inhibitor; ACT, antichymotrypsin; AT, α_1 -antitrypsin; ATIII, antithrombin III; PAI2, plasminogen activator inhibitor type 2; Oval, ovalbumin; Ang, human angiotensinogen.

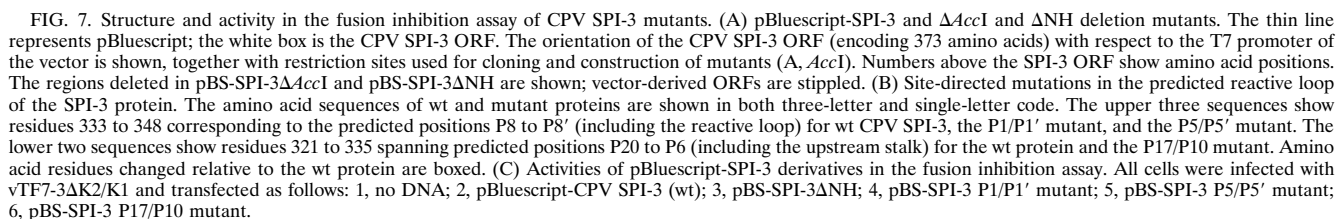
Does SPI-3 protein seem likely to be an inhibitory serpin on the basis of its sequence? The crucial region of serpins for inhibition is the reactive loop and stalk close to the C terminus (7, 9, 32). An alignment of this part of the deduced amino acid sequence of the CPV SPI-3 protein with sequences of other poxvirus and host serpins is presented in Fig. 6B. This alignment indicates that the SPI-3 protein has all of the features which are conserved in inhibitory serpins. The mechanism of proteinase inhibition by serpins involves partial insertion of the stalk of the reactive loop into the β -sheet A (8). All inhibitory serpins show strong conservation of the P17 to P9 residues constituting the stalk or hinge region (32). Note the presence in SPI-3 of a threonine residue at the predicted P14 position, typical of inhibitory serpins, in contrast with the bulky, charged arginine at the corresponding position in the noninhibitory serpins ovalbumin and angiotensinogen (Fig. 6B). SPI-3 also has a glutamic acid at the P17 position, as do almost all inhibitory serpins (32). If SPI-3 functions as a proteinase inhibitor, the P1 residue will be the primary determinant of specificity for target proteinases (9, 32). From the alignment in Fig. 6B, the predicted P1 residue for SPI-3 is arginine.

The predicted reactive loop region of SPI-3 toward the C terminus is not deleted in vTF7-3 Δ SPI-3 (Fig. 1). To prevent any possible recombination in this region of SPI-3 between the infecting virus and transfecting plasmid in the assay, a new derivative of vTF7-3 containing a more extensive deletion of SPI-3 was made. In vTF7-3 Δ K2/K1 (Fig. 1), the C-terminal two-thirds of the SPI-3 gene are removed, together with part of the adjacent nonessential host range gene K1L. This virus fused CV-1 cells readily upon infection (Fig. 7C, panel 1). The SPI-3 C-terminal deletion mutants were assayed for fusion inhibition by using as a vector pBluescript, in which the SPI-3 ORF is transcribed from the T7 promoter but the encephalo-

myocarditis virus leader present in pTM1 is absent. pBluescript was used rather than pTM1 to minimize possible side effects caused by massive overproduction of SPI-3. The level of SPI-3 protein produced from transfection of pBS-SPI-3 into cells infected with vTF7-3 Δ SPI-3 was much lower than that seen for pTM1-SPI-3, as judged by Western blotting (data not shown), although it was somewhat higher than in the natural infection.

Transfection of pBS-SPI-3 DNA into cells infected with vTF7-3 Δ K2/K1 resulted in total suppression of fusion (Fig. 7C, panel 2). The modest level of SPI-3 protein expressed from the pBluescript-derived construct was sufficient for full activity in the assay. pBS-SPI-3 Δ accI (Fig. 7A), a deletion lacking the region (amino acids 218 to 373) coding for the 156 C-terminal amino acids of SPI-3, was completely inactive in the fusion inhibition assay (data not shown), indicating that this truncated form of the SPI-3 protein did not inhibit fusion. A second, smaller C-terminal deletion mutant, pBS-SPI-3 Δ NH (Fig. 7A), deleted of amino acids 304 to 373, which encompassed the predicted reactive loop, again resulted in loss of activity in the assay (Fig. 7C, panel 3). These data were consistent with the idea that SPI-3 protein required the loop region to inhibit fusion. However, the pBS-SPI-3 Δ accI and pBS-SPI-3 Δ NH deletion mutants contained vector-derived sequences in frame with the truncated SPI-3 ORF encoding an additional 51 and 56 amino acids, respectively (Fig. 7A), making definitive conclusions less certain. More precise mutations were therefore introduced into the reactive loop region of the SPI-3 protein to examine whether control of fusion involved proteinase inhibition.

Effect of substitution mutations in the putative reactive loop of the SPI-3 protein. The arginine at the predicted P1 position of wt SPI-3 (Fig. 6B) should control the target specificity if SPI-3 is an inhibitory serpin. The Arg and Ser residues at the



To test the latter possibility, we constructed a second substitution mutant encompassing more residues, from P5 to P5'. The P5/P5' mutant has substitutions at all 10 amino acids from the predicted P5 residue to P5' (Fig. 7B). This SPI-3 mutant was made by introducing a single-base deletion at the P5 codon to generate a frameshift, followed by a single base insertion

The possibility remained that the structural rearrangement seen with inhibitory serpins, namely, stalk insertion (8), was all that was required for fusion inhibition by SPI-3. To address this question, the residues from P17 to P10, inclusive (Fig. 7B), were changed by a single-base insertion in the P17 codon followed by a single-base deletion in the P10 codon. The amino acids changed in this mutant include the glutamic acid residue at the P17 position, which is conserved in at least 40 inhibitory serpins (32), and the critical P14 threonine residue. The P17/

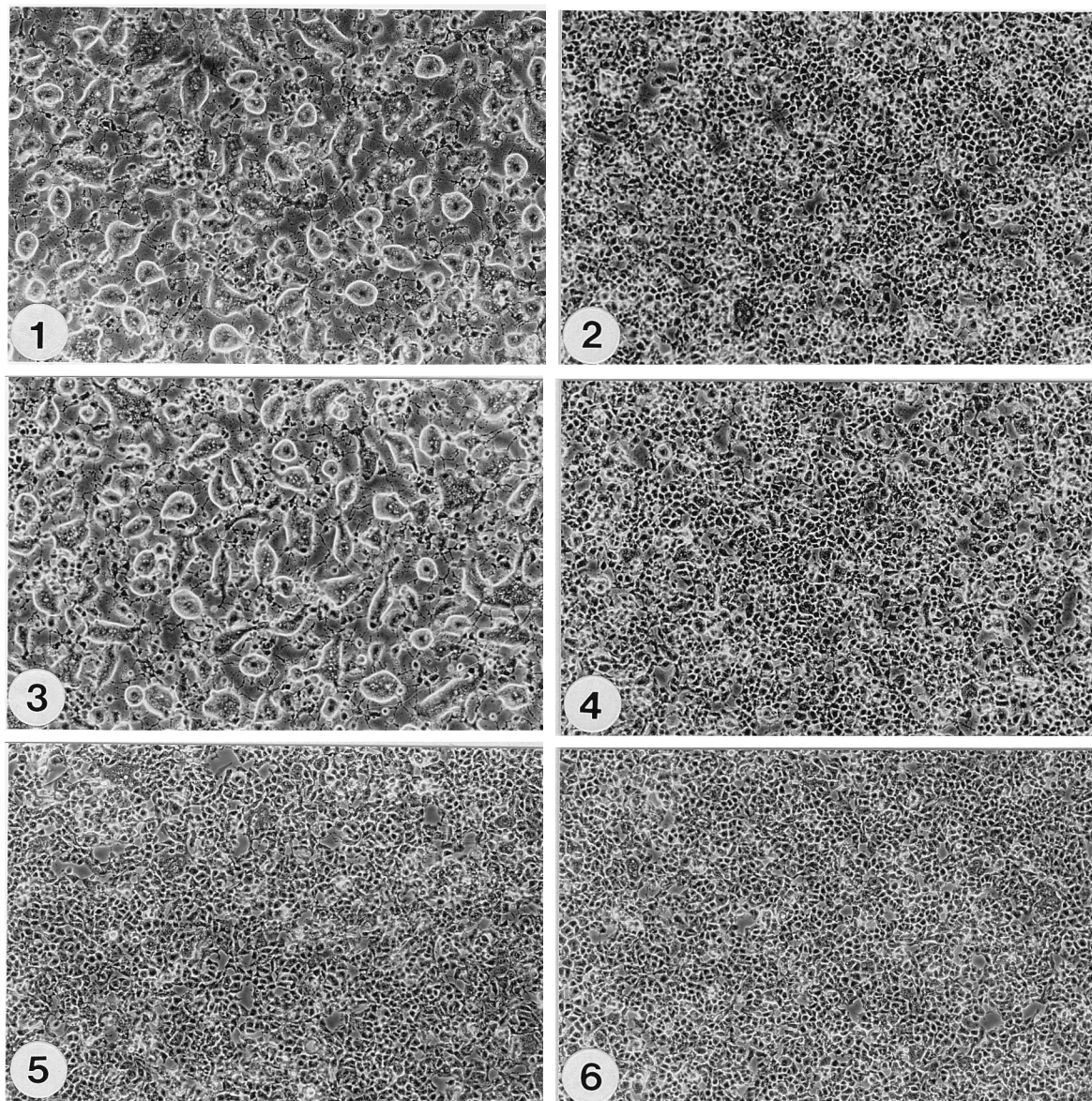
C

FIG. 7—Continued.

P10 SPI-3 mutant was fully functional in fusion inhibition (Fig. 7C, panel 6), suggesting that a serpin-like structural transition is also not required for fusion inhibition. The mechanism by which SPI-3 inhibits cell-cell fusion therefore appears to be unrelated to the serpin-like features of the protein.

DISCUSSION

The CPV SPI-3 protein expressed naturally has an apparent size of 50 kDa (Fig. 4), and overexpression of the SPI-3 ORF resulted in the synthesis of proteins of 50 and 48 kDa (Fig. 3). A smaller 40-kDa form synthesized in the presence of tunicamycin (Fig. 3 and 4) indicates that the CPV SPI-3 protein is N glycosylated. In contrast, the orthopoxvirus serpins SPI-1 and SPI-2 are not glycoproteins (20, 31, 38). In this respect, SPI-3 resembles the leporipoxvirus SERP1 gene (43), whose product is a secreted glycoprotein (23). The 48-kDa form of SPI-3 protein seen following overexpression (Fig. 3) is probably not

fully glycosylated and may result from an inability of the infected cell to completely modify all of the SPI-3 protein produced. The 50-kDa form of SPI-3 (Fig. 3) matches the size seen in wt CPV infection (Fig. 4A) and presumably represents fully glycosylated protein. The mobility of overexpressed CPV SPI-3 protein was unaffected by the O glycosylation inhibitor monensin (data not shown), implying that this protein does not contain appreciable O-linked carbohydrate chains. CPV SPI-3 was expressed early (Fig. 4C), as are CPV SPI-2 (31) and VV SPI-1 and SPI-2 (20); however, the data do not rule out continuing expression of CPV SPI-3 at late times during infection. The myxoma virus SERP1 protein is the only poxvirus serpin known to be synthesized late in infection (23).

The anti-SPI-3 serum was not sensitive enough to allow localization of the SPI-3 protein in wt CPV infection by immunofluorescence. However, when overexpressed, the SPI-3 protein could be visualized in a perinuclear location, consistent with posttranslational modification in the endoplasmic reticu-

lum and Golgi complex (data not shown). Overexpressed SPI-3 protein was not detectable in the medium by immunoprecipitation, suggesting that SPI-3 is not secreted as free protein. Cells overexpressing SPI-3 protein did not have detectable SPI-3 on the surface, as judged from immunofluorescence experiments without permeabilization (data not shown), indicating that SPI-3 was not present on the cell membrane at an appreciable level in a form accessible to antibodies. Nevertheless, SPI-3 protein may be present at the cell surface at low levels or in a masked state that cannot be detected by immunofluorescence with an anti-SPI-3 serum. Glycosylation of SPI-3 implies that the protein is likely to be membrane associated; perhaps SPI-3 is a component of the extracellular enveloped form of virus (EEV). This location would be consistent with the properties of hemagglutinin, the other poxvirus glycoprotein that inhibits cell fusion (19), which is present in EEV (28). In this regard, we have checked the SPI-3 protein by computer analysis for signal sequence motifs but have not found an obvious signal, although the extreme N terminus is hydrophobic (36). SPI-3 and hemagglutinin may be involved with blocking reinfection by preventing fusion of released EEV back to the cell of origin.

The transient assay for fusion inhibition by SPI-3 (Fig. 5) provided a convenient means of testing mutant SPI-3 genes without having to construct virus recombinants. The lack of activity of the truncated gene in pBS-SPI-3ΔNH (Fig. 7C, panel 3) does not necessarily indicate that a domain required for control of fusion is located within the deleted region, as the folding of the remainder of the protein may have been perturbed by the 70-amino-acid deletion and/or the extra 56 amino acids added from the vector. The changes in the P1/P1', P5/P5', and P17/P10 site-directed mutants involved substitution with amino acids that were completely unrelated to the wt residues in terms of charge and size (Fig. 7B). The fact that all were still able to inhibit fusion (Fig. 7C) provides strong evidence that the wt sequence within the predicted reactive loop is not required for fusion inhibition. Why then are the serpin motifs present and conserved? Results of biochemical experiments to identify a target proteinase for SPI-3 have been negative to date. However, the conservation at the predicted P17, P14, and P1 residues in SPI-3 proteins from various orthopoxviruses as diverse as raccoonpox, skunkpox, and volepox (11) argues that SPI-3 may also function as a proteinase inhibitor in some pathway unrelated to control of cell fusion. If so, the intriguing possibility exists that SPI-3 is a bifunctional protein, with one domain involved in regulating fusion and a second domain involved in proteinase inhibition.

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