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Plasma membrane localization and fusion inhibitory activity of the cowpox virus serpin SPI-3 require a functional signal sequence and the virus encoded hemagglutinin

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Abstract

The cowpox virus (CPV) glycoprotein serpin SPI-3, a functional protease inhibitor, and the viral hemagglutinin (HA) are required to prevent fusion of wt CPV infected cells. SPI-3 and HA from CPV infected cells co-localize to the plasma membrane and are found in extracellular enveloped virus (EEV). We also show that an N-terminal SPI-3 signal sequence, but not glycosylation, is required for membrane localization and fusion inhibition. In the absence of HA (CPVΔHA), no SPI-3 is found on the membrane and infected cells fuse. Conversely, HA from both wt CPV and CPVΔSPI-3 infections is on the membrane, indicating a requirement of HA for SPI-3 plasma membrane localization. In the absence of HA, secretion of SPI-3 or SPI-3 N-glyc(−) was markedly enhanced, suggesting HA serves to retain SPI-3 on the plasma membrane,thereby preventing cell fusion.

Keywords: Poxvirus; Serpin; SPI-3; HA; Fusion; Localization; EEV

Introduction

Serine proteinase inhibitors, or serpins, are found in mammals, viruses, and plants, and regulate a variety of biological processes, including inflammation, apoptosis, and coagulation (Silverman et al., 2001). Inhibitory serpins target specific proteinases through recognition of the P1 amino acid in the C-terminal reactive center loop (RCL) of the serpin molecule (Djie et al., 1997; Dahlen et al., 1998). Inhibition of the proteinase is achieved through the formation of a stable covalent complex of the serpin with the proteinase (Wilczynska et al., 1997; Simonovic et al., 2000). Functions other than proteinase inhibition have been ascribed to serpins. For instance, several serpins have binding sites or other activity-associated domains that are not within the RCL, including the nuclear localization signal of MENT (Grigoryev et al., 1999) and the heparin binding site of antithrombin (Whisstock et al., 2000).

Poxviruses are the only virus family known to encode functional serpins. The orthopoxviruses, which include variola, vaccinia, cowpox, and rabbitpox viruses, each encode three serpins, *SPI-1*, *SPI-2/crmA*, and *SPI-3*. The SPI-1 protein has been shown to inhibit cathepsin G in vitro, and this activity is dependent on the P1 amino acid of the serpin (Moon et al., 1999). Deletion of the *SPI-1* gene from rabbitpox virus (RPVΔSPI-1) results in a restricted host-range phenotype whereby the mutant virus will no longer grow on normally permissive cell lines (Brooks et al., 1995). This restricted host range phenotype results in an apoptotic-like process in non-permissive cells (Brooks et al., 1995) but does not involve caspase activation (Moon et al., 1999). A RPV recombinant expressing a *SPI-1* gene with a mutated P1 residue has a host range phenotype similar to a deletion mutant (Moon et al., 1999), indicating that the proteinase inhibitory activity of SPI-1 is required for full host range of RPV.

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The SPI-2/crmA protein from cowpox virus (CPV) has been shown to have anti-apoptotic activity in a number of cell systems (Gagliardini et al., 1994; Ray and Pickup, 1996) and the purified protein has inhibitory activity against several proteinases including caspases-1 and -8 (Ray et al., 1992; Komiyama et al., 1994; Zhou et al., 1997) and granzyme B (Quan et al., 1995). Chicken chorioallantoic membranes infected with CPV deleted for *crmA* (CPVΔcrmA) produce white, inflammatory pocks as opposed to the hemorrhagic, non-inflammatory, red pocks formed by infection with wild-type CPV (Palumbo et al., 1989). Therefore, SPI-2 has both anti-apoptotic and anti-inflammatory activities in vivo.

CPV and other poxviruses deleted for *SPI-3* (CPVΔSPI-3) result in the fusion of infected CV-1 cells, creating polykaryocytes (Turner and Moyer, 1992; Law and Smith, 1992; Zhou et al., 1992). This fusion is indistinguishable from that resulting from the deletion of the CPV hemagglutinin (*HA*) gene (Turner and Moyer, 1992). The fusion which results from disruption of either gene is believed to be dependent on the 14kDa (p14) protein present on the surface of intracellular mature virus (IMV) (Sodeik et al., 1995), as a monoclonal antibody against p14 inhibits fusion caused by deletion of either gene (Turner and Moyer, 1992). Western Blot analysis of HA from CPVΔSPI-3 infected cells demonstrates that the HA is stable and otherwise unaffected by loss of the *SPI-3* gene (Turner and Moyer, 1992). Co-infection of CPVΔSPI-3 with CPV deleted for the *HA* gene (CPVΔHA) dramatically inhibits the amount of fusion. Therefore, the CPV SPI-3 and HA proteins are both involved in the inhibition of fusion in a common pathway which involves p14. HA is present on the surface of extracellular enveloped virus (EEV) of VV strain IHD-J but absent from intracellular mature virus (IMV) (Payne and Norrby, 1976).

The importance of serpin proteinase inhibitory activity on the inhibition of fusion by SPI-3 has been analyzed in an infected cell fusion assay (Turner and Moyer, 1995). Site directed mutations within SPI-3's RCL that included the P1/P1′ residues, P5 to P5′ residues, and P17 to P10 residues had no effect on fusion inhibition. As a result, it is very likely that SPI-3 mediated inhibition of fusion does not require proteinase inhibitory activity.

Subsequently, SPI-3 was purified and shown to effectively inhibit urokinase-type and tissue-type plasminogen activators as well as plasmin in enzymatic assays, with inhibition constants ranging from 0.5–2 nM (Turner et al., 2000). This activity was shown to be dependent on a P1 residue of Arg. Therefore, SPI-3 is a bifunctional protein, having activity against proteinases in vitro and RCL-independent fusion inhibition activity following infection in vivo.

Overall, SPI-3 is biochemically very similar to the SERP1 serpin of myxoma virus (MYX), although their amino acid sequence similarity is fairly low. Both SPI-3 and SERP1 have P1 residues of arginine and have similar proteinase inhibition profiles and kinetic properties in vitro (Lomas et al., 1993; Turner et al., 2000). However, the SPI-3 protein is produced early in infection and is thought to be intracellular, whereas the SERP1 protein is produced late in infection and is secreted (Wang et al., 2000). SERP1 is also a virulence factor in MYX, required for full development of a lethal disease (Upton et al., 1990; Macen et al., 1993). The ability of SPI-3 to substitute for SERP1 of MYX has been analyzed (Wang et al., 2000). The *SPI-3* ORF replaced the *SERP1* ORF in MYX, under the control of the *SERP1* promoter. Similarly, a CPV recombinant was derived where the *SPI-3* ORF was replaced by *SERP1* under control of the *SPI-3* promoter. Infection of CV-1 cells with CPVΔSPI-3::SERP1 caused cell fusion and SERP1 was secreted. Therefore SERP1 cannot substitute for SPI-3 to inhibit fusion.

MYXΔSERP1::SPI-3 infection of rabbits was just as attenuated as MYX deleted for *SERP1*. Surprisingly, when RK-13 cells were infected with MYXΔSERP1::SPI-3, SPI-3 protein was now secreted, in contrast to wt CPV infected cells, where only low levels of SPI-3 secretion are observed. The secretion of SPI-3 by the MYX recombinant was proposed to reflect either a modification of the cell's secretion machinery by MYX, or by the lack in MYX of a protein present in CPV infections that served to retain SPI-3 within the infected cell (Wang et al., 2000).

While the SPI-3 protein has been extensively characterized in vitro, there is little information regarding intracellular localization, and what features of the protein are required for control of fusion. To understand how SPI-3 inhibits fusion, we examined the SPI-3 protein for motifs that might be important in fusion inhibition. SPI-3 protein from infected cells has previously been shown to have N-linked but not O-linked glycosylation (Turner and Moyer, 1995). N-linked sugars have been shown to be critical for a number of protein properties, including proper folding and localization as well as providing contacts for protein-protein interactions (Lis and Sharon, 1993; Dennis et al., 1999). Sequence analysis of CPV SPI-3 indicates the presence of 4 potential N-linked glycosylation sites fitting the consensus sequence N-X-S/T, where X is any amino acid except proline, at positions N161, N188, N232, and N242. In addition to N-linked glycosylation, the first 15 amino acids of SPI-3 comprise a potential signal sequence (SS), as determined by the computer signal sequence prediction program SignalP v1.1 (Nielsen et al., 1997), which if functional, would direct SPI-3 to the secretory pathway. In this paper, we demonstrate that SPI-3 co-localizes to the plasma membrane and to EEV like the viral HA, and that the N-terminal signal sequence, but not N-linked glycosylation, is required for this localization and for fusion inhibition. Surprisingly, the viral HA was needed for normal localization of SPI-3 to the plasma membrane and deletion of HA leads to markedly increased secretion of SPI-3. These results suggest a novel requirement of HA for SPI-3 plasma membrane localization resulting in fusion inhibitory activ-

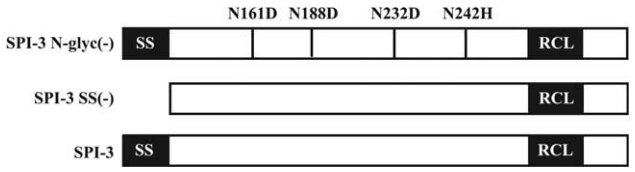

Fig. 1. Schematic representation of the CPV SPI-3 protein. A putative signal sequence (SS) is found from amino acids 1 to 15 at the N-terminus of the SPI-3 protein. Four potential N-linked glycosylation sites that fit the consensus sequence N-X-S/T, where X is any amino acid except proline, are found at the following asparagines (N): N161, N188, N232, and N242. The Reactive Center Loop (RCL), containing the P1 amino acid which determines the specificity of the serpin, is found at the C-terminus of the protein. The SPI-3 N-glyc(−) protein has all four N-linked glycosylation sites mutated as follows: N161D, N188D, N232D, N242H. The SPI-3 SS(−) protein lacks the signal sequence (AA 1–15) which is replaced by an initiating methionine. The SPI-3 SS(−) protein retains the 4 potential N-linked glycosylation sites.

ity. Implications for the localization of SPI-3 on cellular membranes and virions are discussed.

Results

Creation of SPI-3 genes with mutated N-linked glycosylation sites or lacking an N-terminal signal sequence

To investigate whether N-linked glycosylation of SPI-3 was required for fusion inhibition, each of SPI-3's 4 predicted N-linked glycosylation sites (N-X-S/T, where X is any amino acid except proline) was mutated separately by site directed mutagenesis to the following amino acids: N161D, N188D, N232D, N242H. In addition, a final mutant was created which contained mutations in all four potential N-linked glycosylation sites (SPI-3 N-glyc(−)) (Fig. 1). To address the effects of mutations in potential N-linked glycosylation sites, cells were first infected with vTF7-3, a vaccinia virus expressing the T7 RNA polymerase, transfected with plasmids containing a P_{T7}-driven wt or mutant SPI-3 gene, then ^{35}S radiolabeled proteins were resolved by SDS–PAGE (Fig. 2). Mutation of a single glycosylation site would be expected to decrease the apparent molecular weight of SPI-3 by 1.5 kDa to 3 kDa.

Infection with vTF7-3 results in shutoff of host protein synthesis and the appearance of viral specific proteins. This pattern of protein expression is observed when empty vector (control) is transfected into vTF7-3 infected cells (lane 2) compared to uninfected cells (lane 1). After transfection with a plasmid containing the wt *SPI-3* gene, two radiolabeled bands appear at about 51 kDa and 49 kDa (lane 3, asterisks), presumably reflecting complete and partially glycosylated forms of SPI-3 due to overexpression of the protein. It has previously been demonstrated that when SPI-3 is expressed in a similar fashion but in the presence of tunicamycin, an N-linked glycosylation inhibitor, that the 51 and 49 kDa SPI-3 bands are reduced to 42 kDa, approximately the predicted molecular weight of the unglycosylated protein (Turner and Moyer, 1995). Therefore, the higher molecular weight forms seen in lane 3 are a result of N-linked glycosylation. Transfection with each of the *SPI-3* N-linked glycosylation mutant genes N188D, N232D, and N242H (lanes 5–7, respectively) lowers the molecular weight of the two bands seen with wt SPI-3 by approximately 1.5–3 kDa, as expected for the loss of N-linked glycan at the mutated site. The N161D mutation resulted in the appearance of only a single, lower molecular weight band at 48 kDa (lane 4). The reduced SPI-3 molecular weights of each of the single glycosylation mutants (lanes 4–7) are consistent with elimination of a functional N-linked glycosylation site. SPI-3 N-glyc(−) with all four sites mutated (lane 8) runs with the lowest apparent molecular weight (approximately 42 kDa), suggesting loss of all N-linked glycan addition, as this protein exhibits the same electrophoretic mobility as SPI-3 synthesized in vitro where there is no glycosylation (data not shown). In fact, all SPI-3 N-linked glycosylation mutant proteins synthesized in vitro have the same molecular weight by SDS–PAGE as wt SPI-3 protein made in vitro (data not shown). Therefore, the engineered amino acid mutations in SPI-3 do not result in changes in SPI-3 mobility when subject to SDS–PAGE. Since SPI-3 has been shown previously to be N-linked glycosylated when expressed in the context of vaccinia virus, we can conclude that the molecular weight changes observed in Fig. 2 are the result of loss of N-linked glycans. Therefore, this assay demonstrates that all four potential N-linked glycosylation sites of SPI-3 are used in vivo.

Since SPI-3 is glycosylated, it is likely that SPI-3 enters the secretory pathway, which would allow for N-linked glycan addition in the ER/Golgi apparatus. Entry of SPI-3 into the secretory pathway would be facilitated by the presence of a potential N-terminal signal sequence. To address whether SPI-3′s entry into the secretory pathway is neces-

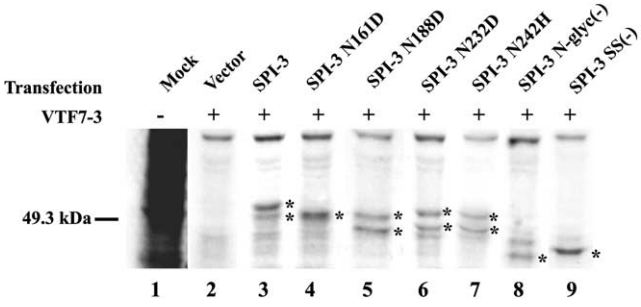

Fig. 2. Utilization of the four potential N-linked glycosylation sites of SPI-3. Cells were mock infected (lane 1) or infected with vTF7-3 (lanes 2–9). Samples in lanes 2–9 were then transfected with plasmid DNAs as follows: lane 2, pTM1 (vector); lane 3, pTM1-SPI-3; lane 4, pTM1-SPI-3 N161D; lane 5, pTM1-SPI-3 N188D; lane 6, pTM1-SPI-3 N232D; lane 7, pTM1-SPI-3 N242H; lane 8, pTM1-SPI-3 N-glyc(−); lane 9, pTM1-SPI-3 SS(−). Radiolabeled proteins in extracts were visualized following SDS–PAGE and autoradiography. Asterisks denote the positions of P_{T7}-driven wild-type and mutant SPI-3 proteins which were readily visible over the background of vaccinia virus proteins.

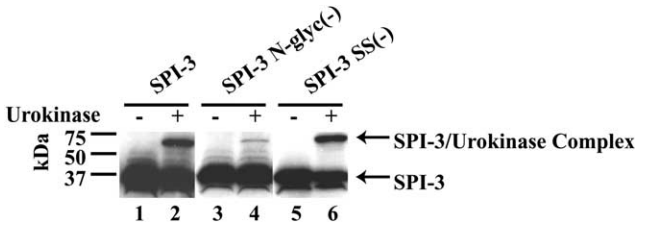

Fig. 3. SPI-3 mutants retain the ability to complex with urokinase in vitro. Radiolabeled wt or mutant SPI-3 protein synthesized in vitro was incubated in the absence (lanes 1, 3, and 5) or the presence (lanes 2, 4, and 6) of human low molecular weight urokinase. Higher molecular weight serpin-proteinase complexes were then visualized following SDS–PAGE and autoradiography. The following plasmids were used to generate radiolabeled SPI-3 protein: lanes 1 and 2, pTM1-SPI-3; lanes 3 and 4, pTM1-SPI-3 N-glyc(−); lanes 5 and 6, pTM1-SPI-3 SS(−). The lower arrow indicates the position of the uncomplexed ^{35}S-labeled SPI-3 protein and the upper arrow indicates the position of higher molecular weight SPI-3-urokinase complexes.

sary for fusion inhibition, a mutant SPI-3 SS(−) was created which eliminated the first 15 amino acids (a predicted signal sequence) and placed a methionine before the 16th amino acid (Fig. 1). Transfection of SPI-3 SS(−) into vTF7-3 infected cells (Fig. 2, lane 9) results in a form of SPI-3 similar to the molecular weight of SPI-3 N-glyc(−) (lane 8), due to lack of glycosylation. Slight differences in molecular weights between these two proteins may be due to differential cleavage of the signal sequence of SPI-3 N-glyc(−) in cells compared to the engineered cleavage of SPI-3 SS(−).

SPI-3 with either mutated N-linked glycosylation sites or lacking the signal sequence retains the ability to complex with urokinase in vitro

Unglycosylated SPI-3 has previously been demonstrated to act as a potent serine proteinase inhibitor in vitro (Turner et al., 2000). To determine whether the mutations in either SPI-3 N-glyc(−) and/or SPI-3 SS(−) affect the ability of SPI-3 to act as a serine proteinase inhibitor, in vitro ^{35}S-labeled wt SPI-3, SPI-3 N-glyc(−) and SPI-3 SS(−) were incubated with human low molecular weight urokinase, and the products analyzed by SDS-PAGE (Fig. 3). Note that labeled SPI-3 in this assay is not glycosylated and therefore any differences in serpin function are due to the engineered amino acid mutations. In the presence of the serine proteinase urokinase, wt SPI-3 forms a readily detectable higher molecular weight complex which is resistant to boiling in the presence of SDS, a characteristic of active inhibitory serpins (lane 2). We quantified the amount of complex formed in the presence of urokinase as a percent of the input serpin without urokinase. In the presence of urokinase, 11.8% of wt SPI-3 is in the higher molecular weight complex. Less SPI-3 N-glyc(−)/urokinase complex is found compared to wt SPI-3 (6.6%), whereas the levels of SPI-3 SS(−)/urokinase complex are somewhat higher (23.0%). Similar percentages of serpin-protease complex were consistently found over 3 different concentrations of urokinase. Therefore, while the mutations made in SPI-3 N-glyc(−) may render the serpin less effective as an inhibitor compared to either wt SPI-3 or SPI-3 SS(−), these mutations do not intrinsically destroy serpin activity.

Characterization of CPV recombinants for expression of SPI-3

To address whether SPI-3 N-glyc(−) or SPI-3 SS(−) retained the ability to inhibit cell-cell fusion, CPV recombinants were generated where the native *SPI-3* gene was replaced with a mutant *SPI-3* gene to generate either CPV SPI-3 N-glyc(−) or CPV SPI-3 SS(−). These constructs were examined by Western Blot analysis to ensure the stability and appropriate molecular weight of the mutant SPI-3 proteins. CV-1 cells were infected with wt CPV, CPVΔSPI-3, CPV SPI-3 N-glyc(−), and CPV SPI-3 SS(−) and infected cell proteins were analyzed by Western Blot using a monoclonal antibody against SPI-3 (Fig. 4). No SPI-3 protein was detected from CPVΔSPI-3 cell pellets (lane 1). There is a background protein of approximately 53 kDa in pellets from CPVΔSPI-3 infected cells. This protein is present in Western Blot analysis of non-infected cell pellets as well (data not shown). SPI-3 protein from wt CPV infected cells (lane 2) presents as a diffuse immunoreactive band between 50 and 55 kDa, indicative of variable N-linked glycosylation as previously described (Turner and Moyer, 1995). SPI-3 protein from a CPV SPI-3 N-glyc(−) infection is a single immunoreactive band (lane 3), at a lower molecular weight than SPI-3 protein from a wt CPV infection, a result of the loss of N-linked glycans. After infection with CPV SPI-3 SS(−), SPI-3 protein was also detected as a single band (lane 4), and migrated at the same position as SPI-3 N-glyc(−) protein (lane 3). With the loss of the signal sequence, we hypothesize that SPI-3 would no longer have access to the secretory pathway, would not be glycoyslated but would exist as a cytoplasmic non-glycosylated protein. Therefore, each SPI-3 protein, either mutant or wild-type, ran at the expected molecular weight based on predicted functions of N-linked glycosylation sites and the signal sequence, in that loss of N-linked glycosylation sites

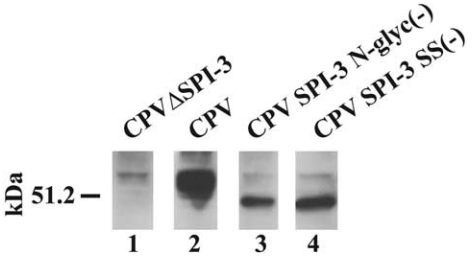

Fig. 4. Western Blot analysis of CPV recombinants expressing mutant SPI-3. Cells were infected with virus as indicated and infected cell proteins were separated by SDS–PAGE followed by Western Blot analysis using an anti-SPI-3 mAb. Lane 1, CPVΔSPI-3 infected cells; lane 2, wt CPV infected cells; lane 3, CPV SPI-3 N-glyc(−) infected cells; lane 4, CPV SPI-3 SS(−) infected cells.

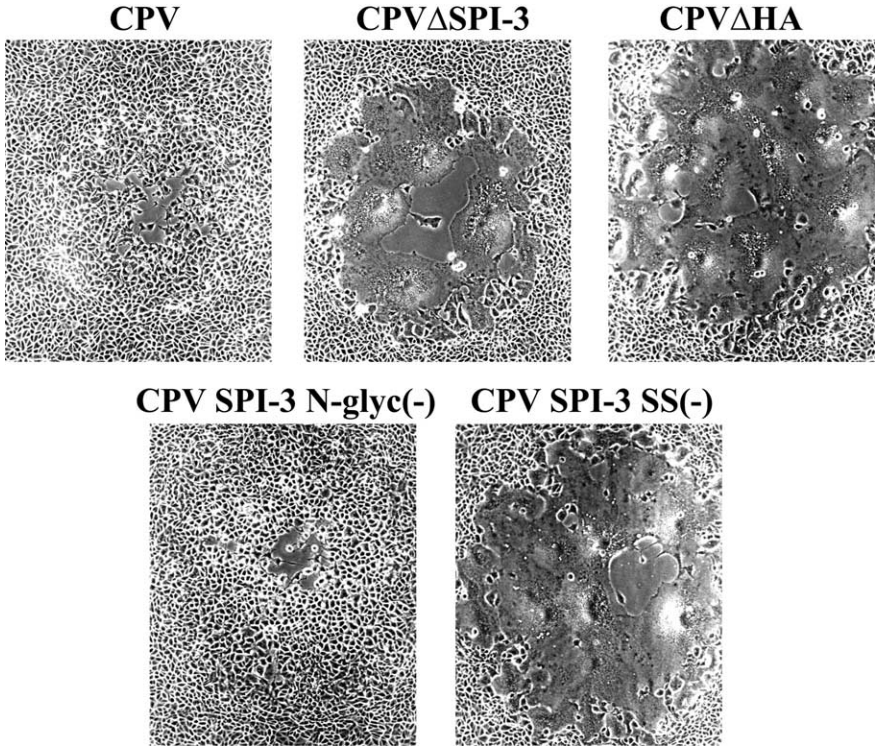

Fig. 5. The effects of SPI-3 mutations on cell fusion. Cells were infected under liquid with the following viruses: wt CPV, CPVΔSPI-3, CPVΔHA, CPV SPI-3 N-glyc(−), CPV SPI-3 SS(−), and photographed at 2 days p.i.

or the signal sequence of SPI-3 resulted in the protein having a lower apparent molecular weight from infected cells.

The signal sequence but not N-linked glycan addition of SPI-3 is necessary for fusion inhibition in vivo

Deletion of either the *SPI-3* or the *HA* gene from CPV results in cell-cell fusion (Turner and Moyer, 1992). The recombinants CPV SPI-3 N-glyc(−) and CPV SPI-3 SS(−) were analyzed for the ability to inhibit fusion by infecting CV-1 cells and evaluating the resulting plaques at 2 days post infection (p.i.) for cell-cell fusion (Fig. 5). Wt CPV infection demonstrated characteristic cytopathic effect with individual cell rounding but no cell-cell fusion. Infection with CPVΔSPI-3 and CPVΔHA gave extensive cell-cell fusion as expected, with syncytial plaques containing giant multi-nucleated cells. CPV SPI-3 N-glyc(−) infection gave no cell-cell fusion, indicating N-linked glycosylation per se is not required for fusion inhibition. However, infection with CPV SPI-3 SS(−) resulted in the formation of syncytia and therefore the signal sequence of SPI-3 is necessary for inhibition of cell fusion. We next studied the effects of the N-glyc(−) and SS(−) mutations of SPI-3 on localization.

SPI-3 localizes to the Golgi apparatus early in infection

The presence of N-linked glycans suggests that SPI-3 must pass through the ER/Golgi apparatus. Therefore, we examined the cellular localization of SPI-3 at different times post infection. Cells were infected with wt CPV and CPVΔSPI-3 and were analyzed by immunofluorescence at 4 or 6 h p.i. (Fig. 6). At 3 h p.i., no expression of SPI-3 was noted from wt CPV infected cells (data not shown). At four h p.i., some cells infected with wt CPV showed detectable levels of SPI-3 protein (green, Fig. 6A), presumably as expression of SPI-3 is just beginning. Cells infected with fusogenic CPVΔSPI-3 have not yet fused at 4 h p.i., and therefore the gross morphology of cells infected with wt and mutant viruses was identical at this time. SPI-3 was present in wt CPV-infected cells at 4 h p.i. in a perinuclear location (Fig. 6A), similar to that of the mannose-6-phosphate receptor (red), a marker for the Golgi compartment (Fig. 6B). In fact, at early times p.i., SPI-3 co-localized with the mannose-6-phosphate receptor as evidenced by the yellow color (Fig. 6C) after merging of the green and red channels. Note that SPI-3 was not detected in cells infected with CPVΔSPI-3, but infected cells still labeled with the mannose-6-phosphate receptor (panels D–E). Cells shown in panels D and E are shown at a lower magnification to demonstrate a greater number of cells that are not positive for SPI-3 at this time point. SPI-3 did not co-localize at 4 h after wt CPV infection with an ER marker, disulfide isomerase (data not shown). All wt CPV infected cells express SPI-3 by 6 h p.i. (panel F) in a manner consistent with not only Golgi localization, but also some plasma membrane localization. Co-localization with the mannose-6-phosphate receptor showed that SPI-3 protein is

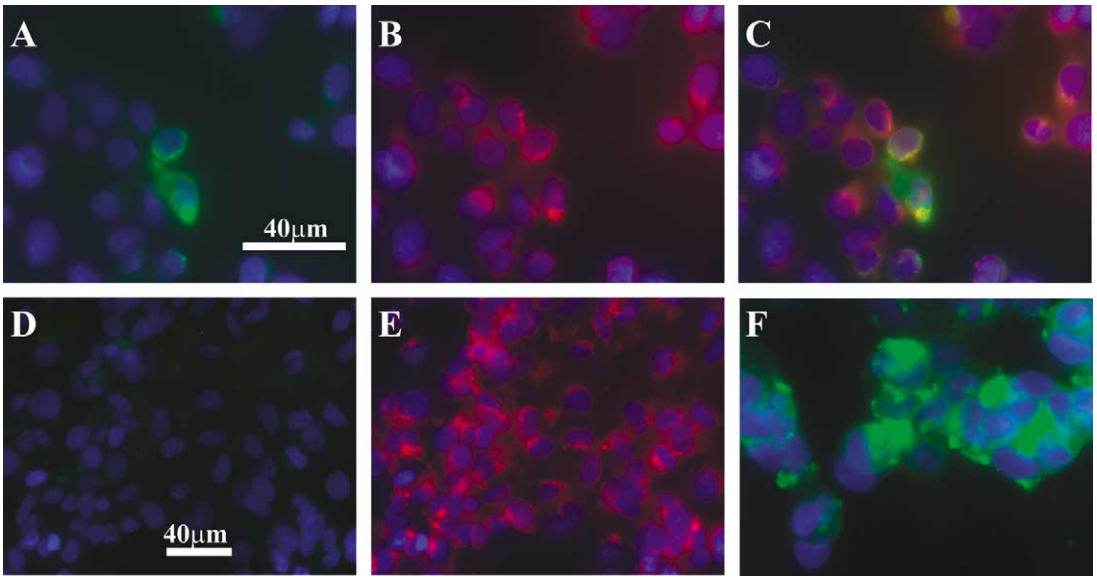

Fig. 6. Immunofluorescent localization of SPI-3 at early times post infection. Cells were infected with wt CPV (panels A–C and F) or CPVΔSPI-3 (panels D–E) and were processed for immunofluorescence at 4 h p.i. (panels A–E) or 6 h p.i. (panel F). All cells were permeabilized with methanol and stained with the DNA stain DAPI (blue) to visualize nuclei. Panels A, C, D, and F show FITC labeling of SPI-3 (green); panels B and E show Texas Red labeling of M6PR, a Golgi marker (red); panel C is a merge of panels A and B (yellow indicates green and red overlap). Size bar in panel A applies to panels A, B, C, and F. Size bar in panel D applies to panels D and E.

present in the Golgi apparatus at early times p.i., as expected for a glycoprotein.

SPI-3 co-localizes with HA to the plasma membrane late in infection

Cell-cell fusion is a late event and can be inhibited with the DNA replication inhibitor AraC (Law and Smith, 1992). Therefore, to understand how SPI-3 inhibits fusion, it is necessary to understand the protein's localization both early and late in infection. We infected CV-1 cells with wt and mutant CPVs and examined them at 16 h p.i. (Fig. 7) for localization of SPI-3. At 16 h p.i. in a wt CPV infection, SPI-3 (green) clearly localizes to the plasma membrane (Fig. 7A). At this time, HA (red) is also present on the surface of infected cells (Fig. 7B). Merging of the images of Fig 7A and B (yellow) shows co-localization of SPI-3 and HA (Fig. 7C). The presence of SPI-3 on the cell membrane is consistent with its role in blocking cell-cell fusion.

Given that CPV SPI-3 N-glyc(−) lacking N-linked glycosylation is able to inhibit fusion, we wanted to determine the localization of the SPI-3 N-glyc(−) protein. Immunofluorescence demonstrated that SPI-3 N-glyc(−) was also present at the surface of infected cells (Fig. 7D). The amount of SPI-3 was somewhat reduced for CPV SPI-3 N-glyc(−) (Fig. 7D) compared with wt CPV (Fig. 7A), suggesting that less protein was present on the surface, although this apparent difference could also reflect more efficient recognition of wt SPI-3 by the monoclonal antibody than the SPI-3 N-glyc(−) mutant. HA was present on the cell membrane following CPV SPI-3 N-glyc(−) infection (Fig. 7E), and SPI-3 N-glyc(−) and HA again co-localized (Fig. 7F). The fact that both wt SPI-3 and SPI-3 N-glyc(−) are at the cell surface is consistent with the inhibition of cell-cell fusion by CPV SPI-3, and demonstrates that SPI-3 can be localized correctly despite a complete absence of N-linked glycosylation.

In cells infected with CPV SPI-3 SS(−), SPI-3 did not appear on the membrane (Fig. 7G–I). The merged red and green channels (Fig. 7I) suggest that HA is at the surface but SPI-3 is present in the cytoplasm as might be expected for a protein lacking a signal sequence. The results of Fig. 7G–I indicate that the SPI-3 signal sequence is required for membrane localization. As previously noted, cells infected with CPV SPI-3 SS(−) fuse (Fig. 5), again consistent with the hypothesis that membrane localization of SPI-3 appears to be necessary to prevent infected cell fusion. At late times p.i., both fusion inhibitory proteins SPI-3 and HA localize to the plasma membrane, even in the absence of N-linked glycosylation of SPI-3. Finally, note that localization of HA at the cell surface occurs in the absence of plasma membrane SPI-3 (Fig. 7H).

Like HA, SPI-3 is a component of extracellular enveloped virus (EEV) but not intracellular mature virus (IMV)

Since both SPI-3 and HA function to inhibit fusion and we have demonstrated both proteins co-localize on infected cells, we next asked whether SPI-3, like HA, could also be found on extracellular enveloped virus (EEV). Poxvirus virions are known to exist in several forms in the infected cell. First, viral DNA and virion proteins are wrapped by membrane to form intracellular mature virus (IMV), which is fully infectious (Hollinshead et al., 1999; Griffiths et al.,

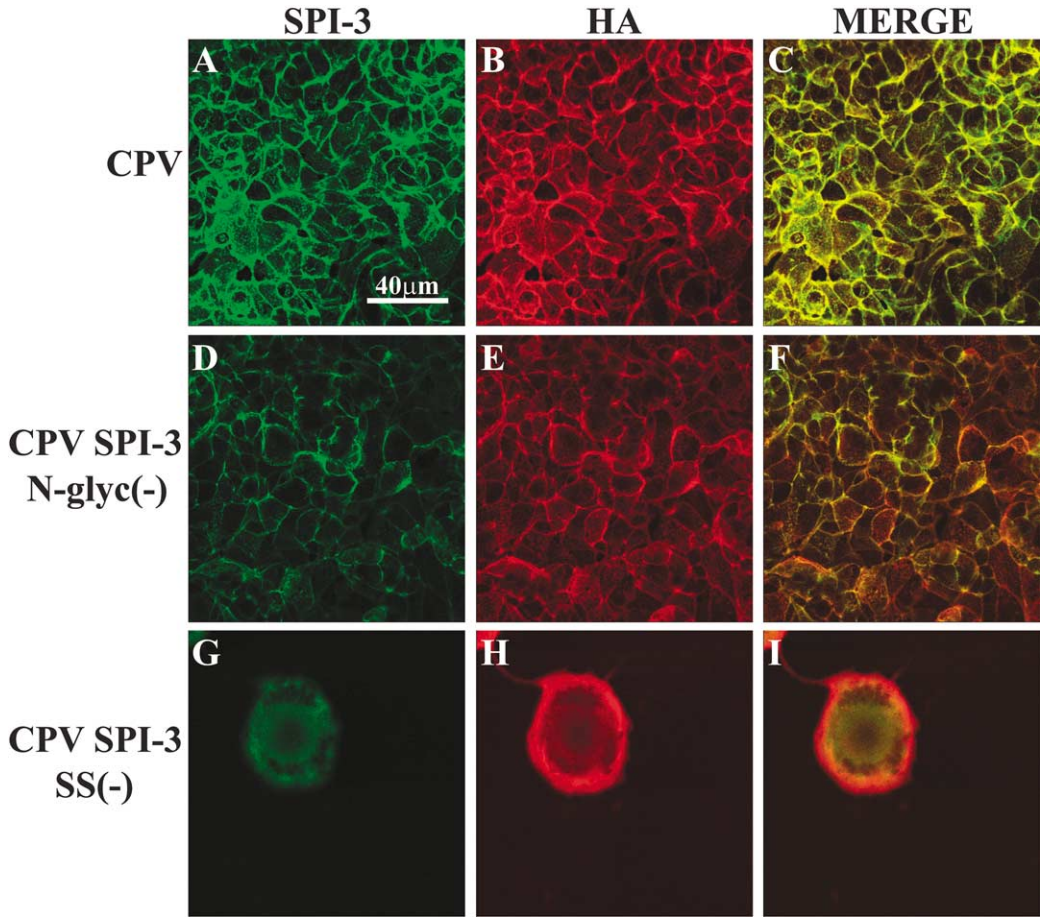

Fig. 7. SPI-3 and HA co-localize to the membrane late in infection. Cells were infected with wt CPV (panels A–C), CPV SPI-3 N-glyc(−) (panels D–F), or CPV SPI-3 SS(−) (panels G–I) for 16 h, then processed for immunofluorescence. Green color, FITC labeling of SPI-3; red color, Texas Red labeling of HA; yellow color, overlap of green and red in merged image.

2001a; Griffiths et al., 2001b). Some IMV are further enveloped by membranes from the trans-Golgi to form intracellular enveloped virus (IEV) (van Eijl et al., 2000). These virions are transported to the cell surface where they fuse with the plasma membrane to form extracellular enveloped virus (EEV) (Smith and Vanderplasschen, 1998). Since the HA protein has previously been shown to be present in vaccinia virus EEV but absent from IMV (Payne and Norrby, 1976), we asked whether SPI-3 could also be found on EEV.

To address this question, EEV and IMV from CPV and CPVΔSPI-3 infected cells were tested for the presence of SPI-3, B5R, a known EEV protein (Martinez Pomares et al., 1993; Mathew et al., 1998), or the 14kDa (p14) protein, a known IMV protein (Sodeik et al., 1995) using an electron microscopy immunogold technique (Fig. 8). EEV prepared from the medium of cells infected with wt CPV shows the presence of the B5R protein (panel C), as expected. SPI-3 was present in wt CPV EEV (panel A), but not in wt CPV IMV (panel D) nor on EEV or IMV purified from CPVΔSPI-3 (panels B and E, respectively). On the other hand, wt CPV IMV shows the presence of the 14 kDa protein (panel F), as previously described (Sodeik et al., 1995). Since EEV and IMV virions labeled with the known EEV protein B5R and the known IMV 14kDa protein, respectively, we can conclude that SPI-3, like the viral HA, is a component of EEV, but not IMV.

Localization of SPI-3 to the plasma membrane requires the HA protein

HA and SPI-3 both localize on the membrane of infected cells as well as EEV and function to inhibit fusion (Figs. 7 and 8). We next analyzed the localization of each of these proteins in the absence of the other. CV-1 cells were infected with CPVΔSPI-3 or CPVΔHA, and examined at 16 h p.i. (Fig. 9). SPI-3 (green) was not detected following infection with CPVΔSPI-3 (data not shown) and large multinucleated cells were evident by DAPI staining (panels A, C). The HA protein (red) localized as expected to the plasma membrane of these giant cells in the absence of SPI-3 protein (panels B and C), in much the same fashion as seen for wt CPV infected cells (Fig. 7B). When CV-1 cells were infected with CPVΔHA, cell-cell fusion again results (panels D–F). As expected, HA protein was not detectable by immunofluorescence (data not shown). However, in the

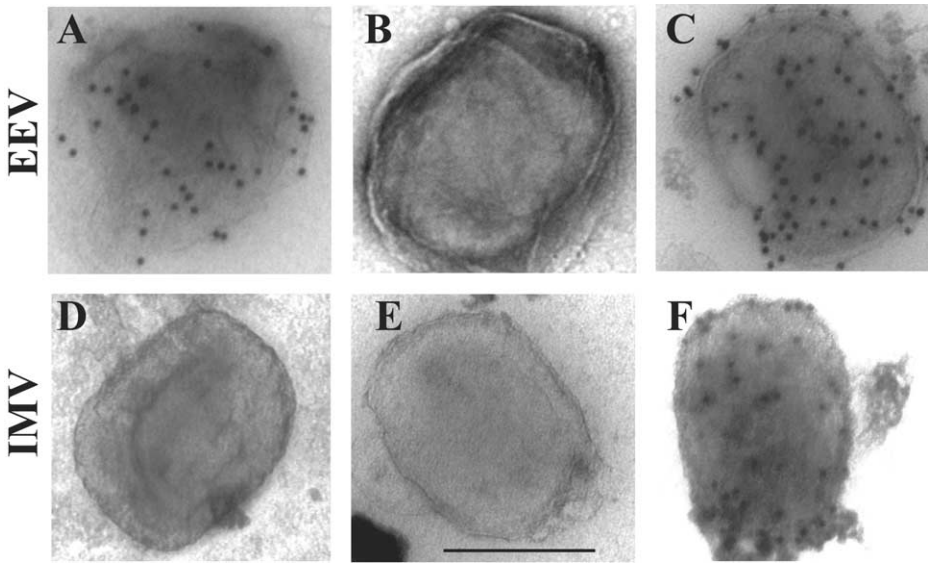

Fig. 8. Detection of SPI-3 in CPV virions by immunogold electron microscopy. EEV (panels A–C) or IMV (panels D–F) of wt CPV (panels A, C, D, F), or CPVΔSPI-3 (panels B, E) were probed with anti-SPI-3 mAb (panels A, B, D, E), anti-B5R mAb (panel C), or anti-14kDa mAbC3 (panel F), treated with a 12 nm-gold-labeled secondary antibody, and then visualized by EM.

absence of HA, SPI-3 is clearly not present at a significant level on the surface of unpermeabilized cells, but instead is present in punctate clumps of unknown localization (panels E and F).

These results suggest that the correct localization of SPI-3 on the plasma membrane directly or indirectly requires the presence of HA protein. The HA might be necessary for one of two functions. Either HA acts as a chaperone to help SPI-3 get from the Golgi to the plasma membrane, or HA is required to retain SPI-3 on the surface of the infected cell. If HA acts to retain SPI-3 on the cell surface, we would expect to see enhanced secretion of SPI-3 into the supernatant of infected cells in the absence of HA.

The viral HA is required for retention of SPI-3 on the plasma membrane of CPV infected cells

To determine if deletion of HA from CPV results in enhanced secretion of SPI-3, CV-1 cells were infected with

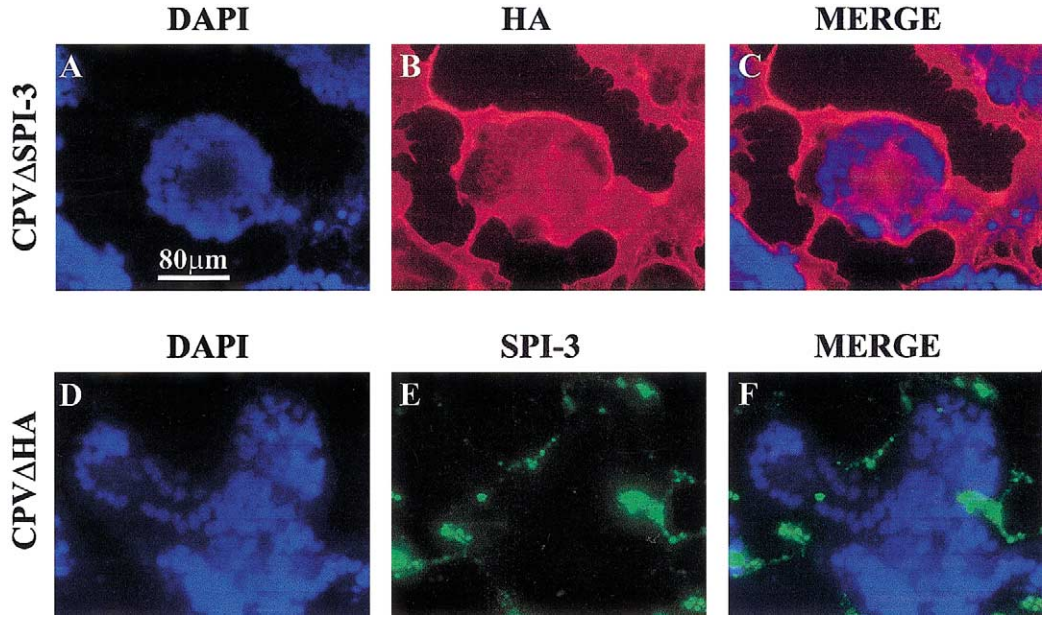

Fig. 9. SPI-3 plasma membrane localization requires the HA protein. Cells were infected with CPVΔSPI-3 (panels A–C) or CPVΔHA (panels D–F) for 16 h and processed for immunofluorescence. All cells were stained with DAPI to visualize nuclei prior to analysis by fluorescence microscopy. Green color, FITC labeling of SPI-3; red color, Texas Red labeling of HA; yellow color, overlap of green and red in merged images.

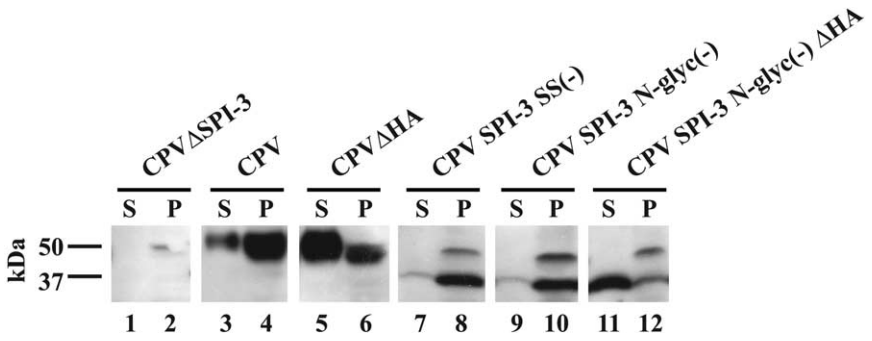

Fig. 10. HA is required for retention of SPI-3 on the plasma membrane. Cells were infected with the viruses indicated and cell equivalents of supernatant (S) and pellet (P) were separated by SDS–PAGE followed by Western Blot analysis using an anti-SPI-3 mAb. Lane 1, supernatant from CPVΔSPI-3 infected cells; lane 2, cell-associated pellet from CPVΔSPI-3 infected cells; lanes 3 and 4, supernatant and pellet respectively from wt CPV infected cells; lanes 5 and 6, supernatant and pellet from CPVΔHA infected cells; lanes 7 and 8, supernatant and pellet from CPV SPI-3 SS(−) infected cells; lanes 9 and 10, supernatant and pellet from CPV SPI-3 N-glyc(−) infected cells; lanes 11 and 12, supernatant and pellet from CPV SPI-3 N-glyc(−) ΔHA infected cells.

wt CPV, CPVΔSPI-3, CPV SPI-3 N-glyc(−), CPV SPI-3 SS(−), and CPVΔHA, and both supernatant (S) and cell pellet (P) fractions were analyzed by Western Blot using monoclonal antibody against SPI-3 (Fig. 10) (Materials and Methods). No SPI-3 protein was detected from CPVΔSPI-3 supernatant or cell pellets (lanes 1 and 2), although there is a background band at approximately 50 kDa in pellets from CPVΔSPI-3 infected cells. This band is present even following Western Blot analysis of non-infected cell pellets (data not shown). SPI-3 protein from a wt CPV infection was found in both supernatant (low levels) and cell pellets, indicating that SPI-3 is poorly but partially secreted (lanes 3 and 4). SPI-3 protein from a CPVΔHA infection is of a wild-type molecular weight distribution. However, far more SPI-3 was secreted into the supernatant than from a wt CPV infection (lanes 5 and 6). This result was independently verified in three replicate experiments. These data suggest that SPI-3 secretion or release from the membrane is inhibited by HA.

After infection with CPV SPI-3 SS(−), SPI-3 SS(−) protein was predominantly detected in the cell pellet (lane 8) as was SPI-3 N-glyc(−) protein following CPV SPI-3 N-glyc(−) infection (lane 10). While both wt SPI-3 and SPI-3 N-glyc(−) are found on the membrane, it is noteworthy that release of SPI-3 N-glyc(−) into the supernatant is very low (compare lanes 3 and 9).

When the HA gene is deleted from CPV SPI-3 N-glyc(−), SPI-3 is no longer found on the membrane by immunofluorescence (data not shown) and instead is now efficiently released from the surface of the infected cell (compare Fig. 10, lanes 9–12). The collective results of Figs. 9 and 10 suggest that HA is necessary not for trafficking of SPI-3 to the membrane but rather for retention of SPI-3 at the surface of the infected cell. In the absence of HA, SPI-3 is not retained at the surface and the secretion of SPI-3 becomes quite efficient and reminiscent of the extensive secretion of SPI-3 seen when cells are infected with myxoma virus containing SPI-3 (Wang et al., 2000).

Discussion

We demonstrate here that late in infection, SPI-3 is found on the plasma membrane, consistent with its role in blocking cell-cell fusion. Cell surface localization of SPI-3 and fusion inhibition occurred normally in the complete absence of N-linked glycosylation, but both were abolished by deletion of the SPI-3 signal sequence. This result supports our hypothesis that amino acids 1–15 of SPI-3 constitute an active signal peptide that directs the protein to the secretory pathway, and is consistent with our inability to purify SPI-3 by attaching a His tag to the extreme N-terminus (PC Turner, unpublished results). When the SS was removed, N-tagged His_{10}-SPI-3 protein could be successfully purified from infected cells (Turner et al., 2000).

The presence of a low level of SPI-3 in the supernatant of wt CPV infected CV-1 cells (Fig. 10) is somewhat different from the result obtained by Wang et al. (2000), where no secretion of SPI-3 was demonstrated from RK-13 cells that had been infected with a vaccinia virus overexpressing SPI-3. However, in the earlier work, SPI-3 was overexpressed and a polyclonal antisera was used rather than the monoclonal antibody used here. Precedence for partial secretion can be found among a number of serpin family members, including the mammalian serpin maspin (Pemberton et al., 1997). The orientation of SPI-3 on the plasma membrane was not determined; however, we hypothesize that the majority of membrane associated SPI-3 protein is outside the cell, as our monoclonal antibody recognizes SPI-3 on the surface of unpermeabilized infected cells. Experiments with Triton-X114 indicate that SPI-3 partitions with the detergent phase (data not shown) and therefore may be an integral membrane protein. However, the SPI-3 protein lacks a convincing transmembrane sequence by available prediction programs, unlike HA, which definitely appears to be an integral membrane protein.

When the SPI-3 gene is deleted, the HA protein localizes to the plasma membrane normally, but when the HA protein

is deleted, SPI-3 is no longer found on the plasma membrane (Fig. 8). Deletion of the HA gene did not alter the localization of the viral B5R protein as judged by immunofluorescence (data not shown), indicating that the effect of HA on SPI-3 localization is relatively specific. The greater secretion of SPI-3 from cells infected with CPVΔHA compared with wt CPV leads us to hypothesize that HA is specifically required for retention of SPI-3 on the plasma membrane. A similar phenomenon was seen when the extent of SPI-3 secretion was compared for CPV SPI-3 N-glyc(−) and its HA^- derivative. The high level of SPI-3 in the supernatant from cells infected with CPV SPI-3 N-glyc(−) ΔHA indicates that secretion does not require glycosylation.

The increased secretion of CPV SPI-3 in the absence of HA may explain the results of Wang et al. (2000), who inserted the SPI-3 ORF into MYX in place of the native serpin SERP1. Expression of SPI-3 from MYX gave efficient secretion into the supernatant of infected cells. The genomic sequence of MYX does not contain a homolog of the orthopoxvirus HA protein (Cameron et al., 1999), and we hypothesize that the lack of HA causes a failure to retain SPI-3 at the surface of cells infected with MYX-SPI-3 and instead produces secretion.

The role of SPI-3 present in EEV is unknown; however, it is interesting to note that IMV contains the 14kDa fusion protein on its outer surface whereas EEV contains the anti-fusion SPI-3 and HA proteins. EEV and IMV enter uninfected cells by different mechanisms, although the exact steps involved have not been determined (Locker et al., 2000). Based on plaque development, SPI-3 does not seem to be required for the infectivity of EEV in CV-1 cells. It remains to be seen whether SPI-3 has a role in the specific entry of EEV into certain cells. Another possibility is that the presence of SPI-3 on EEV may prevent fusion of newly made virions back to the infected cell. According to this hypothesis, SPI-3 would be necessary to prevent superinfection of CPV infected cells. Experiments are currently underway to address this question.

Finally, our results suggest how SPI-3 and HA might function to inhibit infected cell fusion. Since cells infected with CPVΔSPI-3 fuse, yet the HA protein is on the surface, we can conclude that HA alone cannot act to inhibit fusion. SPI-3 may be the actual anti-fusion protein, with HA merely acting to ensure the correct localization of SPI-3 on the surface of infected cells. According to this model, fusion observed in the absence of HA may result from the failure to retain SPI-3 at the cell surface. Alternatively, both SPI-3 and HA may be required at the cell surface for fusion inhibition. Both models imply that HA and SPI-3 may directly interact with one another, with HA acting as an integral membrane protein anchor to retain SPI-3 on the surface of the infected cell. We are currently investigating the direct association of SPI-3 and HA within CPV infected cells.

Materials and methods

Cells and viruses

CV-1 cells were obtained from the American Type Culture Collection (ATCC CCL-70) and were routinely maintained in GIBCO-BRL minimum essential medium (MEM) with Earle's salts supplemented with 5% fetal bovine serum, 2 mM glutamine, 50 U of penicillin G per ml, 50 μg of streptomycin per ml, 1 mM sodium pyruvate, and 0.1 mM MEM nonessential amino acids (GIBCO-BRL, Rockville, MD). wt CPV (Brighton Red strain) was obtained from David Pickup (Duke University Medical Center). CPVΔSPI-3 and CPVΔHA have been described previously (Turner and Moyer, 1992).

Production of a monoclonal antibody to SPI-3

Purification of His-tagged SPI-3 protein lacking the N-terminal signal sequence was carried out as previously described (Turner et al., 2000). Monoclonal antibodies were generated by the Hybridoma Core at the University of Florida's ICBR facility according to established protocols. Monoclonal antibody 4A11-4A3 against SPI-3 was chosen for use in all following experiments.

Site-directed mutagenesis of SPI-3

The four potential N-linked glycosylation sites (N161, N188, N232, and N242) were mutated using the Altered Sites mutagenesis system (Promega Corp.) following the manufacturer's instructions. The fully sequenced CPV *SPI-3* gene was cloned into the pAlter-Ex1 vector using *Eco*RI and *Hind*III. The following 5′-phosphorylated oligos were used to mutate each of the four N-linked glycosylation sites, with mismatched bases indicated by underlining. FS55 (5′-pCTACTATGCTCGAGGATAATACTCTATG) was used to mutate asparagine 161 to aspartic acid (N161D). Note that for screening purposes the neighboring aspartic acid 160 was changed to glutamic acid 160. This primer introduces a new *Xho*I site that was used for screening purposes. FS56 (5′-pGATATCACTAAAACGCGCGATGCTAGTTTTAC) was used to mutate asparagine 188 to aspartic acid (N188D) and introduces a *Hha*I site used to screen for mutants. FS57 (5′-pCGTATAAGGATGCTGATATCAGTATGTACCTGGC) was used to mutate asparagine 232 to aspartic acid (N232D) and adds a new *Eco*RV site. FS58 (5′-pGGCAATAGGTGATGATATGACTCATTTC) was used to mutate asparagine 242 to histidine (N242H) and introduces a *Nde*I site used for screening purposes. These mutagenic primers were used to construct individual mutants of SPI-3 deficient in each of the four N-linked glycosylation sites. To construct a mutant deficient in all four N-linked glycosylation sites, the following strategy was used. FS55 and FS56 were used to create pAlter-Ex1 SPI-3 N161D N188D. FS57 and FS58 were used to create pAlter-Ex1 SPI-3 N232D N242H. Both plasmids were cut with *Sal*I and the 1.1 kb DNA fragment from pAlter-Ex1 SPI-3 N161D N188D was ligated to the 0.824

kb piece of DNA from pAlter-Ex1 SPI-3 N232D N242H to generate pAlter-Ex1 SPI-3 N-glycosylation (−). SPI-3 N-linked glycosylation mutants were subcloned from pAlter-Ex1 into pTM1-SPI-3 using *BsrG*I and *Bgl*II. To generate a mutant of SPI-3 lacking the N-terminal signal sequence, SPI-3 from pAlter-Ex1 was amplified using oligos FS252 (5′GCATGCCATGGCATATCGTCTGCAAGGATTT-ACC3′) and FS256 (5′GCGCGCGGATCCTTAAG-GAGATTCCACCTTACC3′). FS252 eliminates 15 N-terminal amino acids of SPI-3 and introduces an NcoI site. This PCR product as well as pTM1 were digested with *Nco*I and *Bam*HI and ligated to generate pTM1-SPI-3 signal sequence (−). All plasmids were sequenced to verify appropriate nucleotide changes.

In vitro transcription, translation, and complex formation with urokinase

Production of ^{35}S labeled SPI-3 protein derivatives was carried out using the TNT T7 Quick Coupled Transcription/Translation system (Promega Corp.) as directed by the manufacturer using pTM1-SPI-3, pTM1-SPI-3 N-glyc(−) and pTM1-SPI-3 SS(−) as the DNA template. To analyze the ability of radiolabeled SPI-3 protein to complex in vitro with urokinase, 3 μl of ^{35}S labeled SPI-3 protein made in the TNT system were incubated with buffer alone or with 0.25 μl (7.5 U) of human urine low molecular weight urokinase (Calbiochem, La Jolla, CA) in a total volume of 12 μl at 37°C for 30 min. Reactions were stopped by addition of 6× SDS sample buffer plus BME and boiling for 3 minutes. Samples were then separated on SDS–10% polyacrylamide gels which were fluorographed with Amplify, and the radiolabeled proteins visualized by autoradiography. Intensity of bands was quantified using ImageQuant 5.0 on a Molecular Dynamics STORM phosphorimager.

Expression of SPI-3 site directed mutants in the vaccinia virus T7 system

To demonstrate that all 4 potential N-linked glycosylation sites were used in SPI-3, we made use of a vaccinia virus infection/transfection system. Specifically, CV-1 cells were infected with media alone or with vTF7-3, a vaccinia virus expressing the T7 RNA polymerase from the poxvirus $P_{7.5}$ promoter (Fuerst et al., 1986), at an m.o.i. of 10. After a 1 h adsorption, virus was removed and cells were washed 2 times with 3 ml of media minus serum. 1 ml of media minus serum was then added to cells. 5 μg of DNA (wild-type or mutant SPI-3 in pTM1 vectors) was mixed with 12 μl of LipofectACE (GIBCO-BRL) in a total volume of 100 μl. The transfection mixture was added to cells and cells were returned to 37°C. Media was removed at 17 h p.i. and 300 μl of Eagle's MEM without cysteine or methionine (Sigma, St. Louis, MO) and 4.8 μl of 5M NaCl were added for 20 min to starve the cells of cysteine and methionine. To radiolabel proteins, 30 μCi of ^{35}S were added per sample and cells were pulse-labeled for 30 min at 37°C. Media was then removed and 1 ml ice-cold PBS was added to cells. Cells were scraped into PBS and spun for 1 min at 8,200 *g* in a microcentrifuge to pellet cells. Supernatant was discarded and cells were resuspended in 100 μl of lysis buffer (100 mM Tris–HCl pH 8.0, 100 mM NaCl, 0.5% Triton X-100) plus 0.2 mM PMSF and incubated on ice for 5 min. Lysed cells were spun for 5 min at 11,500 *g* in a microcentrifuge and supernatant was removed for analysis. 10 μl of extracts were mixed with 6× SDS–PAGE sample buffer including 100 mM BME and boiled for 5 min. Samples were run on a SDS-polyacrylamide gel. Proteins were enhanced using Amplify (Amersham Pharmacia Biotech, Piscataway, NJ) and detected by autoradiography.

Construction of recombinant CPV expressing mutant SPI-3 proteins

A CPV derivative containing a SPI-3 gene lacking the signal sequence was isolated by cloning DNA upstream from the SPI-3 gene into a plasmid containing SPI-3 SS(−), and then inserting a $P_{7.5}$-gpt cassette (confers resistance to mycophenolic acid) in the plasmid vector to allow for transient dominant selection (Falkner and Moss, 1990). Specifically, SPI-3 SS(−) was excised from pTM1-SPI-3 SS(−) by digestion with *Nco*I and *Pst*I, and inserted into pGEM5Zf(+) (Promega Corp.) digested with the same enzymes. The flank upstream from SPI-3 was PCR amplified from genomic wtCPV DNA with primers FS363 (5′-GAC-CTGCATGCTATACTGCGACGAGATACAACC-3′) and FS356 (5′-GTCTAATTTTGCAGCCGTG-3′), digested with *Sph*I and *Bsp*HI, and inserted into the *Sph*I and *Nco*I sites of pGEM5Zf(+) SPI-3 SS(−). Finally a *Bam*HI fragment containing $P_{7.5}$-gpt was inserted into the *Bam*HI site of the resulting plasmid to give pGEM5Zf(+) SPI-3 SS(−) $P_{7.5}$-gpt. To create recombinant virus, CV-1 cells were infected with wt CPV and transfected with pGEM5Zf(+) SPI-3 SS(−) $P_{7.5}$-gpt in the presence of 2.5 μg/ml mycophenolic acid (Sigma). The resulting recombinant virus CPV SPI-3 SS(−) was plaque purified three times and verified by PCR analysis and sequencing.

The CPV SPI-3 N-glyc(−) recombinant was obtained by infecting cells with CPVΔSPI-3, transfecting with pTM1-SPI-3 N-glyc(−), and selecting for viruses that had lost the gpt cassette by reverse gpt selection (Isaacs et al., 1990). HGPRT(–)D98R HeLa cells (Kerr and Smith, 1991) were used for this purpose, with 6-thioguanine at 50 μM. The resulting viruses were plaque purified three times and were verified by PCR analysis and sequencing to confirm that all four glycosylation site mutations, which lie within the region deleted in CPVΔSPI-3, were present.

CPV N-glyc(−)ΔHA was constructed by infecting CV-1 cells with CPV N-glyc(−), transfecting with pHGN4.1, a plasmid containing gpt inserted into the HA gene, and selecting for viruses that were resistant to 2.5 μg/ml mycophenolic acid. Recombinant virus was plaque purified three times. Loss of the HA gene was confirmed by analyzing

infected CV-1 cells for the loss of the ability to agglutinate chicken red blood cells.

Immunofluorescence

CV-1 cells were grown on glass slides and were infected with media alone or infected with CPV, CPVΔSPI-3, CPV SPI-3 N-glyc(−), CPV SPI-3 SS(−), or CPVΔHA at an m.o.i. of 10. Virus was adsorbed for 1 h, removed, then replaced with media. At the appropriate time p.i., culture media was removed and cells were rinsed twice with PBS. Cells were then fixed by addition of 4% paraformaldehyde for 10 min at room temperature. To permeabilize cells, methanol was added for 2 min at room temperature followed by 2 washes with PBS. (Note that cells were only permeabilized in Fig. 6 to enhance access to intracellular compartments. All other immunofluorescence experiments were done without permeabilization.) Cells were then blocked by addition of PBS plus 3% BSA for 10 min at room temperature. The following primary antibodies were then added to cells either singly or in combination for 1 h at room temperature and if necessary, were diluted into PBS plus 3% BSA. Monoclonal antibody 4A11-4A3 which recognizes SPI-3 protein was used as undiluted hybridoma supernatant. Polyclonal antibody to the mannose-6-phosphate receptor (Dr. William Dunn, University of Florida) was diluted 1:100 in PBS plus 3% BSA for single labeling or in MAb 4A11-4A3 for double labeling. Polyclonal antibody to the vaccinia virus HA protein (Dr. Samuel Dales) was diluted 1:200 in PBS plus 3%BSA for single labeling or in MAb 4A11-4A3 for double labeling. After primary antibody incubation, cells were washed 3 times with PBS. Cells were then stained by addition of goat anti-mouse conjugated to FITC and/or goat anti-rabbit conjugated to Texas Red (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) diluted 1:100 in PBS plus 3% BSA for 30 min at room temperature. Cells were then washed 3 times with PBS and mounted with Vectashield with or without 1.5 μg/ml DAPI (Vector Laboratories, Burlingame, CA). Coverslips were added to slides and sealed prior to microscopy. Samples were examined with a Zeiss Axioplan 2 Fluorescence Microscope or a Bio-Rad MRC 1024 confocal laser scanning microscope.

Preparation of infected cell extracts and supernatants for Western Blot analysis

CV-1 cells (2.6×10^6 cells per well) were mock infected with medium alone or infected with wt CPV or various CPV mutants at an m.o.i. of 10. Virus was adsorbed for 1 h at 37°C. Inoculum was removed, 1 ml of media minus serum was added per well and dishes were returned to 37°C. At 20 h post infection, media was removed, and spun for 5 min at 2,500 *g* to pellet cells. Supernatant was then spun for 20 minutes at 16,000 rpm in a Beckman JA-20 rotor to pellet EEV. Processed supernatant was transferred to a new tube. To process infected cells, 1 ml of PBS was added to the monolayer and cells were scraped into the buffer. Cells were pelleted for 5 min at 2500 *g*. PBS was removed and 50 μl of cell lysis buffer (100 mM Tris–HCl pH 8.0, 100 mM NaCl, 0.5% Triton X-100) plus 0.2 mM PMSF was added. Cells were vortexed and placed on ice for 20 min to lyse cells. Solution was spun for 15 min at maximum speed in a microcentrifuge to pellet insoluble material. The remaining soluble infected cell extract was removed to a new tube. For Western Blot analysis, cell equivalents of supernatant and pellet were mixed with 6× SDS loading buffer including 100 mM BME, boiled for 5 min and run on a 10% SDS-polyacrylamide gel. Gels were transferred to Nitrobind nitrocellulose (MSI, Westborough, MA) and blocked overnight in blocking solution (5% non-fat dry milk, 0.1% Tween in TBS: 150 mM NaCl, 20 mM Tris pH 8.0) at 4°C. Membranes were probed with a 1:50 dilution of SPI-3 monoclonal antibody 4A11-4A3 in blocking solution for 1 h. Blots were washed for 1 h with 6 changes of wash buffer (0.1% Tween in TBS) then incubated with a 1:2000 dilution of HRP-conjugated goat anti-mouse secondary antibody (Sigma) in blocking solution for 1 h. Again, blots were washed for 1 h with 6 changes of wash buffer (0.1% Tween in TBS) and membranes were developed using the ECL plus system (Amersham Pharmacia Biotech, Inc., Piscataway, NJ) following manufacturer's instructions.

Electron microscopy

CV-1 cells were infected with wt CPV or CPVΔSPI-3 at a m.o.i. of 5, and unabsorbed virus removed after 2 h by two washes with serum-free medium before the addition of medium containing serum. EEV was recovered at 18 h p.i. by removing the medium gently, spinning twice at low speed to remove cells, and then pelleting the extracellular virus by centrifugation at 15,000 rpm in a Beckman SW 28 rotor for 1 h at 4°C. Extracellular virus was partially purified by spinning through a 17 ml 36% sucrose cushion at 13,200 rpm in an SW28 rotor for 1.5 h at 4°C. Intracellular virus was prepared by harvesting infected cells at 18 h p.i., washing in PBS, and swelling on ice in 10 mM Tris–HCl, pH 8.0 before Dounce homogenization with 20 strokes. Nuclei were removed by centrifugation for 5 min at 500 *g*. Intracellular virus was partially purified from the supernatant by spinning through a sucrose cushion as above.

10 μl drops of virus containing approximately 10^7 pfu were placed on Parafilm and adsorbed for 10 min to carbon-coated 400-mesh grids. The grids were washed by floating on 4 drops (50 μl) of PBS/0.2% glycine for 8 min total, blocked in PBS/1% BSA for 10 min, and then reacted with the primary antibody by floating on a 25 μl droplet of PBS/1% BSA containing a 1:50 dilution of purified anti-SPI-3, anti-B5R (mAb 20), or anti-14kDa (mAb C3) for 2 h. Following 6 washes with PBS over 10 min, the grids were floated on a 25 μl droplet of a 1:30 dilution of 12 nm gold-conjugated goat anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories, Inc.) for 1 h, washed in 6 drops of PBS, fixed in 2% paraformaldehyde for 10 min, washed in H_2O for 5 min, and stained in 0.5 % uranyl acetate for

30 s. The whole-mount grids were viewed with a Hitachi H-7000 transmission electron microscope.

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