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The cowpox virus fusion regulator proteins SPI-3 and hemagglutinin interact in infected and uninfected cells

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Abstract

The serpin SPI-3 and the hemagglutinin (HA) encoded by cowpox virus (CPV) block cell–cell fusion, and colocalize at the cell surface. wtCPV does not fuse cells, but inactivation of either gene leads to fusion. SPI-3 mAb added to wtCPV-infected cells caused fusion, confirming that SPI-3 protein at the cell surface prevents fusion. The SPI-3 mAb epitope mapped to an 85-amino acid region at the C-terminus. Removal of either 44 residues from the SPI-3 C-terminus or 48 residues following the N-terminal signal sequence resulted in fusion. Interaction between SPI-3 and HA proteins in infected cells was shown by coimmunoprecipitation. SPI-3/HA was not associated with the A27L "fusion" protein. SPI-3 and HA were able to associate in uninfected cells in the absence of other viral proteins. The HA-binding domain in SPI-3 resided in the C-terminal 229 residues, and did not include helix D, which mediates cofactor interaction in many other serpins.

Keywords: Poxvirus; Cowpox virus; Serpin; SPI-3; Hemagglutinin (HA); Protein–protein interaction; Coimmunoprecipitation; Cell–cell fusion

Introduction

Poxviruses have large, complex virions, linear dsDNA genomes, and replicate within the cytoplasm (Moss, 2001). Many aspects of the poxvirus lifecycle have been extensively characterized, including early, intermediate and late transcription, and the assembly of virions (Smith et al., 2002). The most numerous virions produced are intracellular mature virus (IMV), an enveloped form that is fully infectious but not thought to be important in the spread of infection during a natural infection. Extracellular enveloped virus (EEV) contains an additional membrane relative to IMV, and is needed for efficient dissemination within the host (Payne, 1980), despite making up only a small proportion of total infectious virus. The mechanisms by which EEV and IMV enter cells are not completely understood, and remain active areas of investigation.

One of the components of the EEV envelope is the SPI-3 protein. Immunogold EM revealed that SPI-3 was present in EEV but not in IMV (Brum et al., 2003). SPI-3 is one of the three functional *ser*ine *p*roteinase *in*hibitors (serpins) encoded by orthopoxviruses. The other two orthopoxvirus serpins are SPI-2, also known as crmA (cytokine response modifier A), and SPI-1. SPI-2/crmA is a cross-class proteinase inhibitor with both anti-inflammatory and antiapoptosis activity (Turner et al., 1999). SPI-1 is involved in conferring full host-range on wild-type (wt) rabbitpox virus, and in vitro is thought to be active against cathepsin G (Moon et al., 1999). SPI-3 is required to inhibit cell–cell fusion following infection with orthopoxviruses (Law and Smith, 1992; Turner and Moyer, 1992; Zhou et al., 1992), and purified SPI-3 protein inhibits plasmin, urokinase, and tissue-type plasminogen activator in vitro (Turner et al., 2000). For SPI-3, the P1 residue at the center of the reactive center loop (RCL) is arginine, which is consistent with inhibition of proteinases that cleave after basic residues. However, mutations in the RCL of SPI-3 that destroyed proteinase inhibition did not affect control of cell–cell fusion (Turner and Moyer, 1995), indicating that the SPI-3 protein is bifunctional.

The orthopoxvirus hemagglutinin (HA) is required in addition to SPI-3 to prevent cell–cell fusion (Ichihashi and Dales, 1971), and like SPI-3 is also a component of EEV (Payne, 1979). Immunofluorescence studies showed that HA

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and SPI-3 were colocalized at the surface of infected cells (Brum et al., 2003). Although SPI-3 is membrane-associated, the protein does not contain any predicted transmembrane domain. HA and SPI-3 are components of EEV, but neither protein is required for virion morphogenesis or for EEV infectivity. Despite the conservation of SPI-3 in orthopoxviruses, animal experiments have not shown any pronounced attenuation of CPV or vaccinia virus (VV) mutants lacking SPI-3 (Law and Smith, 1992; Thompson et al., 1993). In contrast, deletion of HA does attenuate vaccina WR (Flexner et al., 1987; Lee et al., 1992; Shida et al., 1988).

The biological significance of cell–cell fusion following poxvirus infection is unclear. Presumably, fusion between infected cells reflects the entry of poxvirus virions into cells by a process involving fusion between the envelope of IMV and the cell membrane. Currently, EEV is thought to dissociate at the cell surface by a non-fusogenic process to release IMV (G.L. Smith, personal communication). IMV then enters the cell by direct fusion at neutral pH (Carter et al., 2005; Doms et al., 1990; Janeczko et al., 1987). IMV added to cells in large quantities and then treated briefly at acid pH can induce cell–cell fusion known as "fusion from without" (Gong et al., 1990). Antibody mAbC3 against the A27L protein, a component of IMV, neutralizes IMV (Rodriguez et al., 1985) and blocks cell–cell fusion (Rodriguez et al., 1987), and based on this finding A27L has been referred to as the 14 kDa fusion protein. mAbC3 blocked fusion of labeled IMV with cells in an assay involving dilution of the fluorescent probe R18 (Doms et al., 1990). However, a VV recombinant with an IPTG-inducible A27L gene was able to form fully infectious IMV in the absence of IPTG (Rodriguez and Smith, 1990), suggesting that entry of IMV does not require A27L. More recent data using vYFP-A4/ΔA27 with a complete deletion of A27 (Ward, 2005) showed that IMV lacking A27 added directly to cells was still able to mediate fusion from without (Senkevich and Moss, 2005), demonstrating that A27 is not required for cell–cell fusion. The A28 protein, a component of the IMV membrane (Senkevich et al., 2004a), is absolutely required for virus entry (Senkevich et al., 2004b). Interestingly, A28 is needed both for fusion from without (by added virions) and for fusion from within (by virions produced from infected cells) (Senkevich et al., 2004b), which strongly suggests that fusion and virus entry are closely related processes. A second protein, H2, has almost identical properties to A28, and forms a complex with A28 that may mediate virus fusion with cells (Senkevich and Moss, 2005).

HA and SPI-3 are both glycoproteins. The SPI-3 protein of cowpox virus (CPV) has four N-linked glycosylation sites that are all utilized, but glycosylation is not required for the inhibition of fusion by SPI-3, or for localization of SPI-3 to the cell membrane (Brum et al., 2003). Retention of SPI-3 at the cell surface was, however, abrogated by inactivation of HA (Brum et al., 2003), implying that there may be an interaction between the two proteins. We show here that SPI-3 is retained at the cell surface by binding to HA, and that the interaction between HA and SPI-3 does not depend on any other viral proteins. SPI-3 mutants that are inactive as proteinase inhibitors are still able to associate with HA, consistent with earlier data that such mutants are still capable of fusion inhibition (Turner and Moyer, 1995).

Results

SPI-3 monoclonal antibody induces fusion in cells infected with wild-type cowpox virus

In view of reports that the addition of an anti-HA monoclonal antibody was able to cause fusion during infection with wild-type (wt) VV strain IHD-J (Seki et al., 1990), we tested the SPI-3 monoclonal antibody 4A11-4A3 generated against purified SPI-3 protein (Brum et al., 2003) for its ability to induce cell–cell fusion in wtCPV infection. SPI-3 mAb at 10 μg/ml had no effect on a confluent monolayer of uninfected CV-1 cells over a period of 3 days (data not shown). CV-1 cells infected with wtCPV gave a cytopathic effect with individual cell rounding, but no evidence of fusion between infected cells (Fig. 1A). The presence of SPI-3 mAb at 10 μg/ml throughout infection with wtCPV resulted in extensive cell–cell fusion (Fig. 1B) resembling that seen on infection with a CPV or VV mutant deleted for SPI-3 (Law and Smith, 1992; Turner and Moyer, 1992; Zhou et al., 1992). With reduced input of wtCPV such that individual plaques were visible, addition of SPI-3 mAb resulted in syncytial plaques (data not shown). Clearly, the epitope for the SPI-3 mAb is accessible on the surface of wtCPV-infected cells, and binding of SPI-3 mAb interferes with the inhibition of fusion mediated by SPI-3.

Mapping of the epitope for the SPI-3 mAb

Nested deletions of SPI-3 were used to localize the epitope for the SPI-3 mAb in order to gain possible insights into the mechanism of fusion inhibition by SPI-3. The deletion mutants (Table 1) were constructed using a PCR-based strategy, and sequenced to confirm that no errors had been introduced. Each deletion mutant was successfully expressed in vitro using a coupled transcription/translation system, and the radiolabeled products tested for the ability to be immunoprecipitated by the SPI-3 mAb. Of the C-terminal deletion mutants C2 through C6, the smallest deletion C2 which lacked only 44 amino acids was not immunoprecipitated, and C3 through C6 which contained longer deletions were likewise not immunoprecipitated (Fig. 2A).

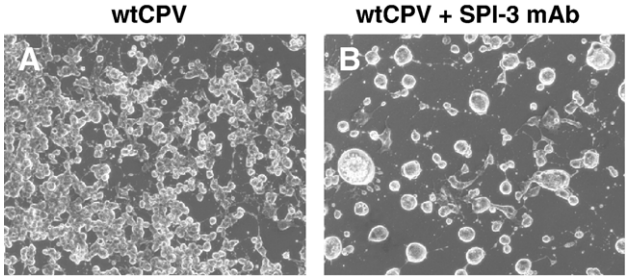

Fig. 1. Adding SPI-3 monoclonal antibody during infection with wild-type CPV induces fusion. CV-1 cells were infected with wtCPV at a multiplicity of 5 in the absence (A) or presence (B) of SPI-3 monoclonal antibody at 10 μg/ml, and photographed at 3 days p.i.

Table 1
SPI-3 deletions

Construct	Residues present
wt SPI-3	1–373
SPI-3 C2	1–329
SPI-3 C3	1–286
SPI-3 C4	1–223
SPI-3 C5	1–159
SPI-3 C6	1–87
SPI-3 7N	71–373
SPI-3 SS-7N	1–22; 71–373
SPI-3 SS-8N	1–22; 145–373
SPI-3 9N	223–373
SPI-3 SS-9N	1–22; 223–373
SPI-3 10N	289–373
SPI-3 SS-10N	1–22; 289–373

On the other hand, the N-terminal deletions 7N (303 residues remaining), 9N (151 residues), and 10N (85 residues) diagrammed in Fig. 2A were all recognized by the mAb, enabling us to localize the epitope to within a C-terminal 85-amino acid region from residues 289 to 373 (Fig. 2B) which includes the RCL. Cleavage of radiolabeled SPI-3 with urokinase, presumably at the P1–P1′ bond in the RCL, resulted in a fragment of approximately 39 kDa which was also immunoprecipitated by SPI-3 mAb. To investigate the possibility that a complex of urokinase and both large and small SPI-3 fragments was being immunoprecipitated by recognition of the smaller SPI-3 fragment, we cleaved unlabeled His-SPI-3 protein with urokinase, and immunoblotted with SPI-3 mAb. Only the larger cleavage fragment was detected by immunoblotting, indicating that the epitope lies within this fragment. The epitope is likely located within the 52-amino acid region of overlap between 10N and cleaved SPI-3 indicated by the thick black line in Fig. 2B. The location of this portion of SPI-3 within the predicted structure is indicated in black in Fig. 2C, and spans part of the RCL at the top of the SPI-3 model, one of the strands in β-sheet A, and a loop at the bottom. In addition, the SPI-3 substitution mutants which alter positions P1/P1′, P5 through P5′, and P17 through P10 (Fig. 2B) were all recognized by SPI-3 mAb, suggesting that the epitope lies to the N-terminal side of P17, although a contribution to the epitope from the P9–P6 residues located in between the P17/P10 and P5/P5′ mutations cannot be ruled out.

The effects of mutations in SPI-3 and HA on fusion inhibition

Although binding of SPI-3 mAb to SPI-3 protein located on the surface of infected cells prevents fusion inhibition, the

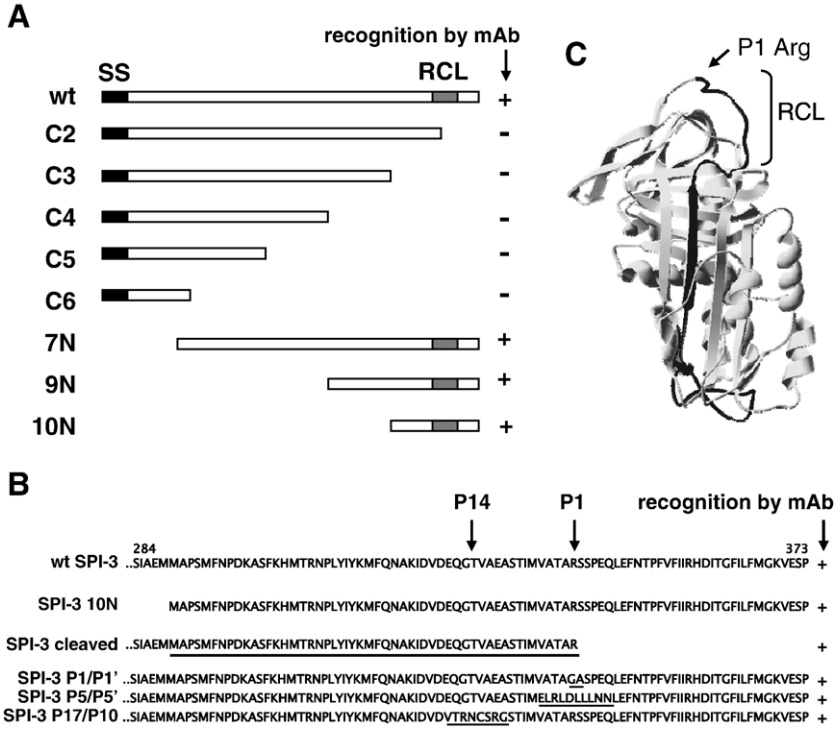

Fig. 2. Mapping of the epitope for SPI-3 mAb. (A) Recognition of SPI-3 C-terminal deletions C2–C6 and N-terminal deletions 7N, 9N, and 10N by SPI-3 mAb. A plus indicates ability of radiolabeled protein to be immunoprecipitated by the SPI-3 mAb. The locations of the signal sequence (SS) and reactive center loop (RCL) in wild-type (wt) SPI-3 protein are indicated. (B) The sequences of SPI-3 mutants near the C-terminus, which are all recognized by the SPI-3 mAb. The top line shows the region at the C-terminus of wt SPI-3, with the locations of the P14 (Thr) and P1 (Arg) residues marked by arrows. The 85-amino acid sequence of SPI-3 deletion 10N is shown, which contains the SPI-3 epitope. The black line indicates the region of overlap between 10N and urokinase-cleaved SPI-3. The sequences of the SPI-3 RCL substitution mutants P1/P1′, P5/P5′, and P17/P10 are shown, with altered residues underlined. (C) Model of SPI-3 structure created with SWISS-MODEL at expasy.org using the crmA crystal structure (Renatus et al., 2000; Simonovic et al., 2000) and other serpins as templates. The P1 Arg residue located within the reactive center loop (RCL, bracketed) at the "top" of the molecule is indicated. The position of the 52-amino acid region underlined in panel B that encompasses the SPI-3 epitope is shown in black, with the remainder of the protein in grey. The black portion includes part of the reactive center loop at the top of the molecule, strand s5A of beta-sheet A, and a loop at the bottom of the molecule.

location of the epitope does not delineate which region of SPI-3 is important for this activity. The P1/P1′, P5/P5′, and P17/P10 substitution mutations in the RCL prevented proteinase inhibition (Turner et al., 2000), but had no effect on fusion inhibition (Turner and Moyer, 1995), suggesting that proteinase inhibition and control of fusion were independent processes. The C-terminal SPI-3 deletion mutants C2 through C6 were tested for fusion inhibition by a transient transfection assay (Turner and Moyer, 1995). Briefly, CV-1 cells were infected with a vTF7-3 (VV-T7 RNA Pol) derivative lacking the SPI-3 gene, and transfected with pTM1 derivatives carrying either a wtSPI-3 control gene or one of the deleted forms of the SPI-3 gene. Transfection with the empty vector pTM1 had no effect on the fusion caused by vTF7-3ΔSPI-3 (Fig. 3B). However, expression of functional wild-type SPI-3 protein from the T7 promoter suppresses the cell fusion that otherwise results from infection with vTF7-3ΔSPI-3 (Fig. 3C). Removal of the 44 C-terminal amino acids in the SPI-3 C2 deletion destroyed the ability of SPI-3 to inhibit fusion (Fig. 3D). Deletions C3 through C6 extending further toward the N-terminus were also inactive (summarized in Fig. 3G). For mutants C2 through C6, the lack of binding to SPI-3 mAb paralleled the inability to inhibit fusion. We were unable to confirm expression of C2 through C6 by immunoblotting with SPI-3 mAb since the antibody does not recognize these deleted proteins, but we did show subsequently that FLAG-tagged versions of C2 and C4 were detectable with FLAG mAb in transfected cells by immunofluorescence (Fig. 10) and by immunoprecipitation (data not shown). The N-terminal deletions 7N (Fig. 3E), 9N, and 10N (data not shown) had no activity in fusion inhibition, as expected since removal of the signal sequence alone (residues 1–15) was sufficient to prevent the inhibition of fusion (Brum et al., 2003). The signal sequence was added to the smallest deletion 7N to create SPI-3 SS-7N, which would be expected to transit through the endoplasmic reticulum and Golgi. The results indicated that deletion of wtSPI-3 residues 23–70 in SPI-3 SS-7N also prevented fusion inhibition (Fig. 3F). SPI-3 SS-8N, SPI-3 SS-9N, and SPI-3 SS-10N with the

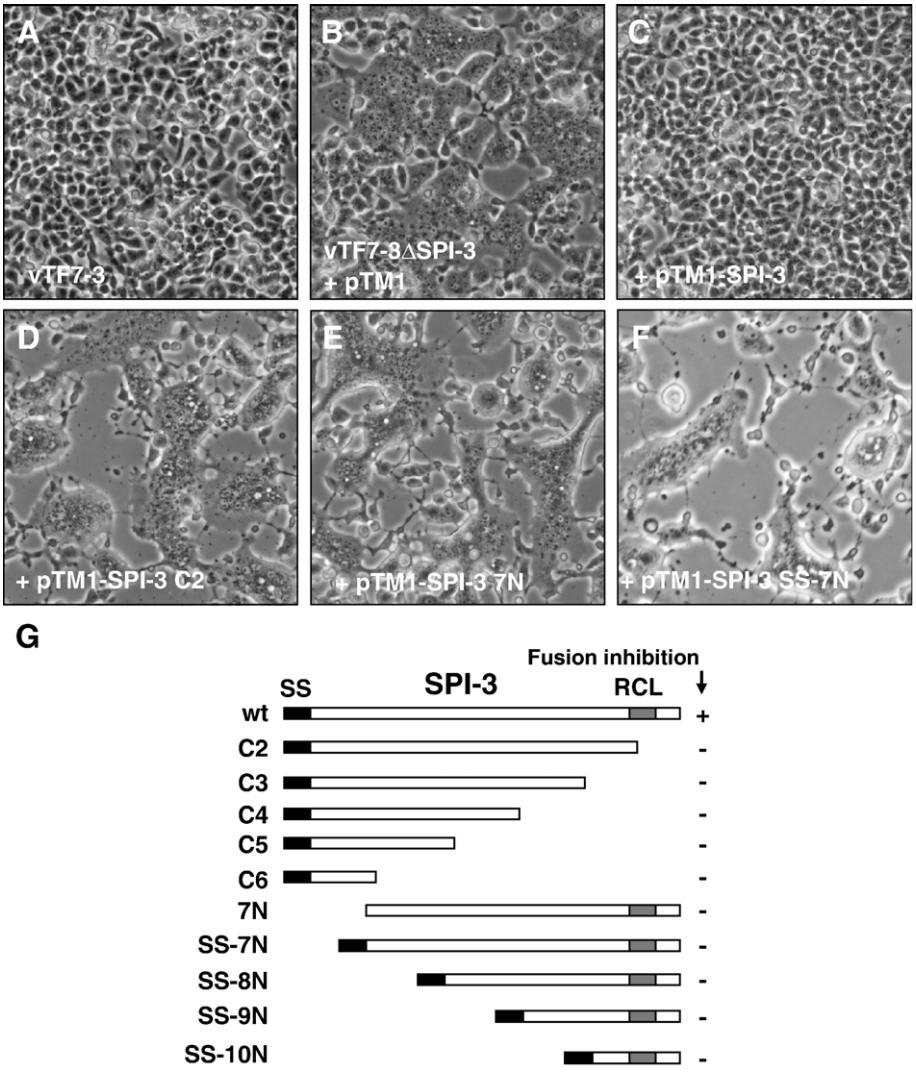

Fig. 3. The activity of N- and C-terminal SPI-3 deletion mutants in fusion inhibition. (A) CV-1 cells infected with vTF7-3. (B–F) Cells infected with vTF7-3Δ SPI-3 and transfected with: (B) pTM1 vector DNA; (C) pTM1-SPI-3; (D) pTM1-SPI-3 C2; (E) pTM1-SPI-3 7N; (F) pTM1-SPI-3 SS-7N. (G) Cartoon summarizing the results of the fusion inhibition assays for SPI-3 deletions.

signal sequence added to the more extensive 8N, 9N, and 10N deletions were similarly inactive in fusion inhibition (Fig. 3G). SPI-3 SS-7N and SS-8N were readily detected in transfected BSR-T7 cells by immunofluorescence (Fig. 10); however, SS-9N (Fig. 10) and SS-10N (data not shown) were not, indicating that the SS-9N and SS-10N deletions had perturbed protein stability. The fact that virtually any deletion of SPI-3 destroys the ability to inhibit fusion suggests that the overall serpin structure is an important component of fusion inhibition.

The HA protein was not subjected to a detailed mutational analysis; however, the effect of removal of the HA transmembrane domain (TM; residues 262–284) was assessed. CV-1 cells infected with CPV HA$^-$ were transfected with pSC65 derivatives carrying wtHA (297 amino acids) or HA(−)TM deleted for residues 262–297 (Fig. 4A). In this instance, the HA gene in pSC65 is under the control of a synthetic early/late poxvirus promoter (Chakrabarti et al., 1997). Compared to the fusion observed with CPV HA$^-$, expression of wt HA from the plasmid completely prevented fusion (Figs. 4B and C), but HA(−)TM lacking the region at the C-terminus including the TM domain had no activity (Fig. 4D). Expression of wtHA and HA(−)TM from the transfected cells was confirmed by immunoblotting cell extracts with HA mAb. In addition, wtHA was absent from the medium, but HA(−)TM was present in significant quantities in the medium, consistent with secretion (data not shown). Anchoring of HA at the cell membrane is presumably required for HA to inhibit fusion.

Coimmunoprecipitation of SPI-3 and HA

Our previous observation that retention of SPI-3 at the cell surface is dependent on the presence of HA (Brum et al., 2003) implies that SPI-3 may interact directly with HA, or that SPI-3 and HA are both members of the same multiprotein complex. In order to investigate association of SPI-3 with HA and with other viral proteins, cells were infected with wtCPV or CPVΔSPI-3, and radiolabeled. The protocol used results in strong labeling of viral proteins, but little labeling of cellular proteins whose expression is rapidly inhibited following infection. Wt CPV extracts immunoprecipitated with SPI-3 mAb gave an intense diffuse band at approximately 55 kDa, corresponding to the expected size for glycosylated SPI-3 protein (Fig. 5, lane 1). In addition, there was a band at approximately 85 kDa (Fig. 5, lane 1) that was a potential interacting protein. Both the 55 kDa and 85 kDa bands were absent in immunoprecipitation of a control extract from infection with CPVΔSPI-3 (Fig. 5, lane 2). The apparent mobility of 85 kDa was the same as the reported size of one of the two observed forms of vaccinia virus HA (Brown et al., 1991), and was consistent with the interacting protein being CPV HA. We obtained both polyclonal serum and mAb against VV HA to test this hypothesis. Immunoprecipitation of a wtCPV extract with a polyclonal HA antibody resulted in the same protein profile (Fig. 5, lane 3) as seen with SPI-3 mAb (Fig. 5,

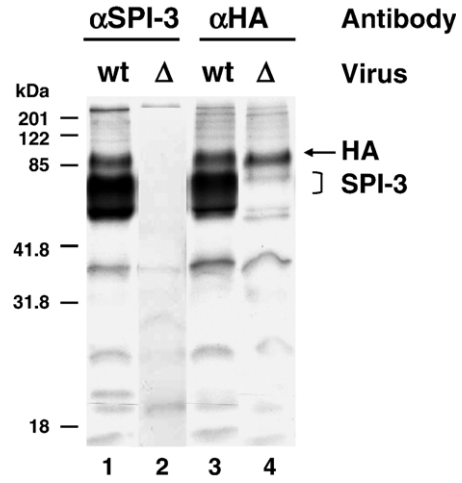

Fig. 5. Coimmunoprecipitation of SPI-3 and HA. CV-1 cells were infected with wtCPV (indicated by "wt" above lanes 1 and 3) or with CPV Δ SPI-3 ("Δ", lanes 2 and 4), labeled with ^{35}S-Met TranSlabel from 3 h to 7 h p.i., and cytoplasmic extracts immunoprecipitated with SPI-3 mAb 4A11-4A3 (lanes 1 and 2) or with polyclonal HA antibody (lanes 3 and 4). The products were subjected to electrophoresis on a 10% acrylamide gel followed by autoradiography. The bracket indicates SPI-3, and the arrow HA.

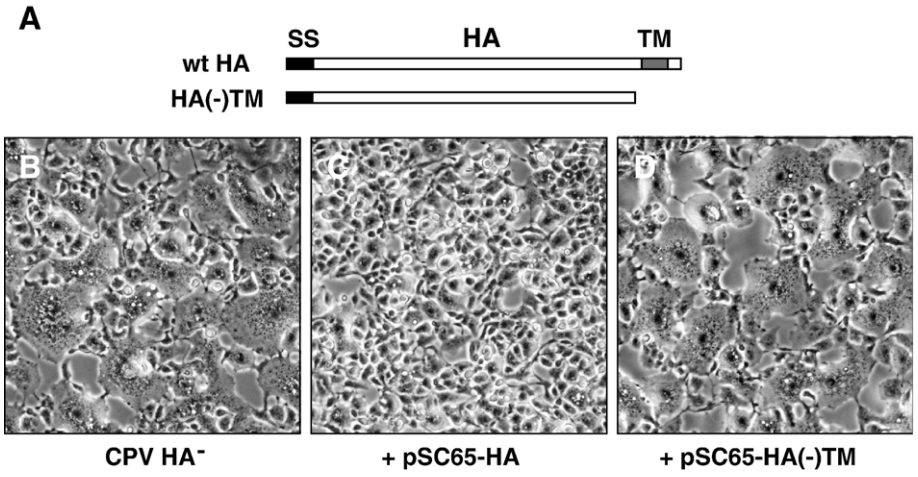

Fig. 4. Removal of the transmembrane domain from HA prevents control of fusion. (A) Location of the signal sequence (SS) and transmembrane domain (TM) in wild-type HA, and removal of the C-terminal region spanning the TM domain in HA(−)TM. (B, C, D) CV-1 cells were infected with CPV HA$^-$ and mock-transfected (B) or transfected with pSC65-HA (C) or pSC65-HA(−)TM (D), and photographed at 24 h p.i.

lane 1), i.e. both the SPI-3 (55 kDa) and 85 kDa bands were recovered. The anti-HA antiserum precipitated only the 85 kDa protein from a CPVΔSPI-3 extract (Fig. 5, lane 4), confirming that the 55 kDa band was SPI-3. Control immunoprecipitation with HA antiserum of extracts from cells infected with CPV HA$^-$ resulted in no detectable protein at 85 kDa (data not shown), consistent with the protein of this mobility being HA. Immunoprecipitation of wtCPV extracts with the mAb 1H831 against HA instead of the HA polyclonal Ab also gave coimmunoprecipitation of SPI-3 (data not shown).

The effect of the SPI-3 RCL mutations on the association with HA was evaluated by coimmunoprecipitation experiments. Since recombinant CPV bearing the SPI-3 RCL mutations were not available, a transfection strategy was employed. A double mutant CPV HA$^-$ SPI-3::gfp was constructed by recombination between CPV HA$^-$ and a plasmid containing SPI-3 with gfp inserted in place of the central portion of the SPI-3 gene. pSC65 derivatives carrying HA and either wt SPI-3, SPI-3 P17/P10 or SPI-3 P1/P1′ were transfected into cells infected with CPV HA$^-$ SPI-3::gfp. The infected and transfected cells were labeled with ^{35}S-Met (TranSlabel), and extracts immunoprecipitated with either SPI-3 mAb or HA mAb. HA protein expressed alone was not immunoprecipitated by SPI-3 mAb, but was precipitated with SPI-3 when HA and SPI-3 were coexpressed (Fig. 6, lanes 2 and 4). HA was not strongly labeled in these experiments and migrated as a diffuse band above the position of SPI-3. The reciprocal experiment indicated that the HA mAb did not immunoprecipitate SPI-3 (lane 11) unless HA was coexpressed (lane 12). HA and SPI-3 P17/P10 were coimmunoprecipitated both by SPI-3 mAb (lane 6) and by HA mAb (lane 14). Similarly, the SPI-3 P1/P1′ mutant protein interacted with wt HA as judged by coimmunoprecipitation with SPI-3 mAb (lane 8) and with HA mAb (lane 16). Comparable results were obtained with the SPI-3 P5/P5′ mutation. The association between SPI-3 and HA thus did not depend on SPI-3 functioning as a proteinase inhibitor. This result is consistent with previous observations that mutations in the SPI-3 RCL did not affect fusion inhibition (Turner and Moyer, 1995).

SPI-3 and HA are not associated with A27L

We looked for an interaction of SPI-3/HA with the A27L product, the 14K "fusion protein", using mAbC3 (Rodriguez et al., 1985). This mAb against the A27 protein is able to block fusion induced by infection with VV WR mutant 87-4 (Rodriguez et al., 1987), by low pH treatment of infected cells (fusion from within) (Gong et al., 1990; Rodriguez et al., 1985), and by infection with VVΔSPI-3 (Turner and Moyer, 1992), suggesting that the A27L protein is required for cell–cell fusion. Radiolabeled extracts of cells infected with wtCPV were immunoprecipitated with mAbC3, revealing a band at approximately 15 kDa marked by the asterisk that was the A27L product, but there was no coimmunoprecipitation of HA or SPI-3 (Fig. 7, lane 2). Immunoprecipitation of the same wtCPV extract with SPI-3 mAb gave detectable SPI-3 and HA (Fig. 7, lane 4, arrows); however, there was no indication that the A27L protein was coimmunoprecipitated. Neither HA nor SPI-3 was immunoprecipitated from an extract of CPVΔSPI-3-infected cells (Fig. 7, lane 5). The SPI-3 mAb did bring down small amounts of proteins at about 200 kDa and 38 kDa (Fig. 7, lane 4), but these proteins were also visible in wtCPV extracts which were incubated with protein A-Sepharose in the absence of antibody (Fig. 7, lane 7), indicating that they were not associated with the SPI-3/HA complex. To date, we have not detected any virus proteins other than HA that interact with SPI-3.

SPI-3 localizes to the surface of BSR-T7 cells expressing HA in the absence of other virus proteins

In order to assess interaction between SPI-3 and HA in the absence of other virus proteins, we made use of BSR-T7 cells that express the T7 RNA polymerase constitutively (Buchholz et

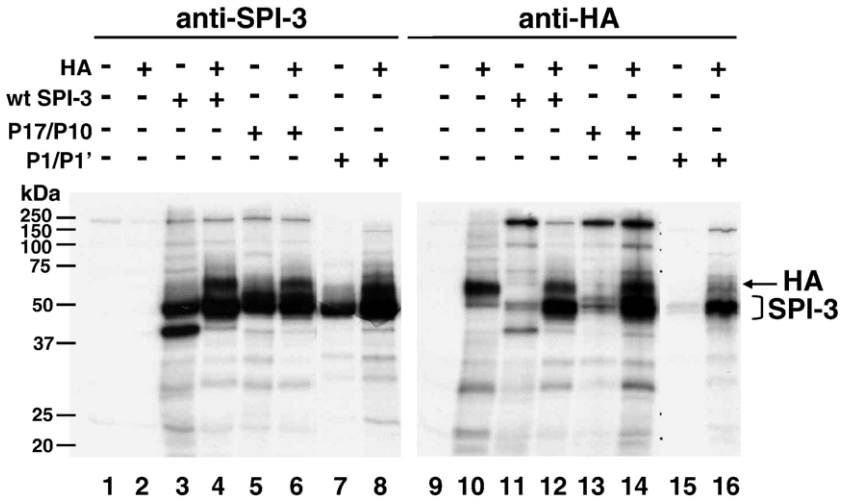

Fig. 6. SPI-3 RCL mutations do not prevent interaction with HA. CV-1 cells were infected with CPV HA$^-$ SPI-3::gfp and transfected with pairwise combinations of pSC65-HA and either pSC65-SPI-3, pSC65-SPI-3 P17/P10, or pSC65-SPI-3 P1/P1′, together with single plasmid controls, as indicated above the lanes. Radiolabeled extracts were immunoprecipitated with SPI-3 mAb (lanes 1–8) or with HA mAb (lanes 9–16). The position of SPI-3 is marked by the bracket and HA by the arrow, as in Fig. 5.

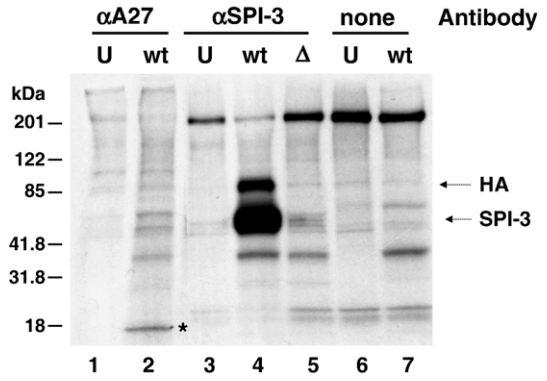

Fig. 7. Immunoprecipitation from wtCPV-infected cells does not reveal any association between SPI-3/HA and A27L (the 14 K "fusion" protein). Extracts were made from CV-1 cells that had been mock-infected (U, lanes 1, 3, 6) or infected with wtCPV (wt, lanes 2, 4, 7) or CPVΔ SPI-3 (Δ, lane 5), and labeled with ^{35}S-Met from 3 h to 7 h p.i. Immunoprecipitation was done with mAbC3 against the A27L 14K protein (lanes 1, 2), with SPI-3 mAb (lanes 3, 4, 5) or with no antibody (lanes 6, 7), and the products visualized by SDS-PAGE and autoradiography. The asterisk indicates the position of the A27L protein in lane 2, and arrows mark SPI-3 and HA bands in lane 4.

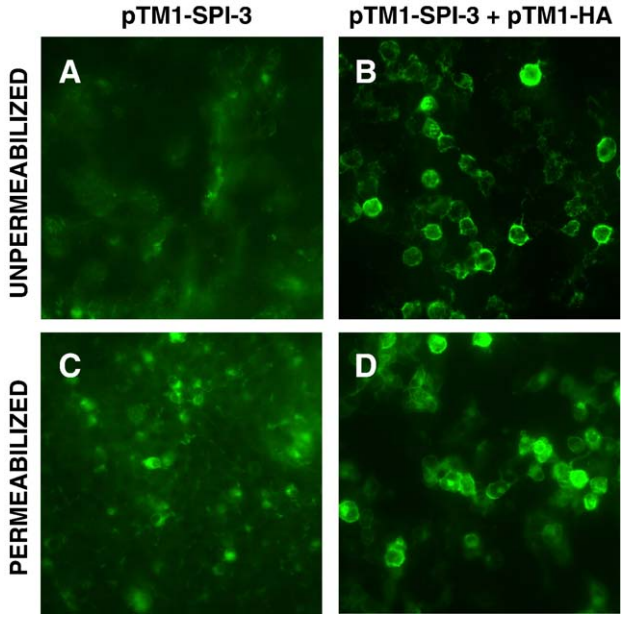

Fig. 8. Surface localization of SPI-3 in transfected BSR-T7 cells in the presence of HA. BSR-T7 cells were transfected with pTM1-SPI-3 (A, C) or with pTM1-SPI-3 and pTM1-HA (B, D). Cells were either washed with PBS (A, B) or permeabilized with methanol (C, D) before addition of SPI-3 mAb. Following PFA fixation, goat anti-mouse FITC secondary antibody was added to visualize SPI-3.

al., 1999). Uninfected BSR-T7 cells were transfected with plasmids containing SPI-3 and HA under the control of the T7 promoter, and the localization of SPI-3 was determined by immunofluorescence. Live, untreated cells were reacted with SPI-3 mAb to detect SPI-3 at the cell surface, or cells were permeabilized with methanol to visualize internal SPI-3 protein. In both cases, transfected BSR-T7 cells were fixed with paraformaldehyde *after* reaction with the primary antibody, to reduce the problem of paraformaldehyde fixation making the cells permeable to primary antibody (M. Law, personal communication). BSR-T7 cells transfected with pTM1-SPI-3 alone gave no detectable SPI-3 at the surface of unpermeabilized cells (Fig. 8A), but intracellular SPI-3 was clearly detectable in permeabilized cells (Fig. 8C). In contrast, cotransfection with pTM1-SPI-3 and pTM1-HA gave strong SPI-3 expression at the surface of unpermeabilized cells (Fig. 8B), as well as internally (Fig. 8D). Retention of SPI-3 at the cell surface therefore did not require any additional virus proteins besides HA, implying that SPI-3 and HA interact directly. Although in BSR-T7 cells SPI-3 required HA to localize at the cell surface, HA was present at the surface both with and without coexpression of SPI-3 (data not shown). These results parallel the situation in infected cells, where SPI-3 fails to localize at the cell membrane in cells infected with CPV HA$^-$, but HA is at the surface in cells infected with wtCPV or with CPVΔSPI-3 (Brum et al., 2003). Although expression of the viral proteins SPI-3 and HA is sufficient for surface localization, we cannot exclude the possibility that a cellular protein is also involved.

Purified SPI-3 protein binds to the surface of transfected cells expressing HA

We were unable to find any association between SPI-3 and HA proteins following coexpression by coupled transcription/translation in vitro, even in the presence of canine microsomal membranes (data not shown). The interaction between the two proteins may take place during their transit through the ER and Golgi. We asked if cells bearing HA on their surface could specifically bind exogenously added SPI-3 protein. HA localizes to the cell surface normally in the absence of SPI-3 in either infected cells (Brum et al., 2003) or in transfected cells (data not shown). Purified SPI-3 protein expressed in the vaccinia/T7 system was tested for binding to BSR-T7 cells that were either transfected with empty vector pTM1 or transfected with pTM1-HA (Fig. 9). Surface-bound SPI-3 protein was visualized by immunofluorescence with SPI-3 mAb using unpermeabilized cells. The added SPI-3 protein carried a His tag at the N-terminus, and lacked the signal sequence. SPI-3 protein added at 20 μg/ml did not bind to BSR-T7 cells transfected with the vector pTM1 alone (Fig. 9A), but did bind to BSR-T7 cells expressing HA (Fig. 9B). SPI-3 can therefore associate with HA post-translationally, and cotrafficking through the ER and Golgi

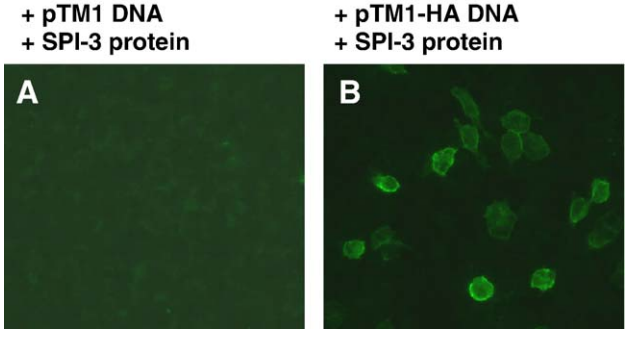

Fig. 9. Purified SPI-3 protein binds to transfected cells expressing HA on the surface. BSR-T7 cells were transfected with pTM1 (A) or pTM1-HA (B), and after 18 h, purified SPI-3 protein was added at 20 μg/ml. After incubation for 2 h, followed by three washes, bound SPI-3 protein was visualized by immunofluorescence using SPI-3 mAb and goat anti-mouse FITC.

is not absolutely required for the interaction. In addition, the purified SPI-3 protein lacked glycosylation since the signal sequence had been removed, and N-linked glycosylation is clearly not required for binding to HA in this instance. This result is consistent with the phenotype of the virus recombinant CPV SPI-3 N-glyc(−), which was engineered to have all 4 N-linked glycosylation sites in SPI-3 inactivated by site-directed mutagenesis, but was still able to inhibit cell–cell fusion, and gave normal cell surface localization of SPI-3 N-glyc(−) following infection (Brum et al., 2003).

The effect of SPI-3 C- and N-terminal deletions on cell surface localization with HA

SPI-3 deletion mutants extending from either the C-terminus or the N-terminus following the signal sequence were both unable to prevent cell–cell fusion (Fig. 3). Was the loss of activity caused by failure to associate with HA at the cell surface, or by correct localization coupled with an intrinsic loss of fusion inhibition? Detection of SPI-3 by immunofluorescence at the surface of unpermeabilized HA-expressing cells provided a convenient assay for interaction between various SPI-3 mutants and HA. BSR-T7 cells were transfected with pTM1-HA and also with plasmids carrying FLAG-tagged versions of SPI-3 deletions C2 and C4. The addition of a C-terminal FLAG tag (DYKDDDDK) was necessary for detection since the C2 and C4 deletions removed the SPI-3 epitope (Fig. 2A). A pTM1 derivative containing full length SPI-3 with the FLAG tag appended at the C-terminus was included as a positive control. SPI-3-FLAG was detectable with FLAG mAb at the surface of unpermeabilized cells that had been transfected with plasmids expressing SPI-3-FLAG and HA (Fig. 10A); however, SPI-3-

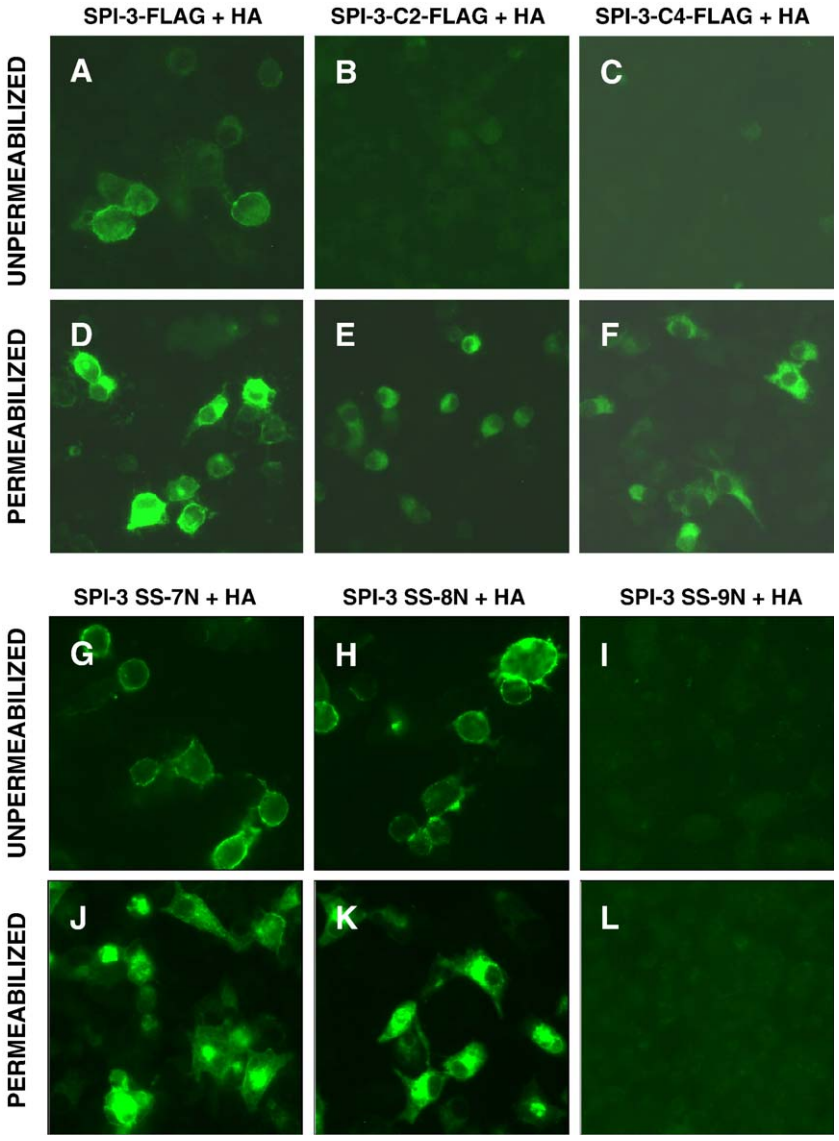

Fig. 10. Effect of SPI-3 deletions on localization in transfected BSR-T7 cells expressing HA. BSR-T7 cells were transfected with pTM1-HA and with pTM1-SPI-3-FLAG (A, D), pTM1-SPI-3-C2-FLAG (B, E), pTM1-SPI-3-C4-FLAG (C, F), pTM1-SPI-3 SS-7N (G, J), pTM1-SPI-3 SS-8N (H, K), or pTM1-SPI-3 SS-9N (I, L). SPI-3 protein was detected with anti-FLAG mAb (A–F) or with SPI-3 mAb (G–L). Cells in panels A, B, C, G, H, and I were unpermeabilized and cells in panels D, E, F, J, K, and L were permeabilized with methanol before addition of mAb. Fixation and detection with goat anti-mouse FITC were as in Fig. 8.

FLAG was not detected on the surface of unpermeabilized cells if HA was absent (data not shown). Therefore, addition of the 8 amino acid FLAG sequence to wt SPI-3 did not perturb SPI-3 localization, and did not affect fusion inhibition in the transfection assay (data not shown). Coexpression of SPI-3-C2-FLAG and HA did not result in localization of the FLAG epitope at the cell surface (Fig. 10B), although SPI-3-C2-FLAG was visible inside cells (Fig. 10E). Similarly, the more extensive deletion SPI-3-C4-FLAG when expressed with HA was only detectable in permeabilized cells (Figs. 10C, F). Radiolabeling of transfected cells confirmed that SPI-3 C2-FLAG and SPI-3 C4-FLAG proteins were expressed and were of the appropriate size (data not shown). Removal of the 44 amino acids from the C-terminus of SPI-3 to create the C2 deletion presumably disrupted the interaction between SPI-3 and HA that is required for SPI-3 to be retained at the cell surface.

The N-terminal SPI-3 deletions SS-7N, SS-8N, SS-9N and SS-10N were tested in a similar manner to determine if the mutant SPI-3 protein was present on the surface of unpermeabilized BSR-T7 cells when HA was coexpressed. Each of these constructs contained the signal sequence at the N-terminus to permit normal trafficking within the cell, and detection in IF was by the SPI-3 mAb 4A11-4A3. SPI-3 SS-7N and SPI-3 SS-8N were readily detectable in permeabilized cells when expressed with HA (Figs. 10J, K). In contrast SPI-3 SS-9N (Fig. 10L) and SPI-3 SS-10N (data not shown) were not visible inside cells by IF, and could not be detected by immunoblotting either in cell extracts or in concentrated supernatants from transfected cells (data not shown). Therefore, no conclusions could be drawn concerning the interaction of SPI-3 SS-9N and SS-10N with HA. However, SPI-3 SS-7N and SPI-3 SS-8N were also visible on unpermeabilized cells (Figs. 10G, H), suggesting that these deleted forms of SPI-3 were still capable of binding to HA on the surface. SPI-3 SS-8N was coimmunoprecipitated with FLAG-tagged HA by FLAG mAb following expression of the two proteins in CV-1 cells infected with vTF7-3ΔSPI-3 (data not shown). The binding site for HA presumably resides in the 229 amino acid residues remaining in SPI-3 SS-8N. The SS-8N deletion removes from SPI-3 helices A, B, C, D and E, two strands of β-sheet A, and one strand of β-sheet B, indicating that the interaction between SPI-3 and HA does not require any of these structural elements. Helix D of antithrombin is involved in binding to the cofactor heparin (Jin et al., 1997), but binding of SPI-3 to HA clearly requires different regions of the serpin.

Discussion

The finding that SPI-3 is physically associated with the membrane protein HA suggests that SPI-3 interacts directly with HA both on the surface of infected cells and on the outer envelope of EEV. Binding of SPI-3 to HA embedded in membranes allows SPI-3 to localize to the surface of virions and cells without possessing a transmembrane domain. All available data from mutants suggest that colocalization of SPI-3 and HA at the cell surface is necessary for fusion inhibition. As would be expected, removal of the SPI-3 signal sequence (Brum et al., 2003) or of the HA TM domain (Fig. 4) causes fusion, as does inactivation of HA, which also results in failure of SPI-3 to localize to the plasma membrane (Brum et al., 2003).

By testing N- and C-terminal deletion mutants of SPI-3, we found that the shortest deletion of each series at either end resulted in loss of fusion inhibition (Fig. 3), and we were therefore not able to define a precise region of SPI-3 required for fusion inhibition. However, deletions at opposite ends of the mature SPI-3 protein had different effects on interaction with HA. Deletion C2 at the C-terminus blocked the association with HA (Fig. 10), which is required for correct SPI-3 localization. In contrast, deletion SS-7N at the N-terminus did not prevent binding of SPI-3 to HA (Fig. 10). Despite the presence of SPI-3 SS-7N at the cell surface, fusion was not inhibited (Fig. 3), suggesting that residues 23–70 of SPI-3 are required for activity as a fusion inhibitor even when the protein is correctly localized. SPI-3 mutants that fail to inhibit fusion fall into two classes: those that are unable to bind to HA and do not localize to the cell surface, and those that bind HA and localize normally.

We attempted to identify other proteins associated with HA and SPI-3 by attaching affinity tags that allowed tandem affinity purification (TAP). The original TAP system (Puig et al., 2001) was devised for use in yeast, and employs two affinity tags, protein A (binds to IgG) and the calmodulin binding peptide (CBP). The addition of these bulky tags at the C-terminus of SPI-3 resulted in loss of fusion control, and this construct was not pursued further. However, fusion of the protein A and CBP tags to the C-terminus of HA did not destroy activity in fusion inhibition, and a recombinant CPV expressing HA with protein A and CBP appended C-terminally was constructed (HA-TAP). When HA-TAP protein was purified by sequential IgG-Sepharose and calmodulin columns, we isolated very pure HA-TAP protein together with small amounts of SPI-3 protein that were identified by immunoblotting, but no other associated protein that could be detected by silver staining (data not shown). Although the system did provide confirmation of the HA–SPI-3 interaction, it did not allow us to detect any other proteins which might also be part of the fusion inhibitory complex.

Evidence that SPI-3 and HA interact in uninfected BSR-T7 cells (Fig. 8) indicates that other viral proteins are not required for association of these two proteins. We were unable to recapitulate the HA/SPI-3 interaction by coexpression in vitro using coupled transcription/translation, even in the presence of microsomal membranes (data not shown). However, purified SPI-3 protein added exogenously to cells expressing HA on the surface was able to bind (Fig. 9), and the association could therefore take place post-translationally. Our data do not address the question of when during infection HA and SPI-3 first associate, and where this occurs. HA is expressed at both early and late times (Brown et al., 1991) and SPI-3 (ORF K2L) is expressed early based on expression of the protein in the presence of cytosine arabinoside, an inhibitor which blocks viral DNA synthesis and late gene expression (Turner and Moyer, 1995). Transcriptional data indicate that SPI-3 is

expressed at both early and intermediate times (Xiang et al., 1998). Presumably both SPI-3 and HA transit through the ER and Golgi, and could interact during this process.

The binding site in SPI-3 for HA is within a 229 amino acid region toward the C-terminus (residues 145–373) based on results with SPI-3 SS-8N (Fig. 10). The fact that SPI-3 RCL substitutions do not affect binding to HA (Fig. 6) makes it likely that the SPI-3/HA interaction differs from the interaction of the serpin with susceptible proteases, which involves the RCL region. Binding of SPI-3 to HA is also different than the serpin antithrombin/heparin complex, which involves binding of the heparin to the serpin helix D (Jin et al., 1997). The mAb epitope for SPI-3 within residues 289–340 (Fig. 2) is still accessible in the HA/SPI-3 complex both in solution (Fig. 5) and when HA is embedded in the membrane (Figs. 1, 8, 9, and 10; Brum et al., 2003). The fact that binding of mAb and HA to SPI-3 is not mutually exclusive strongly suggests that the epitope does not overlap with the binding site in SPI-3 for HA. Based on the model in Fig. 2C, the SPI-3 mAb may be interacting with the front or base of SPI-3, and HA could be binding to the back of the molecule, on the opposite side from β-sheet A.

Binding of SPI-3 mAb to SPI-3 on the surface of wtCPV-infected cells prevents control of cell–cell fusion (Fig. 1), presumably by steric hindrance. However, the mechanism by which the HA/SPI-3 complex regulates fusion has yet to be determined. A28L is needed both for fusion from without and fusion from within (Senkevich et al., 2004b), processes which both apparently depend on virions outside cells. We have been unable to detect any association between SPI-3 and A28 following overexpression in the VV-T7 system. This result is perhaps not surprising given that SPI-3 is present in the outer envelope of EEV (Brum et al., 2003) and A28 is found in the IMV envelope (Senkevich et al., 2004a). How does the presence of associated HA and SPI-3 proteins in the EEV envelope and on the surface of infected cells regulate cell–cell fusion mediated by the IMV A28 and H2 proteins? One explanation is that the presence of the HA/SPI-3 protein complex makes the EEV envelope less likely to rupture to release the fusogenic IMV. Another possibility is that cells expressing HA and SPI-3 may have a reduced ability to fuse with virions. Either of these scenarios could minimize superinfection of virus-producing cells by released virions, which is an attractive potential reason for the conservation of HA and SPI-3 in orthopoxviruses. We are designing experiments to address the mechanisms by which HA and SPI-3 regulate cell–cell fusion.

Materials and methods

Cells and viruses

CV-1 cells (ATCC CCL-70) were maintained in GIBCO Minimum Essential Medium with 5% fetal bovine serum (FBS) and BSR-T7 cells (Buchholz et al., 1999) in Dulbecco's Modified Eagle Medium with 8% FBS, with 0.6 mg/ml active G418 added at alternate passages to maintain expression of T7 RNA Polymerase. The Brighton Red strain of CPV was the parent of CPVΔSPI-3 and CPV HA$^-$ (CPV HA::gpt) (Turner and Moyer, 1992). vTF7-3ΔSPI-3 (Turner and Moyer, 1995) was derived from vTF7-3 (Fuerst et al., 1986).

Antibodies

SPI-3 mAb 4A11-4A3 (Brum et al., 2003) was used as a hybridoma supernatant for immunofluorescence, and as purified mAb to assess the effect on infected cells and for immunoprecipitation. Polyclonal rabbit anti-HA antibody (Dr. Samuel Dales) and mouse mAb 1H831 (Drs. H. Shida and Y. Ichihashi) were used to immunoprecipitate HA. mAbC3 against A27L (Rodriguez et al., 1985) was from Dr. M. Esteban. Anti-FLAG mAb M2 was from Sigma.

Plasmid constructs

SPI-3 was inserted into pTM1 (Moss et al., 1990) downstream from the T7 promoter and EMCV IRES for expression of wt SPI-3 (373 aa) (Turner and Moyer, 1995). SPI-3 C-terminal deletions C2 (44 aa removed), C3 (87 aa removed), C4 (150 aa removed), C5 (214 aa removed), and C6 (286 aa removed) were constructed by PCR, cloned into pTM1, and sequenced to confirm the absence of PCR errors. pTM1-SPI-3-FLAG, pTM1-SPI-3-C2-FLAG, and pTM1-SPI-3-C4-FLAG were generated by subcloning into pTM1-FLAG, a vector designed to add the FLAG tag at the end of the inserted gene. The N-terminal SPI-3 deletions 7N (70 aa removed), 9N (222 aa removed), and 10N (288 aa removed) were created similarly, together with derivatives containing the 22 aa N-terminal region of SPI-3 encompassing the signal peptide (predicted to be residues 1–15). pTM1-SPI-3 SS-8N was deleted for residues 23–144 of wt SPI-3.

The wt HA gene and HA(−)TM lacking residues 262–297 at the C-terminus including the transmembrane domain were PCR amplified and inserted into pSC65 (Chakrabarti et al., 1997) downstream from $P_{E/L}$, a synthetic early/late promoter.

Isolation of virus recombinants

A cassette containing gfp linked to $P_{E/L}$ was inserted into SPI-3 to create pBS-SPI-3::gfp, with residues 182–266 of SPI-3 deleted. Cells infected with CPV HA$^-$ were transfected with pBS-SPI-3::gfp DNA, and green plaques of recombinant CPV HA$^-$ SPI-3::gfp isolated and purified.

Transcription/translation in vitro

Radiolabeled SPI-3 protein derivatives were made using the Promega TNT T7 Quick Coupled Transcription/Translation System, with expression driven by the T7 promoter of pTM1. For each 50 μl reaction, 30 μCi Tran^{35}Slabel (MP Biomedicals) containing 70% ^{35}S-Met and 15% ^{35}S-Cys was used. The reaction was supplemented with 0.5 mM magnesium acetate.

Metabolic labeling

Infected cells were starved for 20 min in Met- and Cys-free Eagle MEM (Sigma), then labeled with 50 μCi Tran^{35}Slabel per 35 mm well. Cells were harvested in PBS, and lysed in 100 mM NaCl, Tris–HCl, pH 8.0, 0.5% v/v NP-40 (IP buffer) supplemented with 0.2 mM PMSF.

Immunoprecipitation

Radiolabeled protein made in vitro or from metabolic labeling was incubated overnight at 4 °C with mAb in a total volume of 200 μl IP buffer. Protein A-Sepharose (Amersham Biosciences) was added for 1 h at 4 °C with continuous rotation of the tubes. The protein A-Sepharose pellet with bound antibody/antigen complex was washed three times in 1 ml IP buffer, and labeled proteins released by heating at 95 °C for 5 min in SDS-PAGE sample buffer containing DTT. Proteins were visualized by PAGE on 10% acrylamide gels, using fluorography with Amplify (Amersham Biosciences) to reduce exposure time to film.

Fusion assay

The transient assay for fusion inhibition was as described (Turner and Moyer, 1995) except that Lipofectin (Invitrogen) was the transfection reagent. CV-1 cells in 6-well plates were infected with vTF7-3ΔSPI-3 (moi = 0.2) and transfected with 4 μg plasmid DNA plus 12 μl Lipofectin, in a total volume of 1 ml medium without FBS. Medium with 5% FBS (2 ml per well) was added at 5 h p.i., and fusion scored at 22 h p.i.

Immunofluorescence

Cells in 8-well slides (Lab-Tek) were transfected with plasmid DNA (0.5 μg per well for one DNA, or 0.3 μg + 0.3 μg for two DNAs) using 2 μl LipofectAMINE 2000 (Invitrogen) per well. The next day, cells were permeabilized with methanol for 2 min, or left unpermeabilized. The cells were blocked with PBS/3% BSA for 10 min, then reacted with the primary antibody in PBS/3% BSA for 1 h at RT. SPI-3 mAb was used at 10 μg/ml, and FLAG mAb at 7 μg/ml.

After 3 PBS washes, cells were fixed with fresh PBS/4% paraformaldehyde for 10 min, washed with PBS, reacted with goat anti-mouse FITC secondary antibody (Jackson Immunoresearch Laboratories) at 1:100 for 30 min, then washed with PBS three times before examination with a Zeiss Axiovert 200M inverted microscope.

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