The acidic C-terminus of vaccinia virus I3 single-strand binding protein promotes proper assembly of DNA–protein complexes

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The vaccinia virus I3L gene encodes a single-stranded DNA binding protein (SSB) that is essential for virus DNA replication and is conserved in all Chordopoxviruses. The I3 protein contains a negatively charged C-terminal tail that is a common feature of SSBs. Such acidic tails are critical for SSB-dependent replication, recombination and repair. We cloned and purified variants of the I3 protein, along with a homolog from molluscum contagiosum virus, and tested how the acidic tail affected DNA–protein interactions. Deleting the C terminus of I3 enhanced the affinity for single-stranded DNA cellulose and gel shift analyses showed it also altered the migration of I3-DNA complexes in agarose gels. Microinjecting an antibody against I3 into vaccinia-infected cells also selectively inhibited virus replication. We suggest that this domain promotes cooperative binding of I3 to DNA in a way that would maintain an open DNA configuration around a replication site.

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Introduction

Poxviruses are large DNA viruses that replicate in discrete cytoplasmic foci, or factories. The prototypical poxvirus, vaccinia virus (VACV), encodes most of its own proteins required for DNA replication. These include a DNA polymerase (E9) which associates with two additional proteins (A20 + D4) in a trimeric complex (Stanitsa et al., 2006), a primase/helicase (D5) (De Silva et al., 2007), a DNA ligase (A50) (Kerr and Smith, 1991), enzymes catalyzing dNTP biogenesis (J2, F2, F4/I4, A48R) (Beaud, 1995; Gammon et al., 2010; Weir and Moss, 1983), a primase/helicase (D5) (De Silva et al., 2007), a primase as well as a DNA binding protein (I3) (Rochester and Traktman, 1998; Tseng et al., 1999), a primase/helicase (D5) (De Silva et al., 2007), a DNA ligase (A50) (Kerr and Smith, 1991), enzymes catalyzing dNTP biogenesis (J2, F2, F4/I4, A48R) (Beaud, 1995; Gammon et al., 2010; Weir and Moss, 1983), a Holiday junction resolvase (A22) (Garcia et al., 2000), a single-stranded DNA binding protein (I3) (Rochester and Traktman, 1998; Tseng et al., 1999), a flap endonuclease (C5) (Senkevich et al., 2009), and other proteins of less certain function such as H5 (Boyle et al., 2015; Kay et al., 2013). Replication is also linked to virus recombination and uses a single-strand annealing reaction catalyzed by E9 and I3 (Gammon and Evans, 2009; Willer et al., 2000).

Several models have been proposed to explain the mechanism of poxvirus DNA replication with a “rolling hairpin” mechanism being long favored as a way of explaining the origins of concatemeric replication intermediates (Moyer and Graves, 1981). However, this model does not explain why VACV encodes a protein with DNA primase activity (De Silva et al., 2007). The fact that VACV encodes a primase as well as a flap endonuclease (Senkevich et al., 2009) suggests that it could also use a discontinuous mode of DNA replication requiring leading and lagging strand DNA synthesis.

Although these matters remain to be resolved, it is well established that single-stranded DNA (ssDNA) substrates are critical replication, recombination, and repair intermediates and ssDNA is always sequestered in complexes composed of the DNA plus single-strand DNA binding proteins (SSBs). VACV also encodes a SSB, called I3, encoded by the I3L gene (Rochester and Traktman, 1998). I3 is an essential gene product that exhibits early biological activity (Greseth et al., 2012). Although the gene cannot be disrupted, RNA interference shows that I3 is needed for both virus replication and recombination in vivo (Gammon and Evans, 2009; Greseth et al., 2012).
The DNA-binding properties of I3 are reminiscent of the different DNA-binding modes exhibited by other SSBs even though there are no obvious similarities between poxviral and other bacterial, viral, or phage SSBs. Moreover, I3 encodes a patch of ~40 negatively charged residues at the C-terminus, a feature that is characteristic of many phage and bacterial SSBs and which serves several different functions. For example, the T4 bacteriophage GP32 protein also encodes a 46 aa acidic C-terminal tail. The tail can be excised by limited proteolysis to create a form called GP32\(^*\), which binds more tightly to ssDNA than the native protein and acquires the capacity to melt duplex DNA (Lonberg et al., 1981; Moise and Hosoda, 1976). When DNA binds to native GP32, it displaces the C-terminus from where it occludes the DNA-binding cleft, and the displaced and exposed end can then serve as a binding site for other proteins (Kowalczykowski et al., 1981; Krassa et al., 1991; Lonberg et al., 1981).

Interestingly, the C-terminus of GP32 (~26 aa) also encodes a critically important aromatic amino acid residue and, if this domain and phenylalanine are deleted, it disrupts GP2.5 binding to the T7 DNA polymerase/thioredoxin complex and primase-helicase, as well as increasing the affinity of the truncated protein for ssDNA. Deleting the tail also prevents GP2.5 dimer formation by interfering with a domain swap interaction. A scheme very similar to the DNA binding and C-terminal tail displacement model, originally proposed for T4 GP32, has been suggested to explain these features of T7 GP2.5 (He et al., 2003; Hyland et al., 2003; Marintcheva et al., 2006, 2008).

**Fig. 1.** Poxvirus single-strand binding proteins and the clones used in this study. Panel A shows an alignment of the vaccinia virus I3 protein with the homologs found in viruses representing other principle Chordopoxviruses. The asterisks (*) show trypsin hypersensitive sites that were previously mapped in I3 (Tseng, 2000). The figure also shows the sequences deleted from two sub-clones of the VACV I3L and molluscum contagiosum virus 046L genes. These encompass most of the acidic C-terminal tails of the two proteins. Panel B shows the purified recombinant proteins used in these studies, analyzed using SDS-PAGE. Note that although I3L encodes a 30 kDa protein, it generally migrates in SDS-PAGE gels at ~35 kDa (Tseng et al., 1999). Native MCV 046 protein (31.5 kDa) seems to exhibit the same property. Panel C illustrates the specificity of monoclonal antibody 10D11 determined by western blotting. It recognized an epitope deleted in form I31–244.
Lastly, these schemas are not restricted to phage SSBs. *Escherichia coli* SSB encodes a highly conserved short C-terminal peptide composed of acidic and hydrophobic residues. Like GP2 and GP2.5, this element mediates interactions with many proteins including those involved in DNA replication (e.g. the χ subunit of polymerase III), recombination (e.g. RecQ), and repair (e.g. exonuclease I and polymerase II) [reviewed in Shereda et al. (2008)]. Likewise, a 42–62 aa peptide encoding the C-terminus of *E. coli* SSB is rendered more protease sensitive by DNA binding and deleting the C-terminus enhances the helix-destabilizing activity of the protein (Williams et al., 1983).

In contrast to these well characterized systems, little is known about the purpose served by the acidic terminus of I3, beyond the fact that it may also promote binding to the viral ribonucleoside diphosphate reductase (Davis and Mathews, 1993). In this article we explore how the I3 C-terminus affects ssDNA binding, protein–protein interactions, and virus replication. Our results show that the I3 C-terminus modulates DNA binding and homotypic interactions in a manner similar to the role served by the same element in bacterial and phage SSBs. However, we can find no evidence of a stable interaction with other proteins mediated by the I3 C-terminus.

**Results**

**Cloning of poxvirus SSB proteins**

The PCR and different oligonucleotide primers were used to prepare bacterial clones expressing a full-length (I31–270) and a C-terminal truncated (I31–244) form of the VACV I3 SSB. The deletion boundary was located on the C-terminal side of a small, highly conserved "EED" motif, and causes the deletion of a 25-residue peptide bearing 9 aspartic or glutamic acid residues (Fig. 1A). The choice of this site as the deletion boundary was based upon a previous study, where we treated full-length N- and C-terminal tagged I3 with trypsin in the presence of ssDNA. Using MALDI-TOF mass spectrometry we detected the rapid production of three different large peptides, all sharing a common C-terminal cleavage site at I3 residue R245 (Tseng, 2000). PCR was also used to prepare full-length (0461–288) and truncated (0461–265) clones encoding the MCV MC046L gene product. This strategy deletes a similar 23-residue peptide bearing 7 acidic residues. All four proteins were expressed in *E. coli* and purified to ≥90% purity using affinity chromatography, as measured by SDS PAGE gels (Fig. 1B). We tried to produce recombinant forms of more distantly related Orf and fowlpox virus I3 homologs, but these expressed too poorly in *E. coli* to be used (data not shown). We also used site-directed mutagenesis to incorporate neutral and positive charged residues in the I3 C-terminal tail, but bacterial proteases rapidly converted these mutant forms of the full-length I3 protein into smaller proteins resembling the I31–244 form in size (data not shown).

As part of this study, we also pursued a number of crystallization trials. A C-terminal his-tagged form of the VACV protein (I315–244–his) rapidly formed crystals in the presence or absence of DNA, and under various buffer conditions. However, despite extensive screening for better growth conditions, the crystals were too disordered to permit further X-ray analysis.

**Deletion of the C-terminal tail from I3 enhances its affinity for ssDNA**

Affinity chromatography was used to test how deleting the C-termini from the VACV and MCV proteins affected ssDNA binding. The purified proteins were bound to ssDNA cellulose resin in a low salt (50 mM) buffer and then eluted in a stepwise manner with increasing amounts of NaCl. The amount of eluted protein was then determined by imaging the stained protein on SDS PAGE gels. The full length VACV I31–270 protein exhibited an “elution concentration 50%” (EC50) value of 0.44 M NaCl (95% c.i. = 0.3–0.6 M) whereas the I31–244 form exhibited a significantly higher affinity for ssDNA with the EC50 = 0.71 M NaCl (95% c.i. = 0.7–0.8 M) (Fig. 2). This same trend was observed with the MCV SSB. The full-length 0461–288 form eluted at EC50 = 0.21 M NaCl (95% c.i. = 0.1–0.3 M) whereas the truncated 0461–265 form eluted at a 3-fold higher salt concentration [EC50 = 0.79 M NaCl (95% c.i. = 0.7–0.9 M)] (Fig. 2). Deleting the acidic tail from two different Chordopoxivirus SSBs enhances the affinity for ssDNA, just as it does when the C-terminus is deleted from phage and bacterial SSBs.
Deletion of the C-terminus from either VACV-I3 or MCV-046 affects the structure of the protein–DNA complexes

Electrophoretic mobility shift assays were used to see what effect these alterations had on the structures of complexes formed by wild-type and mutant poxvirus SSBS. Single-stranded circular ϕX174 DNA was bound to different quantities of each of the SSBS in a low ionic strength buffer (50 mM NaCl) and then the DNA–protein complexes were size fractionated using agarose gel electrophoresis and visualized with a DNA stain. As one would predict, all of the proteins progressively decreased the rate of migration of the DNA as the protein-to-DNA ratio was increased (Fig. 3). At higher protein amounts the intact I31–270 form still bound the DNA, and all DNA entered the gel, whereas the truncated I31–244 form trapped the DNA in the wells in high-molecular aggregates (Fig. 3, arrows). A similar effect was seen with the MCV proteins although the wild-type O461–280 species exhibited some natural tendency to form aggregates at the highest concentrations tested. This property was greatly exacerbated when the C-terminus was deleted in form O461–280 (Fig. 3, arrows). These data suggest that the structures of the complexes formed by SSBS lacking the C-terminal tail are quite different from normal complexes. Moreover, the propensity of the mutant proteins to form such aggregates could be a factor contributing to the higher observed DNA binding affinity (Fig. 2).

We also investigated what effect mixing the wild-type and mutant VACV proteins together would have on these gel-shifted complexes. To detect the anomalous structures that are characteristic of those formed by I31–244, we used a DNA–protein ratio that can generate well-bound aggregates (Fig. 3). As the proportion of the truncated I31–244 form was increased relative to the wild-type protein, it caused a gradual increase in the electrophoretic mobility of the DNA–protein complexes (Fig. 4). However, as the ratio of the two proteins started to exceed 50%, the aggregated form again appeared. Thus the mutant has the capacity to interfere with normal protein activity and might be expected to exhibit a dominant negative phenotype in virus-infected cells. This point is explored further below.

The VACV-I3 C-terminal tail is accessible for binding to other proteins in DNA–protein complexes

We next examined whether the I3 C-terminus would be exposed for binding to other potential protein partners upon DNA binding. To test this hypothesis, we took advantage of the observation that our 10D11 monoclonal anti-I3 antibody reacts very well with the I31–270 protein, but cannot detect the I31–244 protein (Fig. 1C). It also detects a C-terminal histidine-tagged form of I3 in western blots (data not shown). This indicates that that antibody 10D11 recognizes an internal epitope within the I3 C-terminus. We then used a gel “supershift” analysis to determine whether the C-terminal epitope can be detected in DNA–protein complexes. To perform the experiment we assembled DNA–protein complexes composed of ϕX174 DNA plus 1–2.5 μg of I31–270 or I31–244. We then incubated these complexes with up to a 5-fold molar excess of the 10D11 antibody or an isotope control antibody targeting VACV E9 protein. The products were size fractionated using gel electrophoresis (Fig. 5). The I3-specific antibody selectively supershifted the I31–270–DNA complexes relative to the shift observed with just I3 added alone, and in a manner dependent upon both I3 and antibody concentration (Fig. 5A). This large shift was not observed when the I3-specific antibody was added to complexes composed of DNA plus the I31–244 protein (Fig. 5B).
although some background of non-specific binding was detected that was comparable to that seen in reactions containing added E9 control antibody (Fig. 5, panels C and D). Some antibody-dependent aggregation of the DNA-protein complexes was also seen, but only in reactions containing the 10D11 antibody and wild-type I3. These studies showed that the VACV I3 C-terminal is accessible for binding to other proteins in DNA-protein complexes.

The C terminal tail of I3 alters the stability of both I3:I3 and I3:DNA interactions

To gain some insights into the nature of the DNA–protein complexes formed by I3–270 and I3–244 proteins, we used gel filtration in combination with multi-angle laser light scattering analysis. Multi-angle light scattering methods are often used to measure the size of protein complexes and rest upon theoretical foundations established by Stokes and Svedberg. The method can determine the mass of macromolecular complexes to an accuracy approaching ± 3%. One can gain insights into the composition of such complexes by testing the fit between an observed mass and a hypothetical mass obtained by adding together different possible combinations of masses of components of known molecular weights. The purified I3 proteins were initially applied to a Superose 6 column connected to a Wyatt Systems instrument operating in isocratic mode, in a buffer containing 0.15 M NaCl, and the molecular weight of the eluting proteins determined using ASTRA software. This ionic strength reflects biologically relevant salt conditions while still favouring DNA binding (Fig. 2). These proteins were then recovered, concentrated, incubated with a ~1:1 mol ratio of 30-mer poly-dT oligonucleotide (Tseng et al., 1999), and reapplied to the system. Under these conditions I3–270 eluted as a single peak with a molecular weight of 38 kDa (Fig. 6A). When bound to dT30, I3–270 produced two elution peaks, the first exhibiting a molecular weight of 134 ± 7 kDa and the second at 61 ± 5 kDa (Fig. 6B). The behavior of the DNA-free protein is difficult to reconcile with it being either a monomer (30.0 kDa) or a stable dimer (60.0 kDa), and is suggestive of a protein exchanging rapidly between these states during chromatography. The mass of the larger species formed in the presence of 9.1 kDa dT30 molecules is most compatible with an I3 tetramer bound to one molecule of dT30 (129 kDa) whereas the smaller species exhibits a size closely predicted for a protein dimer (60.0 kDa) or possibly a dimer plus DNA (69.1 kDa). Deleting the I3 C-terminal tail significantly altered the behavior of the protein and the complexes it forms. The pure protein exhibited
Microinjection of anti-I3 monoclonal antibodies into virus-infected cells decreases the size of viral factories

Although these studies suggested that the I3 C-terminus regulates the structures of I3-DNA complexes, they provide no insights into whether these observations have any relevance to virus biology. The I3L gene is essential, and has proven difficult to mutate (Greseth et al., 2012), and so using a genetic approach was unlikely to be productive. Indeed, our repeated efforts to rescue a second gene encoding the I31–244 allele into a pure recombinant I3L+ virus failed (data not shown). Instead, we tested whether the 10D11 monoclonal antibody, which targets the C-terminal epitope in DNA–protein complexes (Fig. 5), would have any effects on VACV replication in cells injected with the antibody. This can be easily assayed, because replicating poxviruses produce characteristic structures in the cytoplasm of infected cells (Fig. 7). These factories can be stained with DAPI and the amount of virus DNA quantitated using fluorescence microscopy. BSC-40 cells were microinjected with the 10D11 antibody (Fig. 7), careful measurement of either the size (Fig. 8A) or the fluorescence intensity (Fig. 8B) demonstrated that they were significantly smaller than the factories formed in either buffer-injected cells (p = 0.001) or anti-hexahistidine-injected cells (p < 0.0001) (Fig. 7). However, the difference was not large; the factories in anti-I3-treated cells were only about 2-fold smaller than the factories in the control cells.

We also repeated these experiments using cells micro-injected with 5 mg/ml N30-mer or S30-mer peptides. Both appeared to be inhibitory compared to buffer-injected control cells (p < 0.05) although again the magnitude of the effect was very limited (data not shown). Nevertheless these experiments showed that interfering with processes involving the I3 C-terminus has deleterious effects on virus DNA replication.

I3 does not interact with the small (R2) subunit of VACV during virus infection

There have been at least two reports that I3 can bind to other protein partners (besides itself (McCrainth et al., 2000)). These include elf4G (Zaborowska et al., 2012) as well as the small (R2) subunit of VACV ribonucleotide reductase (Davis and Mathews, 1993). The VACV F4L gene encodes the R2 protein, and the later paper is of particular interest since the F4–I3 interaction has been proposed to involve the I3 acidic C-terminus (Davis and Mathews, 1993). Thus by disrupting such an interaction, one could perhaps explain the result shown in Fig. 8. To explore this matter further, we prepared a plasmid encoding an N-terminal Flag-tagged form of I3 under control of a VACV promoter and transfected the plasmid into VACV-infected BSC-40 cells. The infected-cell proteins were then retrieved using anti-Flag magnet beads and fractionated using SDS-PAGE. For controls we either omitted the VACV or transfected the infected cells with an empty (pSC66) vector. Some of the gels were stained to detect total protein, while others were Western blotted using antibodies directed against the cellular and viral ribonucleotide reductase R1 and R2 subunits. Although the reagents can readily detect the VACV F4 (R2) and I4 (R1) proteins in cell-free extracts, and can also retrieve Flag-tagged I3 from the transfected extracts (Fig. 9A), we were never able to detect an
interaction between Flag-tagged I3 and either of the proteins comprising the VACV ribonucleotide reductase complex (Fig. 9A). As an alternative approach we hypothesized that if I3 can interact with F4, it should recruit at least some F4 to the sites of virus replication where I3 is found. We used immunofluorescence microscopy to map the I3 and a his6-tagged form of F4 (Gammon et al., 2010) in VACV-infected cells and as a control used a virus bearing a N-terminal Flag-encoding form of I3L under control of a poxvirus early/late promoter (lanes 1 and 3). The cells were cultured for 24 h and the N-Flag-tagged I3 recovered using anti-Flag antibodies cross-linked to magnetic beads. The bound proteins were then size fractionated using SDS-PAGE and processed by western blotting to detect the VACV ribonucleotide reductase subunits (encoded by the I4L and F4L genes) or the Flag-tagged and native forms of I3. Panel B, Immunofluorescence analysis of the distribution of the VACV F4 protein in VACV-infected cells. BSC-40 cells were infected (or mock-infected) with the indicated viruses and then fixed and stained to detect DNA, I3, and a his6-tagged form of F4. Note that the ΔF4L mutation retards virus replication. As a consequence the factories are smaller and more compact than in cells infected with virus encoding wild type or his6-tagged F4.

Fig. 9. Analysis of proteins associated with VACV I3 protein. Panel A. BSC-40 cells were infected (lanes 1–2) or mock infected (lane 3) with VACV and transfected with either the empty vector (lanes 2) or a plasmid bearing an N-terminal Flag-encoding form of I3L under control of a poxvirus early/late promoter (lanes 1 and 3). The cells were cultured for 24 h and the N-Flag-tagged I3 recovered using anti-Flag antibodies cross-linked to magnetic beads. The bound proteins were then size fractionated using SDS-PAGE and processed by western blotting to detect the VACV ribonucleotide reductase subunits (encoded by the I4L and F4L genes) or the Flag-tagged and native forms of I3. Panel B, Immunofluorescence analysis of the distribution of the VACV F4 protein in VACV-infected cells. BSC-40 cells were infected (or mock-infected) with the indicated viruses and then fixed and stained to detect DNA, I3, and a his6-tagged form of F4. Note that the ΔF4L mutation retards virus replication. As a consequence the factories are smaller and more compact than in cells infected with virus encoding wild type or his6-tagged F4.

distributed background staining resembling that seen in mock-infected cells. In contrast, this antibody detected a much more intense signal in cells infected with virus expressing his6-tagged F4. The his6-tagged F4 was widely distributed throughout the cells, while I3 was uniquely located in the DAPI-stained virus factories. We also determined the Pearson coefficient of correlation, testing how often red (I3) and green (his6-F4) pixels overlapped, and observed values varying from $r=0.07$ to $r=0.16$, providing little support for there being any clear co-localization of the two antigens in infected cells. Although we cannot preclude the possibility that the I3 acidic tail serves some role as a protein-binding site, our data mostly support the hypothesis that its primary function is to modulate the structure of I3-DNA complexes in infected cells.
Discussion

Although I3-like genes are found only in poxviruses, they share a common acid C-terminal tail with some bacterial and phage counterparts (Fig. 1). This protein element plays an important role in regulating ssDNA binding as well as helping recruit DNA replication, recombination and repair proteins to sites where the ssDNA is exposed. The purpose of this study was to examine whether two exemplars of these poxvirus SSBs, cloned from two different Chordopoxviruses (MCV and VACV) exhibit properties similar to these phage and bacterial SSBs.

Pro tease sensitivity is a characteristic feature of these SSB acidic tails and we had previously noted that the C-terminus of VACV I3 protein is similarly protease sensitive (Tseng, 2000). Tryptic rapidly excises a 25 aa peptide comprising most of I3's acidic C-terminus, although a 30-residue patch extending towards the N-terminus and highly-conserved EED, DDD or EEE motifs probably better defines the boundaries of this element (Fig. 1). Computational modeling of this 30-mer (Neron et al., 2009) suggests that the central acidic portion would likely fold into an α-helix with an unstructured C-terminus. This peptide also clearly encodes the epitope recognized by the 10D11 monoclonal antibody (Fig. 1C). However, we have not screened other I3 antibodies so it is difficult to say whether this is just a coincidence, or because the I3 C-terminus, like the T4 GP32 C-terminus (Krassa et al., 1991), is an immunodominant epitope.

Further parallels between the virus and phage SSBs are observed when one deletes the acidic tails from the VACV and MCV proteins. The recombinant proteins still appeared to retain the ability to bind DNA even when one deletes the acidic tails from the VACV and MCV proteins can bind strongly to ssDNA, it is also clear that most of their his6-tagged and DNA-free I3 exhibited the same hydrodynamic dimensions as ovalbumin (44 kDa) with small amounts of high-molecular-weight complexes or protein aggregates also eluting near the void volume. This is consistent with our data. Although MALLS measures both the hydrodynamic shape and molecular weight of proteins, the protein Greseth et al. saw co-eluting with ovalbumin is most likely DNA-free I3 undergoing rapid monomer-dimer exchange (Fig. 6). In the presence of ssDNA, native I3 is recruited into larger complexes, which exhibit masses compatible with a molecule of DNA in stable association with 2 or 4 molecules of I3. Interestingly, deleting the C-terminus appeared to disrupt the monomer-dimer equilibrium (Fig. 6), and stabilized a tetrameric DNA protein complex.

The MALLS (Fig. 6) and gel shift data (Fig. 3) collectively suggest a model in which the acidic tails serve at least two important functions. First, they may promote cooperative binding of I3 to DNA through a swapped dimer interaction. This would minimize the risk of forming inter-strand links between SSB monomers that have initially bound randomly to different sites in the substrate. Secondly, the C-terminal tails may destabilize the DNA–protein complexes enough to permit sliding of these structures (perhaps as dimers) along the DNA. This would ensure that the SSB molecules are distributed properly along the length of the substrate, in the process generating a more open and accessible structure. In the absence of these tails, the SSB molecules would likely bind randomly to many different and relatively stable sites in the DNA. Collectively this random binding of truncated proteins to DNA sites scattered across many different molecules, proteins still capable of forming tetramers in the presence of DNA (Fig. 6), could produce interstrand crosslinks and the aggregates seen in Fig. 3.

I3 is an essential protein and interfering with its expression and with I3–I3 interactions has deleterious effects on virus replication (Gammon et al., 2010; Greseth et al., 2012). Thus, one would predict that if the I3 C-terminus modulated I3 function in vivo, interfering with this function might lead to a decrease in the amount of virus DNA replication. To examine this question we microinjected the 10D11 anti-I3 antibody into VACV-infected cells and used DAPI staining to measure the amount of DNA in virus factories 6 h post-infection. The microinjected 10D11 antibody co-localized with VACV factories and caused a ~2-fold decrease in virus replication compared to an isotype control (Fig. 8). One difficulty with this experiment is that concentrations and volumes limit how much antibody can be microinjected into a cell, and it may be difficult to occlude all of the potential target sites. Quantitative western blotting suggests that I3 protein is expressed at very high levels in VACV-infected cells, with perhaps ~10^9 molecules per cell by 6 h post-infection (data not shown), and this qualitative observation is in agreement with data obtained, from ribosome profiling (Yang et al., 2015), concluding that I3 is a highly expressed protein. Conversely, it is difficult to introduce much more than ~10^6 molecules of antibody by microinjection, so except at early times in the infection, I3 will usually be present in excess over antibody. The fact that one still sees a small but significant decrease in the amount of DNA in cells injected with the 10D11 antibody, even under these circumstances, suggests that the I3 C-terminus is accessible in vivo and that blocking (or perhaps crosslinking) the protein through the C-terminus can inhibit early virus DNA replication in vivo.

The data shown in Fig. 5 also illustrates additional similarities between phage and poxvirus SSBs. DNA binding still leaves the C-terminal epitope sufficiently exposed that it can be bound by a monoclonal antibody (Fig. 5). These observations further support the hypothesis that the I3 C-terminus shares part of the same binding site as does DNA and it is not appreciably occluded in DNA-containing complexes. Parenthetically, the fact that the

10D11 antibody targets an exposed epitope encoded by one of the most highly translated VACV early mRNAs (Yang et al., 2015), explains why many laboratories have found it a very useful reagent for imaging factories (Liu et al., 2015; McFadden et al., 2012; Zaborowska et al., 2012).

The I3 C-terminus also appears to play a key role in organizing the structure of smaller-scale I3-DNA complexes. Using Sephacryl S100 gel exclusion chromatography, Greseth et al. (2012) observed that most of their his6-tagged and DNA-free I3 exhibited the same hydrodynamic dimensions as ovalbumin (44 kDa) with small amounts of high-molecular-weight complexes or protein aggregates also eluting near the void volume. This is consistent with our data. Although MALLS measures both the hydrodynamic shape and molecular weight of proteins, the protein Greseth et al. saw co-eluting with ovalbumin is most likely DNA-free I3 undergoing rapid monomer-dimer exchange (Fig. 6). In the presence of ssDNA, native I3 is recruited into larger complexes, which exhibit masses compatible with a molecule of DNA in stable association with 2 or 4 molecules of I3. Interestingly, deleting the C-terminus appeared to disrupt the monomer-dimer equilibrium (Fig. 6), and stabilized a tetrameric DNA protein complex.
Where poxvirus SSBs differ from other SSBs is that we have not been able to detect any specific binding of other proteins to the C-terminus (Fig. 9A). It has been hypothesized that I3 interacts with the small F4L-encoded subunit of the VACV ribonucleotide reductase (Davis and Mathews, 1993), but we could not detect this using co-immunoprecipitation reactions and antibodies directed against Flag-tagged I3 and native F4. Although it is possible that an I3–F4 interaction is too unstable to be detected by these methods, we also could not detect any selective recruitment of F4 to the factories where I3 is found (Fig. 9B). Collectively these data argue against the hypothesis that I3 is used to recruit significant amounts of the VACV ribonucleotide reductase to sites of viral DNA replication. The I3 tail is clearly exposed in I3-DNA complexes and could still recruit other proteins to sites of replication. However, we favor the hypothesis that the primary function of the I3 tail is to promote the formation of well ordered, and yet still only meta-stable, I3-DNA complexes. The observation that one can transfact DNA encoding an mCherry reporter gene fused to the I3 C-terminus, and detect seemingly normal protein localization in normal virus factories (Greseth et al., 2012) also supports the hypothesis that I3 function is still preserved even if the C-terminus is occluded by something as large as the mCherry protein.

Although this work has focused primarily on the VACV I3 protein, the 046 homolog encoded by MCV also exhibits many of the same basic properties. MCV differs greatly from VACV, in particular the MCV genome is very G+C-rich and MCV lacks so many of the genes encoded by most other poxviruses (including the thymidine kinase and ribonucleotide reductase) that MCV only replicates slowly in vivo and cannot be cultured in the laboratory. Nevertheless, deleting the C-terminus of the MCV 046 protein enhances the affinity for ssDNA and causes the mutant protein to form complexes that exhibit the same altered migration properties as the truncated I3 protein. These data suggest that the biochemical properties of I3 and 046 are probably shared by all poxvirus SSBs, and that the model we have proposed for cooperative recruitment and I3-DNA interactions is likely applicable to all of the Chordopoxvirus SSBs. What remains to be established is how the I3 C-terminus affects the recruitment of I3 onto ssDNA and how this process affects the global structure of larger DNA–protein complexes.

Molecular cloning

The polymerase chain reaction was used to prepare a full-length copy of the I3L gene [encoding I3 residues 1–270 (I31–270)] using a VACV DNA template and forward (5′-CTTGAAGGAGATACTACA-TATGAAATGCTTATAGTAAATGATTTGTTTCTT-3′) and reverse (5′-CTTGTGATT-CACGGGGATCTTCTATATGGATATTTGCTTTT-3′) primers. A C-terminal truncated form of I3L (I31–244) was prepared using the same forward primer and the reverse primer 5′-CTTGTGATT-CAGCCGGATCTTAAAGCAGATCTCTTCTACAT-3′. A full-length copy of the MCV MC046L gene (046L1–288) was prepared using a plasmid template encoding a cloned and codon-optimized copy of the gene and forward (5′-CTTTAAAGGAGATACTACA-TATGAAATGCTTATAGTAAATGATTTGTTTCTT-3′) and reverse (5′-CTTTTGTGATT-CAGCCGGATCTTAAAGCAGATCTCTTCTACAT-3′) primers. A C-terminal truncated form of the MCV MC046L gene (046L1–263) was prepared using the same MC046L forward primer and reverse primer 5′-CTTTTGTGATT-CAGCCGGATCTTAAAGCAGATCTCTTCTACAT-3′ and reverse (5′-CTTTTGTGATT-CAGCCGGATCTTAAAGCAGATCTCTTCTACAT-3′) primers. Plasmids were then used to transform E. coli DH10B, and recombinant clones sequenced. The plasmids were then used to transform E. coli strain DHE142 for proteins of expression purposes.

To make a Flag-tagged form of I31–270 we used a two-step process. First the PCR, a VACV DNA template and forward (5′-GCGAGCTCTATGAAATGCTTATAGTAAATGATTTGTT-3′) and reverse (5′-GCGAGCTCTATGAAATGCTTATAGTAAATGATTTGTT-3′) primers were used to amplify a copy of the I3 gene and incorporate a Flag-tag-encoding sequence at the start of the gene. The PCR product was TOPO-cloned (Invitrogen) and then recloned into pSC66 under a virus early-late promoter.

Protein purification

Bacteria were cultured at 37 °C in 7.5 L quantities of Luria broth with 100 μg/ml ampicillin (Fishier) and 25 μg/ml chloramphenicol (ICN Biomedicals). Protein expression was induced by adding 1 mM isopropyl β-D-thiogalactopyranoside (Fishier) followed by the addition of 50 μg/ml rifampicin (Sigma). The cells were recovered by centrifugation, resuspended in Buffer A [0.2 M Tris·HCl pH 7.8 (MP Biomedicals), 0.1 mM EDTA pH 8.0 (Sigma), 10 mM β-mercaptoethanol (Sigma), 10% glycerol (Sigma), 50 mM NaCl (Fishier)] and stored frozen at −20 °C. The cells were thawed and sonicated, centrifuged at 20,000 g for 30 min and the pellet re-extracted by sonication and centrifugation. The pooled supernatants were applied to a 5 ml Hitrap Heparin HP column (GE Healthcare), washed with Buffer A, and eluted in a stepwise manner using Buffer A supplemented with 0.1–2.0 M NaCl. Protein-containing fractions were dialyzed against Buffer A, and applied to a 10 ml column of single-stranded calf thymus DNA cellulose (Sigma). The column was washed with Buffer A and the protein eluted with Buffer A containing 0.5 M or 2.0 M NaCl.

Protein-electrophoresis and western blotting

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) and a commercial Coomassie stain (Pierce) were used to determine protein purity. Immobilon FL membranes (Millipore) were used for western blotting. A mouse monoclonal antibody directed against the I3 protein (clone 10D11) prepared by ProSci (Lin et al., 2008) as were monoclonal antibodies directed against the VACV F4 protein (clone 4B3) (Gammon et al., 2010) and the E9 protein (clone 4E5) (Magee et al., 2009). All three are IgG,
idiotypes. Other antibodies included a mouse anti-cellular ribonucleotide reductase R1 subunit antibody (Chemicon), a goat anti-cellular ribonucleotide reductase R2 subunit antibody (N-18; Santa Cruz), a mouse anti-Flag antibody (M2; Sigma), and a mouse monoclonal anti-hexahistidine antibody (Roche). Dr. C. Mathews (Oregon State University) kindly provided a rabbit anti-VACV I3 antibody (Slabaugh et al., 1993). Blots were imaged using a LI-COR Odyssey imager and a LI-COR anti-mouse antibody labeled with an 800 CW IR-Dye, or anti-goat and anti-rabbit antibodies labeled with IR-Dye 680.

To determine the amount of I3 in VACV-infected cells, BSC-40 cells were infected for 6 h at a multiplicity of infection of five, and then harvested and known numbers of cells lysed and applied to an SDS-PAGE gel along with known amounts of recombinant I3. The quantity of I3 per cell was then determined using LI-COR software and the computed standard curve.

Electrophoretic mobility shift assays (EMSA)

Each SSB was incubated with 1 μg of φX174 ssDNA for 20 min at 37 °C in a 20 μl reaction containing 12 mM Tris - HCl pH 8.0, 2.4% glycerol, 1 mM EDTA, and 2.5 mM β-mercaptoethanol. Two microliters of loading buffer (50 μM Tris - HCl pH 7.5, 0.6% glycerol) was added to each sample, and the DNA–protein complexes fractionated by gel electrophoresis using gels composed of 0.8% Sea-kem LE agarose (Lonza) and run at 30 V for 17 h at 4 °C in half-strength Tris-acetate buffer. The DNA was stained with Sybr Safe dye (Invitrogen) and imaged. The same methods were used to perform antibody EMSA supershift studies, except that the SSB was bound to the ssDNA for 20 min at 37 °C and then monoclonal antibodies directed against VACV I3 or E9 proteins were added, and incubated for another 20 min at 37 °C, followed by electrophoresis and imaging.

DNA cellulose binding assay

A 1 ml slurry was prepared containing 50% (w/v) of single-strand calf thymus DNA cellulose and 0.6 mg of SSB in Buffer A. The mix was gently mixed by rotation for 1 h at room temperature and then centrifuged for 1 min at 1000g. The supernatant was recovered and the protein was progressively eluted from the DNA by stepwise addition of 0.5 ml of Buffer A containing increasing concentrations of NaCl (0.05–3 M), followed at each step by mixing and centrifugation. The eluted proteins were fractionated using SDS PAGE gels and stained with a Coomassie blue stain (10% phosphoric acid, 0.8 M ammonium sulfate, 0.0012% Coomassie Brilliant Blue G-250, 20% methanol). A BioRad Gel Doc system and Image Lab software were used to determine the quantity of protein eluted in each fraction.

Multi-angle laser light scattering (MALLS)

Bovine serum albumin (BSA), I3, and I3 were chromatographed on a Superdex 200 10/300 GL column (GE Healthcare) equilibrated with Buffer A containing 150 mM NaCl. Peak fractions were pooled and concentrated using Amicon Ultra 10K Ultracel filters (Millipore). An aliquot of each protein (~0.6 mg in 100 μl) was then applied to a Superose 6 10/300 GL column (GE Healthcare) equilibrated with Buffer A containing 150 mM NaCl and connected to a Wyatt Systems REX and DAWN MALLS. Changes in refractive index were used to detect eluting proteins and the data analyzed using ASTRA software. The I3 and I3 proteins were collected as they eluted, mixed with 30-mer poly-dt oligonucleotide in a ~1:1 mol ratio, incubated for 30 min at 37 °C and re-applied.

Microinjection and fluorescence microscopy

BSC-40 cells were cultured overnight on coverslips in MEM and then injected with a mouse monoclonal antibody directed against I3, or an anti-hexahistidine isotype control antibody (Roche), using an Eppendorf Femtojet microinjector system. The antibodies were prepared at 5 mg/ml in buffer containing 0.1 M glutamic acid (Fisher), 0.14 M KOH (Acros), 1 mM MgSO4 (Acros), 1 mM dithiothreitol (Fisher), adjusted to pH 7.2 with citric acid (Acros). We used duplicate coverslips, injecting 250 cells per coverslip. The cells were then infected for 1 h at 37 °C with ASL-YFP VACV, at a multiplicity of infection of 5 in PBS, the buffer was replaced with MEM, and returned to the incubator for 5 h. The cells were then fixed on PBS in 4% paraformaldehyde for 30 min, stained with 1:2000 diluted Cy5-labeled secondary antibody and counterstained with 10 ng/ml 4,6-diamidino-2-phenylindole (DAPI) and 1 nM rhodamine phalloidin as per the supplier’s instructions (Molecular Probes). The samples were imaged using a Quorum WaveFX spinning disc confocal microscope and analyzed using Velocity software (Perkin-Elmer). Student’s t-test was used to compare differences in mean area, p < 0.05 was considered significant.

For ordinary fluorescence microscopy, samples were fixed and stained as described previously (Lin and Evans, 2010), imaged using an Applied Precision DeltaVision fluorescence microscope, and processed using the deconvolution algorithm supplied with softWoRx (v3.7). Photoshop CS6 was used to prepare composite images. All of the images were subjected to the same scaling and background corrections using only a linear gamma factor.

Immunoprecipitation

BSC-40 cells were infected for 1 h with VACV at a multiplicity of infection of 5 in PBS in 150 mm dishes. The virus was then removed and replaced with Opti-MEM (Gibco). One hour later (2 h post-infection), the cells were transfected with 8 μg of plasmid encoding an N-terminal Flag-tagged form of I3 (N-flag I3) using Lipofectamine (Invitrogen). The cells were cultured overnight, harvested, washed with PBS, and lysed by passage through a 21.5 gauge needle in 0.5 ml lysis buffer [0.15 M NaCl, 20 mM Tris - HCl pH 8, 1 mM EDTA, 0.5% NP-40 (Fisher)] supplemented with one protease-inhibitor tablet per 20 ml buffer (Roche). The lysate (~500 μl) was supplemented with 10 μg of a 30-mer poly-dt oligonucleotide, incubated on ice for 1 h, and then mixed with 50 μl of a 50% slurry of mouse monoclonal anti-Flag magnetic beads (Sigma). The mixture was rotated overnight at 4 °C and then the beads were recovered and washed with buffer containing 50 mM Tris - HCl pH 7.4 and 150 mM NaCl. The beads were boiled in loading buffer (3.7% SDS, 0.6 M β-mercaptoethanol, 40% glycerol, 50 mM Tris - HCl pH 6.8, 1 mg/ml bromphenol blue) and the proteins fractionated on SDS PAGE gels prior to staining or western blotting as described above.

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References


