Characterization of the vaccinia virus F8L protein

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Vaccinia virus infection dramatically affects the host actin cytoskeleton by inducing disassembly of actin stress fibres and formation of actin tails which propel the virus intra- and intercellularly. The viral factors responsible for these actin rearrangements remain unknown. Sequence analysis reveals significant homology between the vaccinia F8L ORF and the proline repeats of iActA, the protein which initiates actin tail assembly and motility in the bacterial pathogen Listeria ivanovii. We characterized the F8L gene product to examine its possible role in vaccinia rearrangements of the host actin cytoskeleton. F8L is a ~8 kDa protein expressed early during infection and is found throughout the cytoplasm, with no discernible association with viral or cellular structures. Furthermore, the F8L deletion strain, WRΔF8L, forms particles and actin tails indistinguishable from WR. Our observations demonstrate that F8L is not required for vaccinia virus morphogenesis or the actin rearrangements observed during infection.

Vaccinia virus is a large, complex DNA virus that has two infectious forms, the intracellular mature virus (IMV) and the extracellular enveloped virus (EEV). IMV is characterized by the presence of a membrane cisterna acquired from the intermediate compartment (Sodeik et al., 1993). A small percentage of IMV acquire a second membrane cisterna from the trans-golgi network of the host cell, resulting in a four membrane form of the virus termed intracellular enveloped virus (IEV) (Schmelz et al., 1994). Fusion of IEV with the plasma membrane as the virus exits the cell results in the release of infectious EEV, which retains three membranes. During the complex morphogenesis of vaccinia virus, several dramatic rearrangements of the host actin cytoskeleton occur including the loss of actin stress fibres and the formation of actin tails as well as large viral tipped microvilli-like projections at the cell surface (Cudmore et al., 1995, 1996; Hiller et al., 1981, 1979; Stokes, 1976). Using a combination of mutant and drug studies it has recently been demonstrated that IEV particles are responsible for inducing the formation of both actin tails and microvilli-like projections at the cell surface (Cudmore et al., 1995). IEV particles are propelled randomly throughout the cytoplasm at rates of ~3·0 μm/min using actin polymerization as the driving force (Cudmore et al., 1995). Upon contact with the plasma membrane as IEV particles fuse they are propelled outwards on long microvilli-like projections at a similar rate facilitating their spread into neighbouring cells (Cudmore et al., 1995, 1996). Currently, the viral factors responsible for vaccinia-induced actin rearrangements and motility are unknown. However, the actin tails induced by vaccinia virus are very reminiscent of those observed in a number of motile intracellular bacterial pathogens, including Listeria and Shigella (Cudmore et al., 1995). Extensive studies of Listeria monocytogenes and Shigella flexneri have identified two unrelated bacterial surface proteins ActA and IcsA, respectively, as being both necessary and sufficient for inducing actin tails that are responsible for initiating actin-based motility of these bacteria (Goldberg & Theriot, 1995; Kocks et al., 1995; Smith et al., 1995). Both proteins induce actin tails by recruiting host proteins that are normally associated with the actin cytoskeleton.

Although all the host components involved in motility of Listeria and Shigella have yet to be identified, it is clear that proline-rich sequences play an important role in the process of actin tail assembly (Higley & Way, 1997). In Listeria the central proline-rich repeats of ActA are able to recruit and directly bind to VASP, a host protein normally found associated with actin stress fibres and focal adhesions (Pistor et al., 1995). Deletion of the central proline-rich region of ActA completely blocks the intracellular motility of this bacterium (Goldberg & Theriot, 1995; Kocks et al., 1995; Smith et al., 1995). Although IcsA has not been demonstrated to bind to VASP it has been shown to interact directly with the head domain of the focal adhesion actin-binding protein vinculin, which also contains a proline-rich sequence similar to an ActA repeat (Suzuki et al., 1996; Higley & Way, 1997). Furthermore, microinjection of peptides corresponding to the proline-rich repeats in ActA completely blocks the intracellular motility of Shigella, suggesting that proline-rich sequences also play an important role in the actin-based motility of this bacterium (Zeile et al., 1996).
Western analysis with either antiserum detects a single band of ~ 8 kDa in good agreement with the predicted size of F8L in vaccinia-infected HeLa. The specificity of the F8L antisera was confirmed by expression of the F8L protein in E. coli followed by Western analysis on total protein samples (data not shown). Examination of the sequence upstream of the F8L open reading frame predicts that the protein might be expressed early during infection. In order to determine if this is the case, as well as to find an optimal time-frame in which to examine F8L function during infection, we looked at the kinetics of F8L expression during vaccinia infection. Western blot analysis indicates that F8L expression is first detected at ~ 3 h post-infection and that increasing amounts of protein are detectable throughout infection (Fig. 1b). To localize F8L during infection HeLa cells were fixed 8 h post-infection and labelled with F8L antisera in conjunction with either FITC–phalloidin (Molecular Probes) or anti-P14 (A27L) monoclonal antibody C3, which recognizes all forms of vaccinia virus particles, as described previously (Cudmore et al., 1995). By indirect immunofluorescence, F8L is expressed early during infection, with signal increasing throughout the infection, in accord with the results from Western analysis. In addition, no changes in cellular distribution could be detected at different time-points (2–24 h). At 8 h post-infection, when numerous actin tails have formed, F8L is distributed diffusely throughout the cytoplasm of the host and shows no obvious localization to perinuclear virus factories, virus particles, actin tails or other cellular structures (Fig. 2). Although taken together our observations on F8L distribution show it is not localized to virus particles, as might be expected of a functional homologue of iActA, they do not rule out a possible role for F8L in the breakdown of the actin cytoskeleton before actin tail formation. Indeed F8L expression occurs at the time when actin stress fibres begin to disassemble. Furthermore, micro-injection of proline-rich peptides related to ActA repeats into uninfected cells has been shown to induce actin stress fibre loss (Southwick & Purich, 1994). To examine the possibility that F8L may play a role in stress fibre disassembly we deleted the F8L gene from the genome of the WR strain of vaccinia. The E. coli gpt gene under the control of the I3L early/intermediate vaccinia promoter from the parent vector pEMBlgppt-Kpn-Rl was cloned into the EcoRI and EcoRV sites of pBluescript SKII (Stratagene) to generate BSgpt. The genomic region immediately downstream of the F8L stop codon was amplified by PCR using the DF8LF forward primer (5’GGGCCCAAG-ATCTTCGAGGTATATATATATCATCATTTC3’) and DF8LR reverse primer (5’CCCCGGACTAGTAGCATGCA-CCTCATCAACCG3’). The resulting downstream F8L PCR product was digested with PsI (underlined in the DF8LF primer) and Xbal (a single endogenous Xbal site is located 475 bp from the stop codon of F8L) and cloned into the PsI and Xbal sites of BSgpt to generate BSgptDF8L.

The genomic region immediately upstream of F8L was amplified by PCR using the UF8LF forward primer

**Fig. 1.** (a) Alignment of the first polyproline repeat of iActA (residues 419–460) with F8L (residues 24–63) showing 35-7% identity and 59-5% similarity. Vertical bars and dots indicate identities and similarities, respectively. Alignment was generated using the Align program of the DNASTAR software package. (b) Western analysis shows F8L is expressed early during vaccinia infection. Total protein (50 µg) from vaccinia-infected HeLa cells at the times indicated was separated on a 15% SDS–PAGE gel and transferred to nitrocellulose membrane before being probed with the FBL antisera at a dilution of 1:4000 and processed for ECL detection as described by the manufacturer (Amersham). The position of F8L is indicated by the arrowhead and molecular mass standards are indicated. The blot was stripped and re-probed with monoclonal anti-actin antibody (Clone AC-74) (Sigma) to control for equivalent loadings in each lane. Western blot films were scanned on a Umax Powerlook II scanner, sized and annotated with the Adobe software package.

Given the similarity between actin tails induced by vaccinia and bacterial systems we searched a vaccinia database of all viral open reading frames with the sequences of ActA and IcsA as well as iActA, a functional homologue of ActA from *Listeria ivanovii*, a species related to *Listeria monocytogenes* (Kreft et al., 1995). This analysis identified a 42 amino acid stretch of the 65 amino acid F8L vaccinia protein as having 35-7% identity and 59-5% similarity to a proline-rich repeat of iActA (Fig. 1a) (Gouin et al., 1995). iActA contains seven copies of a proline-rich repeat of 47 amino acids that plays a critical role in initiating actin tail assembly (Gerstel et al., 1996; Gouin et al., 1995). Based on this degree of homology and the polyproline-rich nature of F8L, we wished to characterize the product of the F8L gene to determine whether it plays a role in viral-induced actin rearrangements or actin tail formation. As a first step we generated polyclonal antisera against F8L. Peptides F8LN and F8LC, CGSKRKHDSRRLQEQE and CLKNDYPRISY-NPPPK respectively, corresponding to the N and C termini of F8L were synthesized, coupled to keyhole limpet haemocyanin via an additional cysteine residue at their N termini using the Imject conjugation kit (Pierce), and injected into rabbits. Both antisera were subsequently purified by affinity chromatography on their respective peptides using the sulfolink system (Pierce).
(5’ GGGCCCCTCGAGGCGCGCTCCGAATAACTAACA-3’) and UF8LRH3 reverse primer (5’ CCCCCCAAGCTTTATCGCGATTGCGTAGATGGA 3’). The resulting PCR product was cloned into the Xhol and HindIII sites of BSgptDF8L using Xhol and HindIII sites that were introduced during the PCR reaction (underlined in the primers). The fidelity of the BSUF8LgptDF8L construct was confirmed by sequencing before it was transfected into HeLa cells that had been infected with WR at an m.o.i. of 0:1 p.f.u. per cell 2±5 h earlier. Two days later progeny virus was harvested by rupturing cells by freeze–thawing and used to reinfect confluent HeLa monolayers, for amplification of progeny virus in gpt selection media (DMEM containing 10% FCS, antibiotics, 25 μg/ml mycophenolic acid, 250 μg/ml xanthine and 15 μg/ml hypoxanthine). Amplification was allowed to proceed under gpt selection conditions for 2 days before virus was harvested, serially diluted, used to infect confluent monolayers of RK-13 cells and overlayed with 0:6% agarose containing gpt selective medium. After 2 days, individual plaques were picked and again amplified on confluent monolayers of RK-13 cells for 2 days. Virus was plaque purified twice more on RK-13 monolayers under gpt selection conditions before selection was
removed. The presence or absence of the F8L gene was determined for six individual isolates by PCR, Western analysis and finally by sequencing (Fig. 3). Genomic DNA isolated from recombinant and wild-type virus as described (EMBO Practical Course Manual on Expression of Recombinant Proteins using Vaccinia Virus) was used for PCR analysis with the UF8LF and DF8LR primers. A single product of \( \sim 2000 \) bp consistent with a predicted size for the upstream and downstream F8L flanks together with the gpt gene was amplified from the recombinant isolates 1, 4 and 5 (Fig. 3a). Western analysis on the recombinants confirms that isolates 1, 4 and 5 do not express F8L (Fig. 3b). To confirm that the F8L gene had been deleted without rearrangement of flanking regions the isolate 4 was subjected to PCR with the primers DF8LR and F33FN (5’ CCC CGC GCC CGC AGG AGA GGT TAT AGG GCA GTT 3’), which primes 10 bp upstream of UF8LF. The resulting product was cloned into the SpeI and NotI sites of pBluescript SKII using the restriction sites introduced by the primers (underlined) and sequenced on both strands. The resulting sequence of isolate 4, now termed WRAF8L, shows that the F8L gene has been deleted with no other rearrangements or base pair errors. Deletion of the vaccinia virus F8L gene in WRAF8L does not affect infectivity or formation of infectious virus particles as the strain produces similar size and numbers of plaques as WR (data not shown). By indirect immunofluorescence the F8L deletion strain WRAF8L produces a similar number of actin tails that are indistinguishable from those seen in WR infections although the protein is not expressed (Fig. 3). The fact that actin tails are formed by WRAF8L suggests that IEF particles are formed, which is consistent with the plaque phenotype being identical to WR. By indirect immunofluorescence we could also detect no differences in the onset of disassembly of actin stress fibre and actin tail formation between WRAF8L and WR during infection.

It is clear that the actin cytoskeleton plays an important role in the life-cycle of a number of pathogens, including viruses and bacteria (Cudmore et al., 1997; Theriot, 1995). In the case...
of the bacterial pathogens *Listeria* and *Shigella*, it is clear that polyproline-rich sequences play an important role in the effects of these bacteria on the actin cytoskeleton (Higley & Way, 1997). The aim of this study was to establish whether the F8L protein from vaccinia virus, which shows homologies to the proline-rich repeats of ActA and iActA, was involved in the actin rearrangements induced by the virus during infection. While the role of F8L during virus infection remains to be established our results clearly show that F8L is not involved in vaccinia-induced rearrangements of actin and its deletion does not affect virus assembly or infectivity.

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References


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