The vaccinia virus F17R protein interacts with actin

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Abstract We have examined the possible role of the F17R protein in vaccinia virus-induced rearrangements of the host actin cytoskeleton. F17R is localized to vaccinia-induced actin tails late during infection. The recombinant vaccinia strain vRO11k is able to induce actin tails that are indistinguishable from controls in the absence of F17R expression. The association of vaccinia and myxoma virus F17R with the actin cytoskeleton in the absence of additional viral factors suggests a basic region in the N-terminal half of the protein is important for this interaction. A peptide corresponding to this region efficiently bundles actin filaments in vitro, confirming that the protein interacts directly with actin. Our results show F17R is not required for actin tail formation and highlight the difficulty in discriminating functional actin-binding proteins from those that associate by virtue of their basic nature.

Key words: Vaccinia virus; F17R; Actin binding

1. Introduction

For many years it has been recognized that viral transformation results in disruption of the actin cytoskeleton and the loss of actin stress fibres [1,2]. However, a number of observations suggest that many viruses are able to interact with or modify the actin cytoskeleton of the host for their own purposes [3]. Of all the viruses that interact with the host actin cytoskeleton is vaccinia virus the most studied and characterized [4–12]. Recently original morphological studies on the effects of vaccinia on the actin cytoskeleton have been ascribed a function [9]. The intracellular enveloped form of vaccinia virus is able to nucleate the polymerization of actin filaments from its membrane surface into a structure known as an actin tail [9]. Video analysis shows that viral particles move throughout the cytoplasm at a rate of ~3 μm/min propelled on the tip of intracellular actin tails [9]. Upon contact with the plasma membrane, virus particles extend from the cell on the tips of microvilli-like projections into neighbouring cells to continue their spread [9,10]. Vaccinia-induced actin tails are strikingly similar to those seen in *Listeria, Shigella* and *Rickettsia* infections [13,14], suggesting that pathogens have developed a common mechanism to exploit the actin cytoskeleton to facilitate their spread between cells [9]. In contrast to bacterial systems the viral protein required for the formation of vaccinia actin tails is currently unknown. However, during initial studies on the interaction of vaccinia virus with the actin cytoskeleton, a basic 11 kDa viral protein was identified that associated with the actin cytoskeleton when virus assembly was inhibited [6]. Using a combination of mapping and sequencing, p11 was identified as the product of the F17R open reading frame of vaccinia [15–18]. Given the association of F17R with the actin cytoskeleton, we have examined its possible role in vaccinia actin tail formation.

2. Materials and methods

2.1. Cells, vaccinia virus strains and infections

HeLa cells were infected with vaccinia virus strain WR as described previously [9]. HeLa cells were also infected with the recombinant vaccinia virus strain vRO11k, in which expression of F17R is IPTG inducible [19]. HeLa cells were infected with vRO11k at a multiplicity of infection of 0.1 plaque-forming unit per cell to ensure infection volumes were small to avoid potential carry over of IPTG from the virus stock which is maintained in 5 mM IPTG. The potential carry over of IPTG was calculated to be less than 2.5 μM during infection which is not expected to induce expression of F17R [19,20]. In addition, after infection, cells were washed extensively to remove any contaminating IPTG. In control experiments IPTG was added to the culture medium to a final concentration of 250 μM or 5 mM.

2.2. Antibodies, Western analysis and immunofluorescence

Infected HeLa cells were labelled with the anti-vaccinia C3 antibody [21], anti-p11 kDa antisera [6] or anti-F17R antibody [22]. HA-tagged mF17R was detected with anti-HA monoclonal antibody (clone 12CA5) (Boehringer Mannheim, Germany). Actin filaments were labelled with rhodamine phalloidin (Molecular Probes, Eugene, OR). For immunofluorescence analysis, cells were fixed 8 h postinfection and labelled with the indicated antibody and rhodamine phalloidin as described previously [23]. When cells were pre-extracted, to reduce the cytoplasmic background, they were washed once with PHGM (60 mM K-PIPES, pH 6.8, 25 mM HEPES, 2 mM MgAc and 1 mM EGTA) at 37°C and incubated for 2 min at room temperature in PHGM containing 0.1% Triton X-100 and 5 μM phalloidin. Extractions were also performed in PHGM containing 50–200 mM NaCl and EGTA was replaced by 1 mM $CaCl_2$. In double labeling experiments, phalloidin was replaced by rhodamine-labelled phalloidin at the same concentration. After detergent extraction, cells were washed twice for 30 s in PHGM before fixation for 10 min at room temperature in PHGM containing 3% para-formaldehyde. Fixed cells were quenched in 50 mM NH_4Cl_2 in PBS for 15 min at room temperature, blocked for 30 min at room temperature in 1% BSA in TBS and subsequently processed as described above [23]. Immunofluorescence images were recorded using the EMBL confocal microscope facility and the resulting digital images annotated and merged using the Adobe 3.0 software package.

2.3. Construction of the expression vectors CB6BSKX, CB6NHA and CB6CHA

The *Kpn*I to *Xba*I pBluescript SK II polylinker was inserted into polylinker of CB6 [24] to generate the mammalian expression vector CB6BSKX. The HA protein sequence YPYDVPDYA optimized for mammalian codon usage was inserted into CB6BSKX in the following way. pBluescript SK II was used as a PCR template together with the T3 or T7 vector primer in conjunction with a specific N- or C-terminal HA primer using the EXPAND high-fidelity TAQ polymerase system (Boehringer, Mannheim). The resulting PCR products were cloned into CB6BSKX to generate the N- and C-terminal HA-tagged vectors, CB6NHA and CB6CHA, the polylinker sequence of which were confirmed by sequencing (Fig. 1).

2.4. Construction of F17R and mF17R expression constructs

An in-frame *Kpn*I–Kozak consensus sequence was inserted immediately adjacent to the endogenous initiator MET of F17R or mF17R and a double-stop *Eco*RI site after the last codon by PCR. The PCR

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CB6BSKX

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                        BgL II Kpn I                Xba I Bam HI
CMV PROMOTER  AGATCTGGTACC BS Polylinker TCTAGAGGATCCCGGG  hGH Term and Poly A
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CB6NHA

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          BgL II Kpn I    M  Y  P  Y  D  V  P  D  Y  A   Not I  Xho I                Xba I Bam HI
PROMOTER AGATCTGGTACCACCATGTACCCCTACGACGTGCCCGACTACGCCGGCGGCCGCCTCGAG BS Polylinker TCTAGAGGATCCCGGG hGH Term
                        HA EPITOPE TAG
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CB6CHA

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          BgL II Kpn I                EcoR I  Not I   Y  P  Y  D  V  P  D  Y  A STOPS Bam HI
PROMOTER AGATCTGGTACC BS Polylinker GAATTCGCGGCCGCCTACCCCTACGACGTGCCCGACTACGCCTGATAAGGATCCCGGG hGH Term
                                                HA EPITOPE TAG
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Fig. 1. A schematic representation of the polylinker of the expression vector CB6BSKX and the HA epitope fusion vectors CB6NHA and CB6CHA. The position of the CMV promoter as well as the transcription termination and polyadenylation signals from the human growth hormone gene (hGH term) are indicated. The sequences of restriction sites flanking the pBluescript SK II-derived polylinker and the HA epitope tag are indicated. For CB6NHA the Kozak consensus, initiator ATG and the *Not*I site fusion are indicated in bold while in CB6CHA *Not*I site fusion and termination codons are highlighted in bold.

products from both reactions were cloned into the *Kpn*I–*Eco*RI sites of CB6BSKX. N-terminal HA-tagged mF17R was constructed by inserting an in-frame *Not*I site immediately adjacent to the endogenous initiator MET and a double-stop *Eco*RI site by PCR. The resulting PCR product was cloned into the *Not*I–*Eco*RI sites of CB6NHA. The expression constructs CB6F17R, CB6MF17R and CB6HAMF17R were identified by PCR and their fidelity confirmed by sequencing with vector primers prior to transfection into HeLa using a standard a calcium phosphate method.

2.5. *In vitro actin peptide bundling assays*

The peptide 6/60, corresponding to residues 6–60 of vaccinia virus F17R was synthesised and purified by reverse-phase HPLC. The fidelity of the 6/60 peptide sequence was confirmed by MASS spectrometry. Monomeric non-muscle actin obtained from Cytoskeleton (Denver, CO) was polymerized by a 10-fold dilution in F-actin buffer (10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM $MgCl_2$, 0.1 mM ATP, 0.1 mM DTT, 0.1 mM $CaCl_2$ and 1 mM azide) to a working dilution of 1 mg/ml. The 6/60 peptide was also reconstituted in F-actin buffer

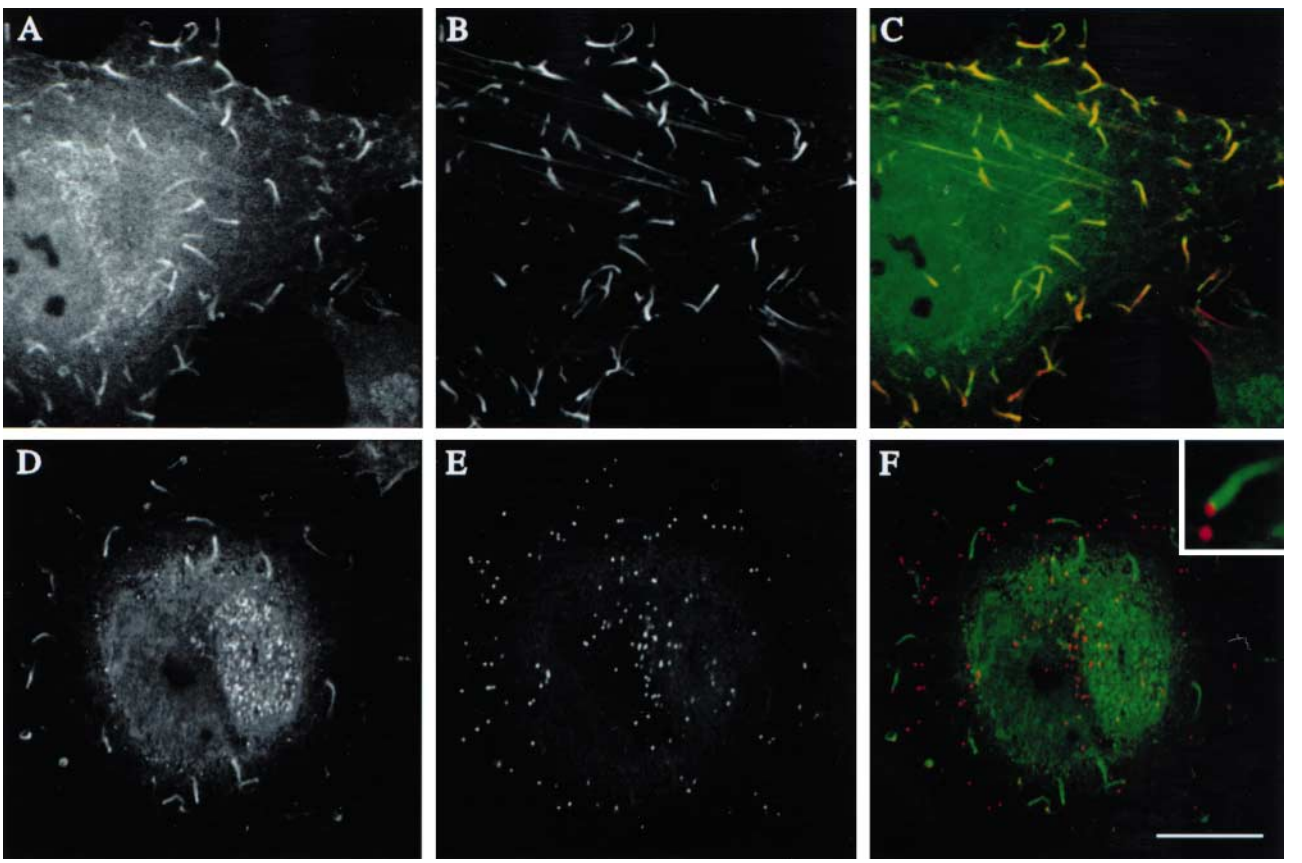

Fig. 2. F17R is localized to vaccinia-induced actin tails. A,D: Localization of F17R with anti-F17R serum; B: Actin filaments visualized by rhodamine phalloidin and E viral particles labelled with anti-P14. C,F: Merged false colour images of F17R (A,D) and F-actin (B) or P14 (E). The insert in (F) shows F17R (green) is only found in the actin tail and not on the surface of the viral particle (red). Scale bar: 20 μm.

to a stock solution of 1 mg/ml (165.6 μM). Actin filaments were diluted to a final concentration of 0.2 mg/ml (4.78 μM) in F-actin buffer containing 0.0, 5.0, 10.0, 20.0 or 40 μM 6/60 peptide. Alternatively non-muscle monomeric actin was polymerized to a final concentration of 0.2 mg/ml by direct addition to 0.0, 5.0, 10.0, 20.0 or 40 μM 6/60 peptide in F-actin buffer. All samples were incubated for 30 min at 4°C prior to absorption to a carbon-coated electron microscope grid followed by negative staining with 2% uranyl acetate. Grids were examined in a Philips 400 at an accelerating voltage of 80 kV.

3. Results

3.1. F17R is localized to vaccinia-induced actin tails but is not required for their formation

By immunofluorescence F17R is undetectable in HeLa cells using either anti-p11 or anti-F17R antibody (data not shown). In vaccinia-infected cells, F17R is found concentrated around the nucleus in viral factories as well as throughout the cytoplasm (Fig. 2). In addition, F17R is localized to vaccinia-induced actin tails in the cytoplasm as well as in projections at the plasma membrane (Fig. 2). Merged images show F17R is localized along the complete length of the actin tail and is not associated with the viral particle (Fig. 2). To address whether F17R initiates actin tail assembly, we examined the ability of the vaccinia strain vRO11k to form actin tails (Fig. 3). As previously observed, there was a severe decrease in the number of particles in the absence F17R expression as judged from anti-P14 immunofluorescence (data not shown). However, those viral particles that did form were able to induce actin tails indistinguishable from controls (Fig. 3). It was noticeable that F17R was localized to viral factories and not actin tails even when present at moderate concentrations in the cytoplasm (Fig. 3). Only in cells with high cytoplasmic concentrations does F17R associate with actin tails suggesting a critical concentration is required for the interaction (Fig. 2).

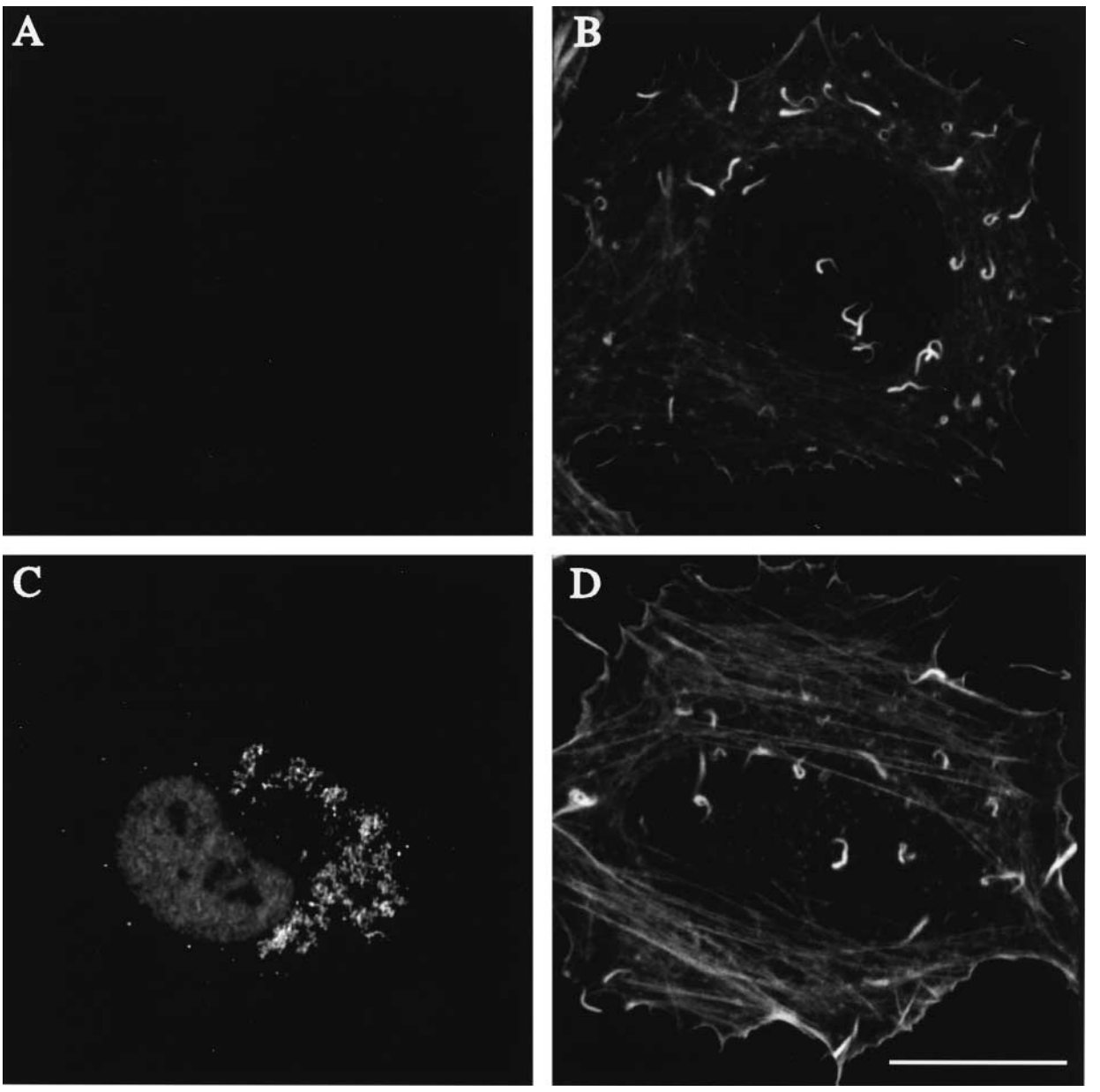

Fig. 3. F17R is not required for actin tail formation by vaccinia virus. In the absence of detectable F17R expression (A) vRO11k induces actin tails (B) in infected HeLa cells. In IPTG-induced vRO11k-infected HeLa cells, F17R is localized in the viral factory region (C) but is undetectable in actin tails (D). Scale bar: 20 μm.

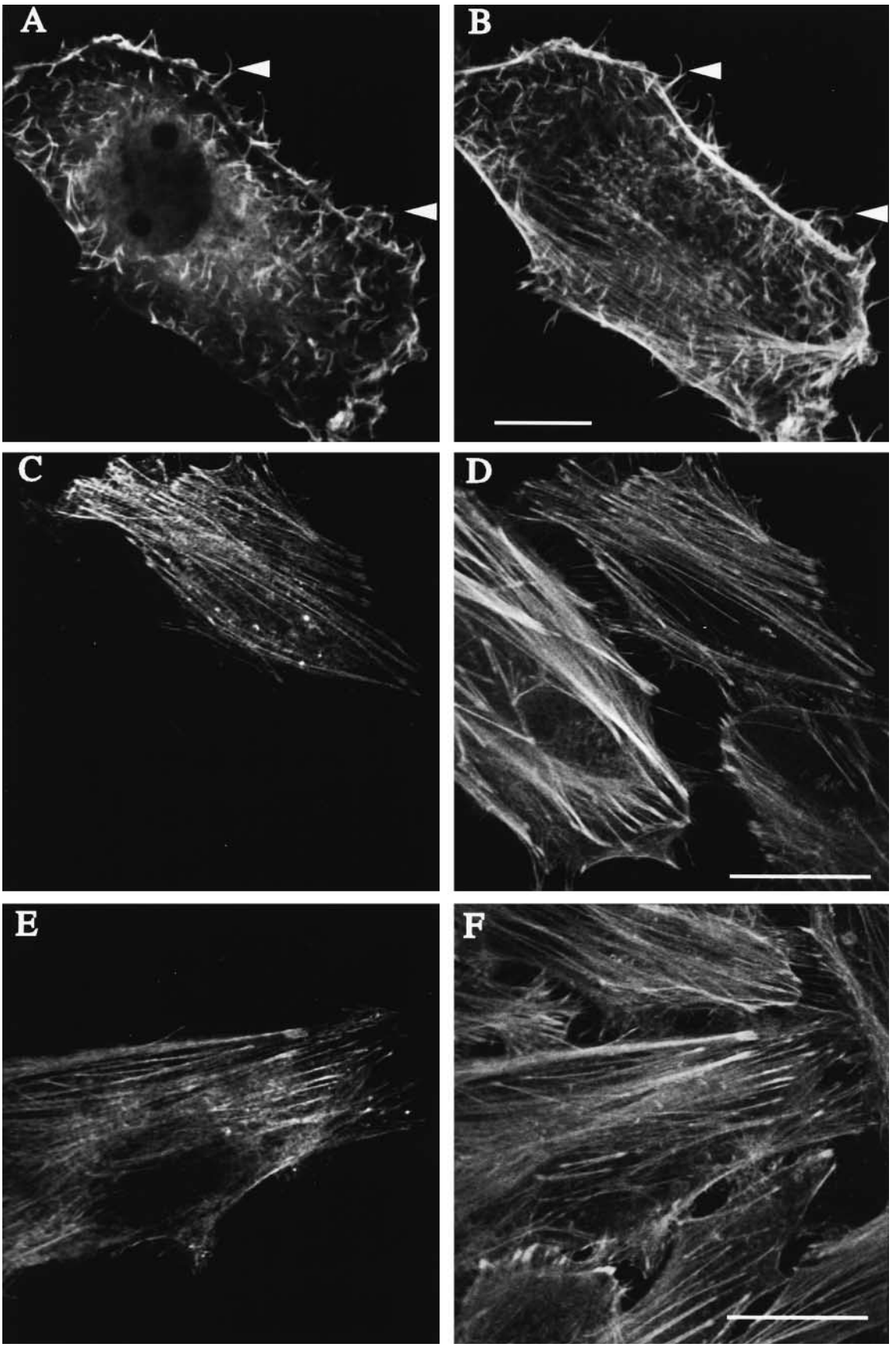

Fig. 4. When expressed in a non-viral background F17R (A) associates with the actin cytoskeleton (B) in actin-rich ruffles and microvilli at the cell surface (white arrowheads). When cells are pre-extracted F17R (C) is seen in association with actin stress fibres (D). HA-tagged mF17R (E) co-localizes with actin stress fibres (F) in an identical fashion to F17R. Scale bar: 30 μm.

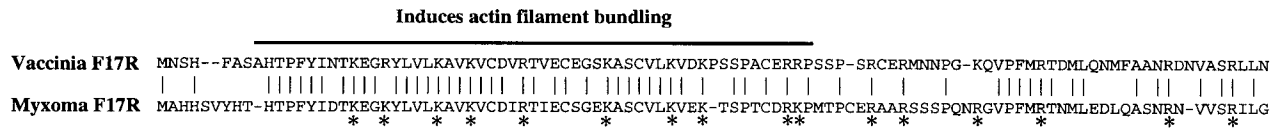

Fig. 5. Shows a sequence alignment between vaccinia virus F17R (top) and the myxoma virus F17R homologue (mF17R) (bottom). Identical residues are indicated by vertical bars, conserved basic residues are indicated by * under the alignment and the conserved region that induces actin bundles in vitro is indicated.

3.2. F17R co-localizes with actin in the absence of other viral proteins

To examine whether F17R is able to associate with actin in the absence of other viral factors, we expressed F17R in HeLa cells. Although F17R is localized throughout the cytoplasm, examination of the cell surface and leading edge showed a strong co-localization of F17R with actin in microvilli, microspikes, filopodia and membrane ruffles (Fig. 4). When cells were pre-extracted before fixation to reduce the cytoplasmic background, F17R was found co-localized with actin stress fibres and at focal adhesions (Fig. 4). F17R remains associated with the actin cytoskeleton when cells are pre-extracted in the presence of calcium or up to 50 mM NaCl but not at 100 mM NaCl. To obtain further insight into the association of F17R with actin we expressed the myxoma virus F17R homologue in HeLa cells. The sequence of the myxoma virus F17R homologue (here termed mF17R) is the most divergent F17R sequence available having only an overall ~48% identity, compared to the F17R sequence from vaccinia virus (Fig. 5). We were unable to detect expression of MF17R in transfected cells with anti-F17R which is not surprising given the sequence differences at the C-terminus (data not shown). To overcome this problem we generated an N-terminal HA-tagged mF17R construct. When expressed in cells HA-tagged mF17R is found co-localized with the actin cytoskeleton in an identical fashion to F17R (Fig. 4). The sequence divergence of MF17R and its association with actin suggests that a basic region in the N-terminal half of F17R, which has no similarity to any known actin-binding protein, is likely to be important for actin interactions (Fig. 5).

3.3. F17R bundles actin filaments in vitro

To address whether F17R binds directly to actin via the conserved basic region in the N-terminal half of the molecule we expressed F17R in *E. coli* to facilitate in vitro actin-binding assays. Although many different growth and induction conditions were examined, we were unable to obtain F17R in a soluble form. As an alternative, we synthesised a peptide 6/60 corresponding the conserved basic region in F17R and mF17R (Fig. 5). Samples of actin filaments in the absence of the 6/60 peptide were in loose tangles or single filaments and no bundles were evident (Fig. 6). Addition of the 6/60 peptide to actin filaments initially resulted in disappearance of single actin filaments and the formation of loose disordered actin bundles. At higher peptide concentrations tight bundles with no obvious order were formed (Fig. 6). This supports the suggestion that the conserved basic region in the N-terminus of F17R is responsible for the association of the protein with the actin cytoskeleton.

4. Discussion

It is clear that the actin cytoskeleton plays an important role during the life cycle of many different pathogens includ-

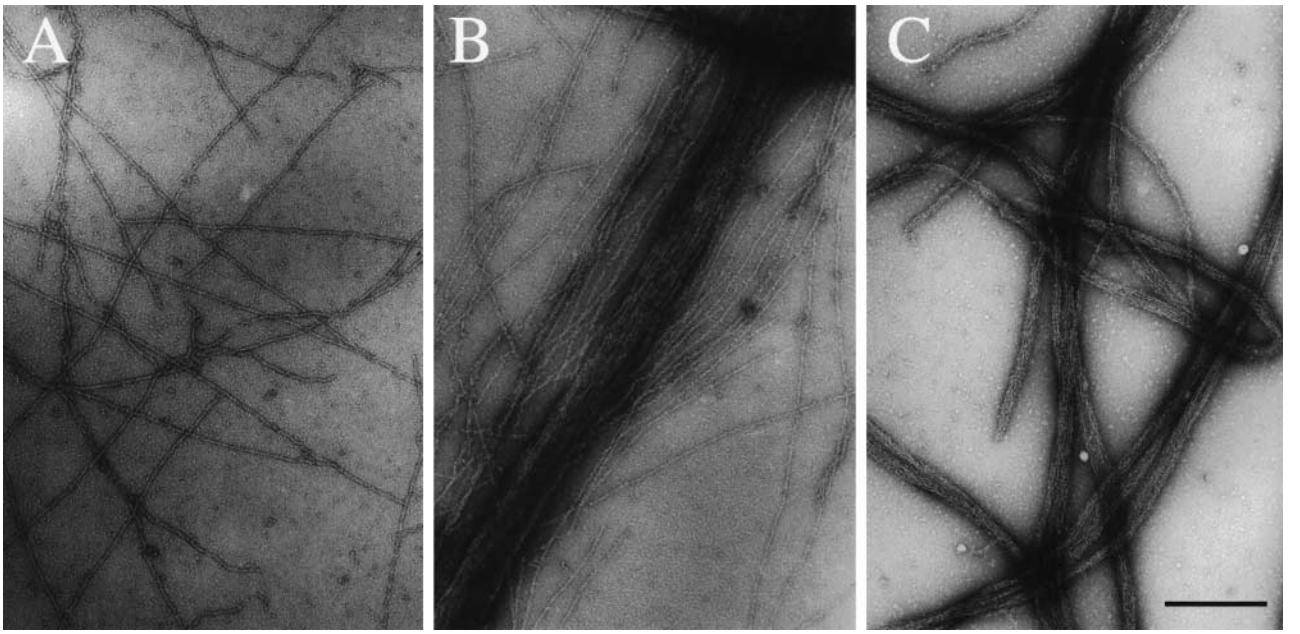

Fig. 6. The 6/60 peptide induces actin filaments to bundle. In the absence of peptide actin filaments appear as single filaments in a loose network (A). A 2:1 molar ratio of 6/60 peptide to actin results in bundle formation but isolated filaments are still visible (B). At a 4:1 molar ratio of 6/60 peptide to actin all filaments have been induced to form compact bundles (C). Scale bar: 100 nm.

ing viruses [3,14,25]. In the most extreme cases, pathogens are able to recruit host cytoskeletal factors which induce the polymerization of actin filaments from their surface into a structure known as a 'tail' [13,14]. Actin polymerization is then used as the driving force to propel the pathogen into neighbouring cells to continue the spread of infection. We have recently shown that the intracellular enveloped form of vaccinia virus is also capable of inducing actin tails that are strikingly similar to those seen in bacterial pathogens such as *Listeria* and *Shigella* [9]. However, in contrast to *Listeria* and *Shigella* the protein required to initiate tail formation is unknown in the vaccinia system. As a first step to identify viral components involved in the actin-based motility of vaccinia we have analyzed the role and actin-binding properties of F17R. Although F17R is associated with vaccinia-induced actin tails our observations with the recombinant vaccinia virus strain vRO11k clearly demonstrate that it is not required for actin tail formation. Given that the primary function of F17R is in viral particle morphogenesis, the exact nature of which remains to be established [19,20], why does the protein associate with the actin cytoskeleton?

Recently, it has been shown that basic polypeptides are able to induce the formation of actin bundles in vitro by virtue of their polyelectrolyte nature in an analogous manner to DNA condensation [26]. We believe our observations with F17R suggest a similar mechanism for its association with the actin cytoskeleton. Firstly, F17R is extremely basic with a predicted isoelectric point (p*I*) of 9.6. Secondly, based on pre-extraction of transfected cells in increasing salt concentrations that are well below physiological levels, F17R has a relatively weak interaction with the actin cytoskeleton in vivo. Consistent with this observation F17R only associates with actin tails when expressed at high levels upon induction in the recombinant vaccinia virus strain vRO11k. Thirdly, mF17R from myxoma virus, which also associates with actin, is extremely basic with a predicted p*I* of 9.1. Finally, in vitro the 6/60 peptide induces actin bundles at a peptide/actin concentration ratio comparable to the values for other positively charged peptides and not to functional actin bundling proteins such as villin and fimbrin [26,27]. The fact that the 6/60 peptide induces actin bundles which are not ordered also reflects a lack of specific contact sites and filament packing constraints that are normally seen with functional actin bundling proteins. Taken together, our observations suggest that the association of F17R with the actin cytoskeleton is a consequence of its basic nature and not through the interaction of a specific binding site. This type of interaction may nonetheless exert an effect on the actin cytoskeleton. F17R would be expected to enhance the density of actin in the tail by promoting bundling between closely opposed actin filaments. We also believe the bundling effect of F17R would account for the increase in actin-rich microvilli, microspikes, filopodia and membrane ruffles seen when the protein is transfected into cells (Fig. 4).

F17R is not the first protein to bind actin in addition to its primary role. Elongation factor EF1-α, which is also basic with a p*I* of ~9.0, bundles actin and associates with the actin cytoskeleton although it is critically required for translation [28]. While further characterization of F17R during viral infection is required to delinate its role in viral morphogenesis, it is clear that the protein plays no role in actin tail formation. Our observations with F17R highlight the difficulty in distinguishing functional actin-binding proteins from those that associate purely by virtue of their basic nature and may offer a possible explaination why so many actin-binding proteins have been identified.

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