Src Mediates a Switch from Microtubule to Actin-Based Motility of Vaccinia Virus.

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Supplementary Online Material

Materials and Methods

Cells, infection and viruses

Chicken embryo fibroblasts (CEF), HeLa, or SYF cells (1) were infected with the wild-type Western Reserve (WR) strain of vaccinia virus or the recombinant virus strains; A36R-YdF (2), A36R-Y132F (3), ΔB5R (4), B5R-P189S and B5R-SCR4 as described previously (5).

Construction of recombinant B5R viruses

A 1580 base pair fragment of genomic WR DNA, encompassing the B5R locus, was amplified by PCR using the primer pair HindIII/LA 5’gggaagcttacatcctttctgtgaagtgcact3’ and XbaI/RA 5’ggtgatggatacacttaatggtatatctagccc3’. The resulting PCR product was cloned into the HindIII and XbaI sites of pBSKII and fidelity of the resulting construct confirmed by sequencing. The Quickchange Site Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) was used to introduce the mutation P189S (CCG>TCG) into B5R to delete the sequence corresponding to the SCR1-3 domains (residues 21-185) of the protein. These constructs were transfected into ΔB5R virus infected cells and recombinant viruses encoding B5R-P189S and B5R-SCR4 isolated by successive rounds of plaque purification as previously described (2). The fidelity of the B5R-P189S and B5R-SCR4 viruses was
confirmed by PCR, sequencing as well as immunofluorescence and immunoblot analyses. The B5R-P189S virus particles was detected with anti-B5R 19C2 rat monoclonal (6) while the B5R-SCR4 virus was detected with anti-A27L (p14) (7) or anti-A36R (8) as it lacks the 19C2 epitope.

In vitro kinase and binding assays
DNA corresponding to amino acids 24-118 of A36R and A36R-Y112F were amplified by PCR and cloned in the Not I –EcoRI sites of the T7 bacterial expression vector pMW-GST. GST, GST-A36R 24-118 and GST-A36R 24-118 Y112F were produced by leaky expression overnight in BL21 (DE3) and purified from the soluble fraction according to the manufacturer’s instructions (Amersham Bioscience, Uppsala, Sweden). The resulting purified GST fusion proteins were dialysed, quantified and rebound to Glutathione sepharose beads prior to in vitro Src kinase assays which were performed according to the manufacturer’s instructions using 0.4-3.6 Units of kinase per reaction (Oncogene, SanDiego, USA). Following phosphorylation, the beads with bound GST or GST-A36R fusion proteins were incubated with soluble E. coli extracts containing His-Nck (3). The beads were subsequently incubated with cell extracts prepared from 293T cells expressing the cargo-binding region of mouse kinesin light chain 2 as a GFP fusion (GFP-TPR) (2). Immunoblot analysis was subsequently performed with anti-His (Sigma, Poole, UK), anti-P-tyr (4G10, Upstate, Milton Keynes, UK) and anti-GFP (3E1, Cancer Research UK, London, UK) antibodies.

Antibodies
Infected cells were labelled with antibodies against the IMV protein A27L (p14) (7) or the IEV proteins A36R or B5R (19C2 rat monoclonal) (6, 8). Rabbit polyclonal antibodies,
were raised against the phosphorylated peptide (EQT1pYQNTT) corresponding to residues 128-136 of A36R, that was coupled, via an N-terminal cysteine-glycine-glycine linker to Keyhole Limpet hemocyanin using the Imject activated immunogen conjugation kit (Pierce Chemical Co, Rockford, IL, USA). Phospho-specific antibodies were affinity purified from the rabbit serum on the phospho-peptide coupled via the N-terminal cysteine to SulfoLink column (Pierce Chemical Co.) after pre-clarification over a similarly coupled non-phospho-version of the peptide. Src was detected with MAb 3-27 (9) or rabbit polyclonal antisera (Sc18, Santa Cruz Biotechnology, California, USA). Cortactin was visualized with MAb 4F11 (Upstate Biotechnology, New York, USA). Phosphospecific antibodies against Src (pY418) and Cortactin (pY421) were purchased from Biosource (California, USA). Conventional kinesin was visualized with monoclonal antibodies MAB1616 (L1), MAB1617 (L2), MAB1613 (H1), MAB1614 (H2), which detect the light and heavy chains of the motor (Chemicon International, Inc. California, USA). F-actin was visualized with Alexa 488- or Alexa 568- phalloidin (Molecular Probes, Eugene, OR, USA).

Immunofluorescence, Src-GFP expression vectors and transfection.

8 hours post infection cells were processed for either immunoblot or immunofluorescence analysis as described previously (8, 10). Extracellular CEV were detected by labelling infected cells with anti-B5R antibody after fixation but prior to cell permeabilization with Triton X-100. Src-GFP, which was kindly provided by Giulio Superti-Furga (EMBL, Germany), was cloned into the BglII/NotI sites of the vaccinia promoter driven pEL expression vector (5). The “dead-open” dominant negative pEL-Src 527Kin− was previously described (5). HeLa cells were transfected with pEL-Src-GFP and pEL-Src 527Kin− 4 hours post infection and fixed 4 hours later for immunofluorescence analysis as previously described (5).
Figures and Legends

Figure S1. Schematic representation of the steps involved in Src-mediated transition between microtubule and actin-based motility.

The events occurring at the plasma membrane are represented in five steps. In the first step, IEV are delivered to the plasma membrane on microtubules in a conventional kinesin-dependent manner. Cell-associated enveloped virus (CEV) are formed on the outside of the cell when IEV fuse with the plasma membrane. During this fusion event, the outer membrane of the IEV becomes incorporated in the plasma membrane to form a specialized micro-domain beneath the CEV. In step 3, CEV particles induce a B5R-dependent outside-in signaling cascade (arrows), which results in phosphorylation and activation of Src. Once activated, Src phosphorylates A36R (arrows), resulting in the release of conventional kinesin.

In the last step, phosphorylated A36R recruits the vaccinia actin tail-nucleating complex (Grb2, Nck, WIP and N-WASP), which leads to localized activation of the Arp2/3 and actin polymerization. Premature phosphorylation of A36R at the site of IEV assembly inhibits kinesin recruitment and blocks movement of virus particles to the plasma membrane (step 1). Alternatively, inhibition of Src signaling prevents release of conventional kinesin at the plasma membrane beneath the CEV (step 4).
References and Notes


Supplementary Figure 1

- **Kinesin**
- **A36R**
- **B5R**
- **Src**
- **PO_4**
- **Arp2/3 et al**
- **Microtubules**
- **F-actin**

**Microtubule transport**

**IEV fusion**

**B5R dependent Src activation**

**Src dependent A36R phosphorylation releases kinesin**

**Src dependent A36R phosphorylation induces actin polymerisation**

- **Premature Src activation blocks kinesin recruitment**
- **Inhibition of Src signalling prevents kinesin release**