

4 Materials and Methods

4.1 Cell culture

HeLa, 143TK human osteosarcoma and BS-C-1 green monkey kidney cells were grown in minimal essential medium (MEM) (Sigma, St. Louis, MO, USA) supplemented with 10% fetal calf serum (FCS) (PAA Laboratories, Linz, Austria), 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine (Sigma) at 37°C, 5% CO₂. Cells were split regularly up to 40 passages before new stocks were thawed. All cell types used could be seeded onto untreated, autoclaved round 11 mm glass coverslips for later use in microscopy. Cells were usually not grown over 100% confluency. The nicest cells for microscopy were obtained at about 70-80% confluency. However, during some experiments, including multiple infections, 100% confluent cells were required to get enough surviving cells for Western or microscopic analysis.

4.2 Infections with vaccinia virus

Wild type vaccinia virus is referred to as WR (Western Reserve strain). Mutant viruses that lack the genes A34R, A36R, B5R, or F13L are referred to as DA34R, DA36R, DB5R and DF13L (vRB12) for simplicity (Blasco and Moss, 1992; Engelstad *et al.*, 1992; McIntosh and Smith, 1996; Parkinson and Smith, 1994). Viral stocks were prepared from 30-35 175 cm² Falcon flasks (Becton Dickinson, New Jersey, USA) containing HeLa infected at a multiplicity of infection (moi) 0.01-0.05 for 48-56 hours. Cells were scraped in 10 mM Tris/HCl pH 7.5 and centrifuged for 10 min at 4°C at 2.000 rpm in 50 ml Falcon tubes. All centrifugation steps below 6.000 rpms were performed in a Heraeus Megafuge 1.0R (Heraeus, Newtown, CT, USA) unless otherwise stated. Cell pellets were resuspended in 10 mM Tris/HCl pH 7.5 and cells were crashed with a gauge 22 needle (Becton Dickinson). Trypan blue staining revealed if all cells were broken. Post nuclear supernatant (PNS) was harvested after cell debris was rotated for 5 min at 3.000 rpm, 4°C in 50 ml Falcon tubes. This PNS was aliquoted, stocked and used for the majority of infections. For cleaner virus preparations PNS was pelleted at 24.000 rpm for 30 min, 4°C in an Beckman ultracentrifuge SW28 rotor through a 36% sucrose cushion in 10 mM Tris, pH 9.

Vaccinia virus stocks were stored at -80°C. Virus titer was determined by infecting HeLa cells with various dilutions of virus stock solution and counting the number of infected cells after labelling for immunofluorescence. 100% of infected cells are referred to as moi 5. The vast

majority of virus preparations were done by Inge Reckmann. All experiments unless otherwise stated were performed by addition of moi 5-10 of virus. Vaccinia virus stock solutions were mixed with 10% volume of 2.5 mg/ml trypsin (Worthington Biochemical Corp., Freehold, NJ, USA), incubated for 30 min at 37°C, and diluted in 9 times volume of serum-free MEM. This secondary virus stock was frozen at -20°C and used for infections after thawing by simply adding the necessary volume of virus to the medium the cells were grown in.

4.3 Preparation of IMV, IEV and EEV particles

EEV was purified from supernatants of cells infected with moi 5 for 24 hours. The highest EEV yield was obtained from confluent RK₁₃ cells infected with the vaccinia virus strain IHD-J. This virus has a single amino acid mutated in the lectin homology domain of A34R, which is thought to be responsible for efficient attachment of the virus to the cell surface (Blasco *et al.*, 1993). Two hours post infection cells were washed three times with PBS and incubated with serum-free MEM. 24 h after infection the culture supernatants were collected and cellular debris pelleted by centrifugation at 2,000 rpm, 4°C for 10 min in 50 ml Falcon tubes. The supernatant was centrifuged for 30 min at 45,000 rpm (100,000 x g), 4°C, and the virus pellet resuspended in 10 mM Tris/HCl, pH 7.5.

For IEV purification twenty 10 cm dishes of 143TK-cells were transferred into twenty 175 cm² flasks one day before infection. Cells were infected with moi 5 for 8 hours and incubated in 1 ng/ml cytochalasin D for a maximum of 30 min, washed with 10 ml of ice-cold PMKV (3 mM MgAc, 10 mM KCl, 1 mM Na₃VO₄ in PBS) scraped in 8 ml PMVK and collected in four 50 ml Falcon tubes. Tubes were fugged with 2,000 rpm (1,000 x g) at 4°C for 10 min, supernatants carefully sucked and cells taken up in 500 ml TKMV (10 mM Tris pH 9, 10 mM KCl, 3 mM MgAcetate, 1 mM Na₃VO₄). Cells were broken with a gauge 22 needle attached to a 10 ml syringe. In a 'one off' experiment I then transferred the needle into my left index finger causing some bad necrotic infection with vaccinia virus. The cell debris was centrifuged at 3000 rpm, 4°C for < 5 min. The middle phase containing most of the viral particles was transferred onto a 22-32% optiprep gradient (Gibco, Karlsruhe, Germany) and centrifuged at 24,000 rpm for 45 min, 4°C in a Beckman ultracentrifuge using a SW40 rotor. Ideally, two distinct bands of virus particles could be

obtained, the lower one consisting of pure IMV while the other was enriched in (mostly not intact) IEV. Pure IMV could also be obtained using cells infected for 48-56 h with moi 0.1.

4.4 Infections with bacterial pathogens

Listeria monocytogenes strain 10403S was a kind gift of Dr. Matt Welch, UC Berkeley and *Shigella flexneri* strains M90T, SC301 and SC570 were kind but dangerous gifts from Dr. Philippe Sansonetti (Institute Pasteur). *Shigella flexneri* strain SC301 differs from the wild type M90T as it includes an ampicillin resistance plasmid containing the gene for E.coli AFAI, a human specific adhesin which allows bacteria to adhere efficiently to human cells. Strain SC570 is modified from SC301 as it lacks the IcsA gene and includes spectomycine resistance (Dehio *et al.*, 1995). *Listeria* were stored on a brain-heart-infusion (BHI) medium (DIFCO Laboratories, Augsburg, Germany) agar plate at 4°C and one colony was incubated in 20 ml BHI-medium at room temperature over night. *Shigella* were stored in soft agar and a small scoop of agar was normally incubated in 20 ml BHI-medium at 37°C over night. Alternatively, if both bacteria were grown over night, *Shigella* were grown at room temperature, which sometimes resulted in delayed growth but did not seem to affect their infectivity. *Listeria* could not be grown at 37°C as this damaged their flagella, which are needed for efficient infection of mammalian cells. 200 ml of the bacterial over night culture was spun at 14.000 rpm in a table top centrifuge (Eppendorf, Engeldorf, Germany) for 10 seconds, washed twice in PBS before taken up in 500 ml PBS.

Mammalian cells were washed three times in serum free and antibiotic free medium before bacteria were added. Of the 500 ml PBS-bacteria solution 1 ml was used to infect cells on one 11 mm coverslip with *Shigella* strains SC301 or SC570 while 50 ml were used when infecting with *Shigella* strain M90T or *Listeria*. In the latter cases cells were monitored every half hour as to see if they show apoptotic blebs. As soon as these could be detected, the latest after 2 hours, the medium was replaced with one containing 5-15 µg/ml Gentamycin (Sigma). Gentamycin kills extracellular bacteria but does not cross the plasma membrane leaving intracellular bacteria unharmed. All plastic that came in contact with bacteria was washed in concentrated Lysoformin (Lysoform, Berlin, Germany) before being disposed as S2 garbage. Erlenmeyer flasks were incubated in concentrated Lysoformin over night before being sprayed with ethanol and disposed for reuse.

4.5 Immunofluorescence analysis

Cells were fixed with either methanol (-20°C) for 1 min or with 3% paraformaldehyde (PFA) (Merck, Darmstadt, Germany) in cytoskeletal buffer (CB) (10 mM Mes pH 6.1, 150 mM NaCl, 5 mM EGTA, 5 mM MgCl₂, 5 mM glucose) for 10 min at room temperature. Brains, livers, spleens and hearts were sometimes fixed with Margaritas or destroyed by B52s (Wittmann, 1999 and Pavel Tomancak and Jamie White, personal experience). PFA-fixed cells were permeabilized for 2 min with CB containing 0.1% Triton X-100 (Sigma). For other fixation methods that were used less commonly see chapter 4.5.1. Non-specific antibody binding sites were blocked with blocking buffer (TBS (20 mM Tris/HCl pH 7.5, 154 mM NaCl, 2 mM EDTA, 2 mM MgCl₂) containing 2% FCS and 1% BSA (Sigma)) for 5 min. Subsequently cells were labelled with a combination of primary antibodies diluted in blocking buffer for 30 min. Cells were then washed three times with TBS and incubated with the appropriate secondary antibodies coupled to fluorescein isothiocyanate (FITC), Texas Red sulfonyl chloride (TR) or 7-amino-4-methylcoumarin-3-acetic acid (AMCA), all obtained from Jackson Laboratories (West Grove, PA, USA). All incubations were carried out at room temperature. F-Actin was visualized with Alexa-488-phalloidin or rhodamine phalloidin (Molecular Probes, Eugene, OR, USA). Alternatively actin was stained with the mouse monoclonal antibody AC-74 (Sigma) when cells were fixed in methanol. Cells were washed three times with TBS and once with distilled water and mounted in MOWIOL (Calbiochem, Bad Soden, Germany) supplemented with 2.5% diazabicyclo[2.2.2]octane (DABCO) (Sigma). Alternatively, MOWIOL was mixed with 1/10 of p-phenylenediamine (Sigma) just before use. Host cell, viral and/or bacterial DNA was visualized by incubating the cells for at least 10 seconds before the final wash in distilled water containing 1 µg/ml 4', 6-diamidino-2'-phenylidole dihydrochloride (DAPI) (Roche Diagnostics, Mannheim, Germany). Mounted coverslips were dried at 37°C for at least 30 min before immunofluorescence analysis.

Images were recorded on a cooled CCD camera with the OpenLab Software (Improvision, Coventry, UK) attached to an inverted Zeiss microscope (Oberkochen, Germany) or using a DMRXA microscope (Leica, Bensheim, Germany) with a high-performance charge-coupled digital camera (Cohu, San Diego, CA, USA or for DM 399.99 at the local Aldi) and NIH image (version 1.62). Weak fluorescence staining was recorded by integrating on chip up to 15 single frames and

averaging 20-30 thus acquired images. Images were processed and annotated with the Adobe software package (Adobe Systems Incorporated, San Jose, CA, USA).

4.5.1 Fixation methods

A number of fixation methods are in common use for immuno-localization studies. The most widely used fixation method is treatment with formaldehyde, freshly prepared from paraformaldehyde (PFA). I used 3% PFA in CB as the standard fixation. However, some epitopes of proteins were not exposed during fixation with PFA and required a different fixation method. Alternative to PFA I used methanol. While cells were fixed in PFA for 10 min at room temperature, I fixed cells in -20°C cold methanol for 0.5 to 5 min. For some antibodies or double labelling experiments it turned out best to use a mix of both fixatives: 2 min prefixation with PFA followed by 1 min of methanol. I also experimented with various mixtures of PFA and glutaraldehyde (GA), some of which included Triton during fixation e.g. 3% PFA, 0.1% Triton or 3% PFA, 0.1% GA, 0.3% Triton or just 1% GA followed by permeabilization with 0.1% Triton. Of these only the PFA-Triton mix gave nice stainings, but one should try out many different fixation protocols before assigning an antibody as "not working for immunofluorescence analysis".

4.6 Video microscopy

For time lapse digital video imaging coverslips of infected cells were placed in an aluminium chamber slide that was fitted for 11 mm coverslips (White *et al.*, 1999). The pH of the medium was adjusted by addition of 10-20 mM HEPES, pH 7.4. Cells were heated to 37°C with an objective heater (Bioptechs, Butler, PA, USA). Images were recorded either on the microscopes described above using NIH image software or with the compact confocal camera on an inverted Zeiss microscope as in White *et al.*, 1999.

4.7 Cell extracts

Infected or uninfected HeLa cells were washed with cold PBS containing 1 mM Na₃VO₄ (PBS-VO), scraped in the same buffer, and centrifuged for 10 min at 2.000 rpm, 4°C. For experiments with "over-stressed" cells, like multiple infections or transfection-infection experiments, cells were not washed, but the medium was simply replaced by ice-cold PBS-VO before scraping. After

centrifugation cells were resuspended in SDS page sample buffer (50% glycerol, 3% SDS, 10% 0.5 M Tris pH 6.9, 2% beta-mercaptoethanol (Sigma) and 35% water mixed with 20% solid bromophenol blue (Kodak, Rochester, NY, USA)) and boiled for 5-10 min. This resulted in obtaining "whole cell lysates". Alternatively cell pellets were resuspended in 30-50 ml ice-cold 2x-extraction buffer (25 mM Tris/HCl pH 7.5, 1 mM EDTA, 1 mM EGTA, 100 mM NaCl, 1% Triton X-100, 0.5% Nonidet P-40) including a protease inhibitor cocktail (0.4 mM phenylmethylsulfonyl fluoride, 20 ng/ml leupeptin, 20 ng/ml chymostatin, 20 ng/ml pepstatin A and 20 ng/ml antipain) (Sigma) and extracted for 1.5 hours to overnight at 4°C. This resulted in obtaining "crude cell extracts". "Crude cell extracts" were centrifuged for 15 min at 14.000 rpm on a table top centrifuge at 4°C. Protein concentrations of the supernatants ("cell extracts") were determined using the BioRad Protein Assay (BioRad, München, Germany) with a dilution series of 1 mg/ml BSA for calibration.

4.8 Immunoprecipitation

Cell extracts of infected or uninfected cells were diluted to a protein concentration of 3-5 mg/ml, and incubated either for 1-2 hours or overnight at 4°C with affinity-purified or commercial antibodies. Protein A or protein G-sepharose beads were added to the extracts and incubated for 1-2 h or overnight at 4°C. After centrifugation at 3.000 rpm on a table top centrifuge for 1 min at 4°C, the supernatant was collected and the beads washed three times with PBS-VO. Both supernatants and beads were supplemented with equal volume of SDS-PAGE sample buffer and boiled for 5-10 min (OK, OK, sometimes over coffee and cake break) before being subjected to SDS-PAGE.

4.9 SDS-PAGE and Western blotting

Cell extracts were subjected to SDS-PAGE using either 10, 12.5, 15% or 8-15% gradient gels (Lämmli, 1970). Nine single 10% running gels were made from 16.6 ml of 30% w/v Acrylamide (National Diagnostics, Hull, UK), 18.6 ml 1M Tris pH 8.9, 0.5 ml of 10% SDS, 14 ml of water and polymerized with 40 ml N,N,N',N'-tetramethylene-diamine (TEMED) (Sigma) and 0.25 ml 10% ammonium persulfate (APS). Single mini gels were run at 6-12 W power in running buffer (1 l 10x stock: 10 g SDS, 30 g Trizma Base (Sigma), 144 g Glycine (Merck)) and then subjected to semi-dry blotting onto nitrocellulose membranes for 1 h at 150 mA (Kyhse-Andersen, 1984). The membranes were then incubated for 1-3 minutes in 3% Ponceau solution (Serva, Heidelberg,

Germany) and washed in distilled water to reveal protein loading. Western blots were first incubated with PBS containing 0.1% Tween-20 (PBS-T) and 5% non-fat dry milk (Blotto) for 10 min at room temperature and then with the appropriate primary antibody diluted in Blotto for 1 hour at room temperature or overnight at 4°C. Subsequently, blots were washed three times with Blotto, incubated for 45-60 min at room temperature with the appropriate secondary antibody coupled to horseradish peroxidase (HRP) (Biorad), and washed at least 5 times with PBS-T for at least 30 min. Western blots were developed using the ECL system according to the manufacturer's instructions (Amersham International, Braunschweig, Germany). Blots could be stripped by incubation in strip buffer (2% SDS and 0.06 M Tris/HCl, pH 6.8 supplemented with 0.78 ml β-mercaptoethanol) for 45 min at 50°C and subsequently reprobed after extensive washing in PBS-T. America's finest strawberry flavoured marshmallow fluff was applied on facial blots and occasionally overlaid with America's finest chocolate fudge brownie mix (see Figure 42+1). Both could be removed by extensive washing with cold or warm water (Figure 42+1).

4.10 Two-dimensional gel electrophoresis

Cell extracts (< 50 µl, < 50 ng total protein) were incubated with an equal volume of IEF (isoelectric focusing) sample buffer (9.8 M urea, 0.8% ampholines LKB 7-9, 0.4 % NP-40, 100 mM dithiotreitol (DTT) (Sigma)) for 15-30 min at 37°C before loading onto IEF gel tubes. "Acidic" gel tubes were prepared from 2.87 g urea (Gibco), 0.67 ml acrylamide stock (4.26 g acrylamide, 0.24 g Bis (N,N'-Methylene-bis-acrylamide) (Biorad) and 14.5 ml water), 0.88 water, 0.139 ml Serva ampholine (Serva), 0.139 ml LKB 5-7 (Serva), 0.101 ml LKB 3.5-10, 1.01 ml 10% NP-40, 8 ml TEMED and 8 ml 10% APS. "Basic" gel tubes were prepared from the same mix using Serva 6-8 instead of Serva 5-7 and LKB 7-9 instead of LKB 5-7. Loaded samples were overlaid with "overlay" buffer (8 M urea, 0.4% ampholines 7-9, 0.5% NP-40, 10 mM DTT) before running at 400 V for 15 min followed by 750 V for 3.5 hours between an upper buffer containing extensively degassed 20 mM NaOH (Merck, Darmstadt, Germany) and a lower buffer containing 10 mM orthophosphoric acid (Merck). IEF worms were soaked for 10 min in 150 µl 2x Lämmli buffer (100 mM Tris-HCl pH6.8, 5 mM EDTA, 100 mM DTT, 4% SDS and 10% (w/v) glycerol). After complete removal of the buffer IEF worms were either frozen at -20°C or directly applied onto an SDS-PAGE gel for electrophoresis of the 2nd dimension.

4.11 Micro-injection

HeLa cells infected for 8-14 hours with vaccinia virus or for 6 hours with *Listeria* were micro-injected using the Zeiss automated injection system (Oberkochen, Germany). During micro-injection cells were kept either at room temperature or at 37°C and the medium was supplemented with 10-20 mM HEPES. For the inhibition of actin tail experiment, cells were injected with antibodies at a concentration of 5 mg/ml in micro-injection buffer (100 mM KCl, 5 mM Na-phosphate, pH 7.5). For the experiment revealing the topology of A36R 12-14 h infected cells were micro-injected with diluted antibody (below 0.1 mg/ml) in micro-injection buffer. Antibodies used for micro-injection were dialyzed into micro-injection buffer (100 mM KCl, 5 mM Na-phosphate, pH 7.5) using Amicon micro-concentrators (Amicon, Witten, Germany). In both cases, cells were processed for immunofluorescence after 1 h recovery at 37°C. Infected cells were identified by labelling with anti-A17L and injected cells by labelling with the respective secondary antibody. In the initial stages of the 'inhibition of actin tails experiment' injected cells were identified by co-injected Texas Red labelled 70 kDa dextran (Molecular Probes, USA).

4.12 Quantification

For each experiment the presence of a single actin tail was scored in injected or transfected versus non injected/transfected cells. The presence of a single actin tail within a cell was scored as positive as it is impossible to quantify a reduction in actin tails as their numbers naturally vary greatly between cells. In one infection the actual actin tail number was determined for 30 cells. The standard deviation from the average numbers of tails per cell was higher than the actual value of tails per cell (7.5 +/- 7.7 of 30 counted cells at 8 hpi with a range from 0 to 28 tails per cell). I am confident that my way of quantification is a stringent approach, as in many experiments only very few cells exhibited only one actin tail, which turned the statistics against slight effects. For example: Of 10 infected control cells all had between 2 and 30 tails averaging 15 tails per cell and of 10 transfected cells in the same field of view 2 cells had each one actin tail. With my method of counting I would score 20% of the control value while counting all tails would give me an effect of 1.3%. The data were normalized to the number of infected cells that were not injected/transfected but showed actin tails. All experiments showing standard deviations from the mean were at least performed three times independently from each other.

4.13 Cloning of pE/L-constructs

A36R expression was placed under the control of the synthetic vaccinia virus early/late promoter (E/L) (Chakrabarti *et al.*, 1997) by PCR using the primers PELA36R For 5' CCGCCGCTCGAGAAAAATTGAAATTTTATTTTTTTTTTTTGGGAATATAAATAAGCTC GAGATCTACCATGATGC T G G T A C C T C T T A T C 3' and A36RH3 Rev 5' GGCCAAGCTTATCACACCAATGATACGACCGATGATTCTAT 3'. The pE/L promotor (Chakrabarti, 1997) is shown in bold while the ATG codons for the first two methionines in the A36R sequence are underlined. The resulting PCR product was cloned into the Xho I - Hind III sites of a Not I deficient pBluescript SKII vector (Stratagene, La Jolla, CA, USA; Reckman *et al.*, 1997) to generate the pELA36R construct.

Eco RI- Bam HI inserts containing the c-Src mutants 527 Kin⁻ and KP Kin⁻ were cloned into the Eco RI- Bgl II sites of the pELA36R vector backbone. EGFP was amplified by PCR from the pEGFP-N1 expression vector (Clontech, Palo Alto, CA, USA) and cloned into the Bgl II- Xho I sites of pEL immediately downstream of the viral promoter. Subsequently, the DNA coding residues 1-397 of rat N-WASP was amplified by PCR and cloned into the Not I- Eco RI sites of the pELGFP vector to generate pELGFP N-WASP-DWA which lacks the WH2 and acidic domains of the protein (Machesky and Insall, 1998). The DNA corresponding to the SH2 domain, residues 275- 373, of human Nck1 as well as the full length protein was amplified by PCR from a HeLa cDNA library and cloned in an identical fashion to N-WASP-DWA into pELGFP. All cloning described in 4.12 was performed by Dr. Michael Way.

4.14 Creation of A36R point mutations

Tyrosine to phenylalanine point mutations of A36R were engineered by "round the world" PCR (Hemsley *et al.*, 1989) using pELA36R as a template. Briefly, 10 ng of pELA36R were incubated in a 100 µl PCR reaction using 1 µl 25 mM NTPs, 50 pmol of each phosphorylated primer (one of which contained the Y to F mutated base triplet in the middle) and 1 µl Taq+Precision (Stratagene, La Jolla, CA, USA) and cycled 30 times (15 sec at 92°C, 15 sec at 55 °C and 4 min at 72°C) after 1 min at 92°C. The reaction was allowed to finish with 7 min at 72°C before the PCR product was purified over a QIAquick PCR purification column (Qiagen, Hilden, Germany). Blunt ends were created using Mung Bean nuclease (1 µl for a 50 µl reaction) at 30°C for 30-60 min. After a second

QIAquick purification the PCR product was ligated using half of the 30 µl eluate with T4 ligase (New England Biolabs (NEB), Beverly, MA, USA) for several hours at room temperature. The 15 µl ligation reaction was expanded to 50 µl for digestion with 2 µl DpnI (NEB) at 37°C for 1-2 hours to remove the methylated template DNA. The pELA36R-YdF construct was engineered in an identical fashion using A36R-Y112F as the template. The fidelity of all pEL constructs used in this thesis was confirmed by sequencing on both strands. All sequencing was performed by the EMBL sequencing service.

4.15 Transfections and "Rescue" assay

DNA was transfected using the calcium phosphate precipitation method. For seven 11 mm coverslips or cells grown in a 3.5 cm diameter culture dish, 0.5 µg of Qiagen midi prep DNA was mixed in a total of 50 µl with 0.25 M CaCl₂ and incubated for about 20 min at room temperature before 50 µl of HBS (280 mM NaCl, 10 mM KCl, 1.5 mM Na₂HPO₄, 12 mM Glucose, 50 mM HEPES, pH 7.05) was added and incubated for another 10-15 min before the mix was put into the medium over the cells. 12-20 hours later cells were washed twice for a total of 15 min in PBS and cells were recovered for various times in MEM. For transfection infection experiments cells were transfected with calcium phosphate over night, recovered for 2-24 hours and infected with the respective pathogen. For infection transfection experiments, cells were infected with vaccinia virus and subsequently transfected with lipofection (Gibco) (1 µg of DNA per coverslip). For assaying the effect on or localization of a protein to vaccinia actin tails infected cells were transfected 4 hpi and fixed 8 hpi. For assaying the effect on or localization of a protein to actin tails of *Shigella* actin tails, vaccinia DA36R infected cells were transfected 1 hpi and super-infected 2-4 hours later with *Shigella* strains expressing AFAI (SC301, SC570) and fixed 4 -5 hours after the super-infection.

4.16 Expression of proteins

Human Nck1 was cloned from pELGFP into the vector pMW172, expressed in BL21(DE3) and the soluble fraction prepared as described previously (Way *et al.*, 1992). The tyrosine phosphorylated and unphosphorylated versions of the peptide CGGAPSTEHIYDSVAGST, corresponding to residues 105-116 of A36R, were coupled via the N-terminal cysteine residue to Sulfolink resin (Pierce, Rockford, USA). The BL21(DE3) soluble fraction containing Nck1 was incubated with

peptide resin in the presence of 1 mM vanadate at room temperature for 30 minutes and washed 5 times with PBS containing 100 mM NaCl, 0.1% Tween and 1 mM vanadate. The washed resin was then incubated with cell extracts prepared as described in 4.7. The peptide was synthesized by Genosys (Sigma), Nck1 cloned, expressed and the peptide crosslinked by Dr. Michael Way.

4.17 Transformation of bacteria

For preparation of calcium competent cells 1 ml of a 5 ml over night culture of *E. coli* strain DH-5a was grown in 100 ml LB to an OD₆₀₀ of about 0.4 -0.8. Cells were fugged at 3000 g, 4°C for 5 min and 50 ml of ice-cold 0.1 M CaCl₂-buffer was added to the pellet and cells left for 30 min to several hours on ice. After centrifugation cell pellets were taken up in 0.1 M CaCl₂-buffer and the now competent cells were stored at 4°C for up to 2 weeks. For transformation half of the respective ligation reaction was mixed with 100 µl of competent cells, stored for 5 min on ice and plated onto one or two prewarmed (37°C) agar plates containing the respective antibiotic (Pope and Kent, 1996).

4.18 DNA purification

For miniprep DNA purification picked colonies were grown over night in 3 ml LB containing the appropriate antibiotic. 1.5 ml of the over night culture was poured into eppendorf reaction tubes and cells pelleted at 14.000 rpm for 1 min on a table top centrifuge. Supernatants were poured off, cells frozen over coffee or lunch at -20°C, thawed and vortexed in a mix of 5 µl preboiled RNase A (10 mg/ml in water, heated to 80°C for 15 min to inactivate DNase) and 150 µl of SET (20% Sucrose, 50 mM Tris pH 8.0, 50 mM EDTA). 350 µl of Alkali Lysis solution (0.2 M NaOH, 1 % SDS) was added and the tubes inverted a few times until the solution became clear. Tubes were placed on ice for 4 min, 250 µl ice-cold 3M Na-Acetate pH 4.8 was added, tubes inverted for a few times and again incubated for 4 min on ice before pelleting at 14.000 rpm. The supernatant was transferred to another tube which was subsequently filled with isopropanol. Again tubes were inverted and the DNA was pelleted for 5 min at 14.000 rpm. The supernatant was poured off and 70% ethanol (-20°C) was added to the pellets. Again tubes were inverted a few times and the DNA was pelleted at 14.000 rpm. Ethanol was decanted and fully removed by vacuum centrifugation for 10-15 min and DNA resuspended in 30 µl TE (10 mM Tris pH 8.0, 0.1 mM EDTA). 2-4 µl of this

DNA was used for digestion using 0.5 ml of restriction enzymes in 10 ml total volume. DNA was resolved by electrophoresis in 1% agarose gels in TBE containing 1 ml 10 ng/ml ethidium bromide (Sigma). Larger quantities of pure DNA for transfection purposes were purified from 100 ml overnight cultures using the Qiagen Midi-prep kit according to the manufacture's description (Qiagen). DNA concentration was measured using the Pharmacia GeneQuant II (Pharmacia, Uppsala, Sweden).

4.19 Antibodies used in this study

Commercially available antibodies were purchased from the following companies: Actin AC-40 (Sigma), actin AC-74 (Sigma), a-actinin (Sigma), cortactin (Upstate Biotechnology, Lake Placid, N.Y., USA), ezrin (Transduction Laboratories, Lexington, Kentucky, USA), Fyn (Transduction Labs), FAK (Upstate and Transduction Labs), Grb2 (Upstate, Santa Cruz and Transduction Labs), gelsolin (Sigma), Lyn (Transduction Labs), Nck (Transduction Labs and Upstate), phosphotyrosine (Upstate, Transduction Labs and Santa Cruz), PI3-kinase (Upstate), paxillin (Transduction Labs), tensin (Transduction Labs), talin (Sigma), VASP (Transduction Labs), vinculin (Sigma), Yes (Transduction Labs), zyxin (Transduction Labs).

Several colleagues provided us generously with antibodies and are therefore enthusiastically acknowledged; if you ever happen to read this thesis I do owe you a beer, and not just an old papercup but a glass of beer: Matt Welch (Arp3), Dorothy Schafer (CapZ), Tom Parsons (Cortactin), Mary Beckerle (Cysteine-rich protein, Talin and Zyxin), Tony Bretcher (Ezrin), Frank Gertler (Mena), Evelyn Friedrich (LPP), Roger Carlsson and Tom Pollard (Profilin), Stefania Gonlfonti and Giulio Superti-Furga (Src), Lan Bo Chen (Tensin), Mario Gimona (Vinculin), Matthias Krause and Jürgen Wehland (Zyxin) and Jacomine Krijnse-Locker for various viral antibodies directed against a protein (A17L) that also becomes phosphorylated during infection, but well, we got scooped on it, bummer (Betakova *et al.*, 1999; Derrien *et al.*, 1999)

Rabbit antisera against the IEV specific proteins A33R, A34R and A36R were produced by Sabine Röttger (read this and get a glass of nice red wine).

Dilutions of working antibodies used for this thesis

Antibody	Western blotting	Immunofluorescence	preferred fixation method
A33R	1:1000	1:50	Methanol
A34R	1:2500	1:10	Methanol
A36R	1:5000	1:750	Methanol
Actin AC-40	1:1000	1:50	Methanol
Actin AC-74	1:1000	1:50	Methanol
a-actinin	1:2000	1:200	PFA
Arp3	1:1000	1:50	Methanol
CapZ (5B12)	1:300	1:100	Methanol
Cortactin (p80/85)	1:2000	1:200	PFA
Ezrin	1:500	1:50	PFA
FAK	1:500	1:50	PFA
Gelsolin	1:1000	1:500	PFA
LPP	1:200	1:5	PFA
Mena	1:500	1:50	PFA
Nck	1:2000	1:100	PFA/Triton
P-Tyr (4G10)	1:1000	1:100	PFA
Paxillin	1:5000	1:1000	PFA
c-Src (327)	1:2000	1:200	PFA
Tensin	1:3000	1:100	PFA
Talin (B11)		1:1000	PFA
VASP	1:500	1:50	PFA
Vinculin	1:1000	1:400	PFA
Zyxin (B38)	1:1000	1:500	PFA