## 2 Results

### 2.1 Localization of focal adhesion and actin binding proteins to actin tails

In uninfected cells VASP is found at focal adhesions. In vaccinia infected cells, VASP is found at the tip of actin tails induced by vaccinia virus as well as throughout the tails (Figure 9, upper panels). In contrast to vaccinia virus, VASP was recruited to *Listeria* but not found in the bacterial induced actin tails (Figure 9, middle panels). VASP was localized throughout *Shigella* actin tails and at the site of actin tail nucleation (Figure 9, lower panels). The observations with *Listeria* and *Shigella* confirm previously reported results (Chakraborty et al., 1995).

Interestingly, in cells infected with either vaccinia virus or *Listeria* VASP localization to focal adhesions was reduced, although only in vaccinia infected cells a clear decrease of stress fibers was detectable (Figure 9). Are focal adhesions disassembled during infections with vaccinia or are only the components needed for the actin-based motility recruited from these complexes? Preliminary studies did not yield conclusive answers to this question, as it is not clear to what level proteins like vinculin and paxillin are still associated with focal adhesion in vaccinia infected cells. VASP staining at focal adhesions was also reduced when cells were infected with vaccinia virus strains that were not able to form actin tails (data not shown). It is clear that vaccinia recruits proteins from focal adhesion, but a definite study investigating the effects of vaccinia infection on focal adhesion complexes is still missing. Interestingly, the focal adhesion protein zyxin is recruited from focal adhesions and accumulates in the nucleus upon infection with vaccinia.

The localization of a number of actin cytoskeleton associated proteins was described for vaccinia, as well as for *Listeria* and *Shigella* induced actin tails (Table 2, Figures 10, 11 and 38). In addition, during these studies caldesmon, filamin, fimbrin, gelsolin, Mena and tropomyosin were localized to vaccinia induced actin tails, while cofilin, CapG, CapZ, focal adhesion kinase, myosin II and spectrin were not recruited. I did not stain *Listeria* or *Shigella* infected cells with antibodies against these proteins, but it would be interesting to repeat at least the staining with the CapZ and cofilin antibodies in *Listeria* and *Shigella* infected cells, as both CapZ and cofilin were reported to be found in both *Listeria* and *Shigella* induced actin tails and to be essential for actin-based motility of these bacteria *in vitro* (David *et al*., 1998; Loisel *et al*., 1999)

Table 2: Localization of proteins to actin tails of pathogens

| | Vaccinia | *Listeria* | *Shigella* |
|---|---|---|---|
| α-actinin | T | B+T | B+T |
| Arp3 | T | B+T | B+T |
| Cortactin | T | B+T | B+T |
| Ezrin | P | P | P |
| N-WASP | V | - | B |
| Paxillin | - | - | - |
| P-Tyr | V | - | - |
| VASP | V+T* | B | B+T |
| Vinculin | - | - | B+T* |
| Zyxin | - | B+T# | B+T# |

T indicates localization to actin tails, B to bacteria, V to viral particles; * indicates very weak staining, # indicated variable staining meaning that in some cells actin tails are labelled while in others they are not, "-" stands for no staining to tails or pathogens. This variation is consistent as several antibodies gave the same result. P-Tyr stands for proteins phosphorylated on tyrosine residues.

The most striking difference to emerge from the comparative immunofluorescence study was the presence of a phosphotyrosine labelling at the site of vaccinia actin tail assembly which was absent in *Listeria* and *Shigella* induced actin tails (Figure 11). To investigate whether the protein(s) underlying this signal play(s) an active role in actin tail formation I mirco-injected anti-phosphotyrosine antibodies into cells infected with vaccinia. As a control I also injected *Listeria* infected cells. As shown in Figure 12 and discussed in chapter 4.12, micro-injection of anti-phosphotyrosine antibodies into cells infected with vaccinia caused a severe decrease in the numbers of cells showing actin tails. However, micro-injecting anti-phosphotyrosine antibodies into *Listeria* infected cells did not affect the ability of the bacteria to induce actin tails (Figure 12). Likewise, injection of anti-IgG antibodies into cells infected with vaccinia virus did not inhibit actin tail formation (Figure 12). These observations suggest that the phosphotyrosine protein(s) plays an important role in actin tail formation of vaccinia. Is this unknown phosphotyrosine protein of viral origin or a host protein?

**2.2 The role of IEV proteins in vaccinia actin tail formation**

As it is the IEV that makes actin tails, a logical conclusion would be that an IEV specific protein should be responsible for actin tail formation. To investigate the role of IEV specific proteins in vaccinia actin tail formation we obtained all available recombinant 'knockout' viruses lacking one of the genes encoding the respective IEV specific protein. I infected BS-C-1, HeLa and 143TK cells with each of the recombinant viruses and stained the actin cytoskeleton with phalloidin to investigate whether they are capable of inducing actin tails. While the absence of A56R, the viral hemagglutinin, did not affect actin tail formation, lack of A34R, A36R, B5R or F13L resulted in an absense of actin tail formation (Figures 13,14). Independent of actin tail formation viral infection still resulted in disassembly of actin stress fibers (Figure 14) and decreased focal adhesion staining of focal adhesion proteins, including VASP and vinculin (data not shown). It was previously shown that F13L was not able to form IEV (Blasco and Moss, 1992; Cudmore *et al*., 1995). The inability to form actin tails may reflect a problem with IEV morphogenesis rather than a direct role for the missing protein in actin tail formation. Careful examination of cells infected for longer periods of time (18-24 hours) revealed that viruses lacking B5R or F13L were still able to assemble low numbers of actin tails (Figure 15). This finding indicates that these proteins are not essential for the formation of actin tails, but that they play a role in the formation of IEVs. To further investigate this possibility, I infected cells with the recombinant viruses and stained with antibodies raised against IEV specific proteins (Röttger *et al*., 1999). Interestingly only viruses lacking A36R were able to induce a similar number of IEVs when compared with WR infected cells (Figure 16). Viruses lacking B5R or F13L made occasional IEVs confirming the observation of rare actin tails in cells infected with these viruses (Röttger *et al*., 1999). Similar conclusions were obtained by Sabine Röttger using electron microscopy (Röttger *et al*., 1999). Viruses lacking A34R were not able to make IEV (Figure 16) and thus actin tails (Figure 13) indicating that this protein is involved in IEV formation. In cells infected with ΔA34R, ΔB5R and ΔF13L, but not ΔA36R or ΔA56R IEV specific proteins were dispersed throughout the cell showing that the normal assembly of IEV is perturbed (Figure 16). These observations leave A36R as the most likely candidate for an IEV protein involved in actin tail formation as it is the only known IEV protein that, when deleted, results in loss of actin tails but not IEV formation (Röttger *et al*., 1999).

## 2.3 The role of A36R in vaccinia actin tail formation

### 2.3.1 A36R is tyrosine phosphorylated

To investigate the role of specific IEV proteins in actin-based motility and to identify the phosphotyrosine protein(s) I tried to purify the IEV form of vaccinia. While EEVs can easily be prepared from the medium of infected tissue culture cells and IMVs from infected cells, I was not able to obtain pure IEVs (see chapter 4.3).

Due to the lack of pure IEVs I decided to check if infection with vaccinia induces tyrosine phosphorylation of host proteins. Comparison of cell extracts obtained from uninfected cells and cells infected with vaccinia virus show that infection results in tyrosine phosphorylation of three or four proteins of approximate molecular mass 50, 80/85, 110 and 200 (Figure 17). Those proteins were termed pTyr50, pTyr80, pTyr110 and pTyr200. In contrast to pTyr50, pTyr80 and pTyr200, pTyr110 was sometimes already phosphorylated in uninfected cells (compare figure 17 and figures 36, 39). Analysis of cell extracts prepared from cells infected with recombinant viruses lacking IEV specific proteins showed that pTyr50 but no other phosphotyrosine protein was specifically absent in cells infected with ΔA36R (Figure 17). Western analysis of two dimensional gels and immunoprecipitation experiments showed that pTyr50 and A36R are the same protein (Figure 18). There is only a small overlap of the signal obtained when the 2-D blot was probed with anti-phosphotyrosine antibody or with anti-A36R antibody (Figure 18). Interestingly, there is also a higher molecular weight band detectable with both antibodies indicating that A36R might form a dimer (Figure 18). These data and the small amount of A36R retained in the immunoprecipitate with anti-phosphotyrosine antibody show that only part of the A36R pool is phosphorylated (Figure 18). Strangely, the previously reported topology of A36R predicted the protein to have only two cytoplasmic residues, both methionines (Parkinson and Smith, 1994). This was not consistent with the fact that A36R is phosphorylated as so far phosphorylation in the lumen of intracellular membrane cisterna has not been reported. It is therefore clear that the previously reported topology of A36R was 'in conflict with my data' and most likely wrong.

Micro-injection of antibodies raised against the predicted lumenal domain of A36R readily recognized the protein localized to the perinuclear region of the Golgi and to IEV that nucleated actin tails (Figure 19). A similar localization was observed with micro-injection of an antibody raised against the cytoplasmic domain of A33R, while antibodies raised against the lumenal

domains of A33R or A34R did not show any localized staining, but dispersed throughout the cytoplasm (Figure 19). These results and more experiments performed by Sabine Röttger including pre-embedding immunolabelling followed by electron microscopy and protease digests of purified EEV demonstrated that A36R is a type 1b membrane protein with a cytoplasmic tail of about 200 amino acid residues (Figure 20 and table 3) (Röttger *et al.*, 1999) .

Table 3 Role of IEV specific proteins in IEV and actin tail formation

| IEV protein | A33R | A34R | A36R | A56R | F13L | B5R |
|---|---|---|---|---|---|---|
| role in IEV formation | yes | yes | no | no | yes | yes |
| ...actin tail formation | no | no | yes | no | no | no |

Compare the summarized data here with table 1 and note the difference between IEV and EEV. We able to show direct involvement of IEV proteins in IEV and actin tail formation only due to the availability of good antibodies against IEV specific proteins were.

### 2.3.2 Phosphorylation of A36R mediates actin tail formation

To study the role of A36R phosphorylation in the actin-based motility of vaccinia one could make a series of recombinant viruses expressing partially deleted or point mutants of A36R. However, as this would be a time-consuming approach we looked for something faster. As vaccinia replicates in the cytoplasm, we first tested whether transfection of A36R would lead to sufficient ectopic expression of A36R in ΔA36R infected cells and if this would rescue actin tail formation. To this end I used a modified pBluescript vector containing the A36R gene under the control of a viral early late promotor (pE/L) (Chakrabarti *et al.*, 1997) (Figure 21). Ectopic expression of A36R resulted in efficient rescue of actin tail formation (Figure 22). In 5 independent experiments the number of transfected cells showing actin tails was 111±36 % compared to non-transfected control cells. Ectopic expression of A36R also resulted in over expression and reappearance of the pTyr50 signal and did not affect phosphorylation of pTyr80 or pTyr200 (Figure 23). I wanted to see which tyrosine is phosphorylated and if this phosphorylation is important for actin tail formation. Therefore I made a series of A36R point mutants where each of the six tyrosine residues in the A36R cytoplasmic domain was individually changed into phenylalanine, which corresponds to the least local change of the protein structure. Ectopic expression of these constructs in cells infected

with ΔA36R showed that mutation of either tyrosine 112 or 132 reduced A36R phosphorylation as judged by Western analysis, suggesting that both of these residues are phosphorylated (Figure 24). I continued to make the double point mutant YdF in which both residues 112 and 132 were changed to phenylalanine. Although expression of A36R-YdF was comparable to the other point mutants, I found no evidence for tyrosine phosphorylation of A36R-YdF (Figure 24). The mobility of A36R-YdF on SDS-PAGE, as detected by Western blotting was slightly faster than the mobility of A36R wild type protein or of the proteins bearing single point mutations (Figure 24). This observation is consistent with an absence of phosphorylation. Immunofluorescence analysis of ΔA36R infected cells transfected with the A36R mutants reveals that only a change of tyrosine 112 to phenylalanine resulted in a dramatic reduction of actin tails (Figure 25). However, rescue of actin tail formation completely failed when A36R-YdF was transfected into ΔA36R infected cells (Figure 25). This lack of rescue was not the consequence of mis-targetting, as A36R-YdF localized to IEV particles (Figure 26).

### 2.4 Actin based motility of vaccinia virus mimics receptor tyrosine kinase signalling

#### 2.4.1 Adaptors bind to phosphorylated A36R and recruit N-WASP

Phosphorylated tyrosine residues often mediate the binding of a protein to another protein containing a src homology 2 (SH2) domain (Songyang and Cantley, 1995). Investigation of the sequence around tyrosines 112 and 132 showed some homology to the sequences of phospho-peptides that were found to bind specifically to Nck and Grb2, respectively (Figure 27). Immunofluorescence analysis technology using antibodies and transfections of WR infected cells with GFP-Nck showed that Nck was localized to the tip of actin tails (Figure 28). The same experiments with antibodies against Grb2 showed that Grb2 was not localized to actin tails. However, using GFP-Grb2 and ectopic expression of the constructs A36R-Y112F, Y132F and YdF, Violaine Moreau in the lab was able to show that Grb2 is present at the site of actin tail nucleation on IEVs containing A36R or A36R-Y112F. Using a similar approach we could also show that Nck is only localized to IEV when tyrosine 112 was present (data not shown). Nck recruitment was dependent on the phosphorylation of tyrosine 112, as it was absent in ΔA36R infections with or without ectopic expression of A36R-YdF (data not shown).

*In vitro* binding assays with peptides corresponding to residues 105-116 of A36R were used to demonstrate that Nck recruitment to the virus occurs via the direct interaction with A36R and is dependent on phosphorylation of tyrosine 112 (Figure 29, upper panel). As both, Nck and Grb2 have been shown to interact with N-WASP (Rivero-Lezcano *et al*., 1995; She *et al*., 1997) and N-WASP is localized to the actin tail of vaccinia (Figure 10), I thought to demonstrate a role for N-WASP in vaccinia actin tail formation. Using the Nck-A36R phosphorylated peptide complex I could show that N-WASP was efficiently recruited from cell extracts (Figure 29, lower panel). I tested whether these molecules play a direct role in the actin-based motility of vaccinia virus by overexpressing putative dominant negative constructs of Nck and N-WASP. Over-expression of the Nck SH2 domain from a pE/L driven promotor transfected 4 hpi into WR infected cells resulted in a reduction of actin tail formation (Figure 30). Overexpressing the Nck SH3 domains, however, did not result in a decrease of the number of cells showing actin tails. As observed with the Nck SH2 domain, overexpression of the dominant negative construct N-WASP-ΔWA, which cannot interact with Arp2/3 complex (Machesky and Insall, 1998), inhibits actin tail formation (Figure 30).

**2.4.2 Src family kinases are involved in actin based motility of vaccinia**

Vaccinia virus encodes two kinases in its genome, B1R and F10L (Lin *et al*., 1992; Traktman *et al*., 1989; Traktman *et al.*, 1995; Wang and Shuman, 1995). One of these, F10L, has recently been shown to possess the ability to phosphorylate serine, threonine and tyrosine residues (Betakova *et al.*, 1999; Lin and Broyles, 1994). Disruption of this kinase results in incomplete viral morphogenesis (Betakova *et al.*, 1999; Lin and Broyles, 1994). To investigate which protein tyrosine kinase is involved in phosphorylation of A36R we compared the sequence around Y112 to known optimal substrate sequences for tyrosine kinases (Songyang and Cantley, 1995). Indeed, there is some homology between the region around Y112F and the sequence for optimal phosphorylation of a peptide by c-Src (Figure 31). I applied the broad range tyrosine kinase inhibitor Herbimycine to infected cells, but this drug inhibited viral morphogenesis and formation of IEV (data not shown). When applied after the formation of IEV was completed (6 hpi) Herbimycine did not abolish actin tails (data not shown). Interestingly, the most specific src family kinase inhibitor PP1 did inhibit actin tails in a dose dependent fashion (Figure 32). However,

although PP1 slows down viral morphogenesis, it does not prevent IEV formation (Figure 33 and data not shown).

The data presented above indicate that src family kinases play a role in actin-based motility of vaccinia virus. Unfortunately I did not succeed in showing a localization of an endogenous src family kinase to vaccinia actin tails using various monoclonal antibodies raised against the ubiquitously expressed Src family kinases fyn, src and yes (data not shown). A functional role for c-Src family members can be observed by over-expressing "dead-open" kinase proteins (Gonfloni *et al*., 1997). "Dead-open" src proteins are able to bind via their SH2 and/or SH3 domain to target proteins but are not able to phosphorylate their substrates as they are catalytically dead. Therefore they block endogenous kinases to bind and phosphorylate their substrates after they are activated (opened). This phenotype is achieved by a deregulating mutation that inhibits the intramolecular interactions which render the src-kinase closed and therefore inactive as well as a mutation in the kinase domain which kills the catalytic activity of the enzyme (Gonfloni *et al.*, 1997). In cells infected with vaccinia strain WR, over-expression of the two "dead-open" src proteins, 527kin$^-$ and KPkin$^-$ inhibited vaccinia actin tail formation (Figure 34). In four independent experiments, expression of 527kin$^-$ and KPkin$^-$resulted in a decrease of cells showing actin tails to 22±10% and 24±10%, respectively. In cells expressing low amounts of "dead-open" src I was able to localize the antibody signal to the site of actin tail nucleation of vaccinia virus (Figure 35).

**2.4.3 Activation of src family kinases by vaccinia virus**

Western analysis of extracts from infected cells treated with PP1 suggests that phosphorylation of A36R is deminished when compared with untreated cells (Figure 36). The level of A36R phosphorylation also seems to be decreased when "dead-open" src constructs are expressed (Figure 36). Interestingly the pTyr80 band is completely gone in extracts treated with PP1 (Figure 36). This effect of a src specific kinase inhibitor suggested that this band could be a src substrate. A well known src substrate of size 80/85 is the actin binding and crosslinking protein cortactin (Wu and Parsons, 1993). Indeed, immunoprecipition experiments and 2-D gel electrophoresis showed that pTyr80 is p80/85 cortactin (Figure 37 and data not shown). Cortactin is localized to actin tails of all intracellular pathogens (Figure 38). This localization is independent of tyrosine phosphorylation of cortactin, as *Listeria* does not induce cortactin phosphorylation (data not

shown). Furthermore, in cells lacking c-Src cortactin does not become phosphorylated but still localizes to vaccinia induced actin tails (data not shown).

Cortactin is phosphorylated by src kinases during the entry of *Shigella* in mammalian cells (Dehio *et al.*, 1995). I therefore investigated if cortactin is phosphorylated during the entry of vaccinia virus. Figure 39 shows that viral entry but not adhesion to cells is needed for stimulation of vaccinia induced phosphorylation (left panel). Interestingly, the level of phosphorylation is not affected by the number of infecting viral particles (Figure 39, right panel). Western analysis of cells infected with drugs blocking early or late vaccinia gene expression shows that it is not the entry process of vaccinia virus that induces phosphorylation events, but that early gene expression is necessary (data not shown). Blocking early gene expression does not lead to the severe changes of the actin cytoskeleton observed 4 hours after infection, when cells retract their lamellipodia and round up (Figure 40). However, inhibition of late vaccinia gene expression with AraC does not inhibit actin cytoskeletal changes (Figure 40, right panel), and still results in tyrosine phosphorylation of proteins (data not shown). This demonstrates that an early expressed vaccinia gene product activates c-Src and possibly other host kinases.

Finally, figure 41 shows a model of the possible interactions of proteins involved in actin tail formation by vaccinia and summarizes the main data presented in this thesis.