1 Introduction

1.1 Smallpox and vaccinia virus

1.1.1 Historical background

Smallpox is widely considered to have been one of the most devastating diseases in human history (Oldstone, 1998). During the hundred years prior to the eradication of smallpox, it killed well over 300 million people (Henderson, 1996). The causative agent of smallpox is variola virus. Variola virus was eradicated through a world wide vaccination effort by the World Health Organization, using various strains of vaccinia virus as the immunizing agents (WHO, 1980). The last case of naturally occuring outbreak of smallpox was recorded in 1977 in Somalia (Moss, 1996; WHO, 1980).

The first documented appearance of smallpox goes back to ancient Egypt. On the mummy of Ramses V, who died from an acute illness in 1158 B.C., typical smallpox lesions are visible. Even earlier recordings by the Hitties indicate an epidemic in 1346 B.C. (Oldstone, 1998). The first attempts of overcoming the disease go back to first century China. During the Sung dynasty (960 - 1280) dried smallpox scabs were converted into powder and inhaled in order to prevent infection by smallpox (Oldstone, 1998). This method of variolation, which still resulted in about 2% fatalities among the recipients of the dried smallpox scabs, slowly spread to western Europe. Dr. Edward Jenner was intrigued by the observation that milking maids, who showed symptoms of cowpox sometime during their life, were not affected by smallpox epidemics. In 1796 he performed an ethically highly questionable experiment by infecting the 8 year old James Phipps first with cowpox and subsequently with smallpox (Oldstone, 1998). As the boy did not succomb to smallpox, Jenner announced a new, apparently safe method to vaccinate and protect people from the disease (Jenner, 1798). Sadly, the scientific establishment of the time, namely the Royal Society of England, who appointed Jenner as a member for his discovery that cuckoo hatchlings eject the other eggs from the nest of their foster parents, did not realize that they witnessed one of the biggest breakthroughs in medical history and blocked the publication of his paper (Oldstone, 1998). Vaccination with cowpox slowly gained pace and finally led to the worldwide push against smallpox by the WHO. During this time it was realized that the vaccinating agent was not cowpox anymore, but a different virus (Moss, 1996; Oldstone, 1998). It is since called vaccinia virus from the latin word vacca for cow, although the origin of vaccinia virus is still a matter of debate (Baxby, 1977).

Like other disease agents smallpox has been used extensively in biological warfare (Oldstone, 1998). Recent revelations showed that the late Soviet Union ran enormous programmes to arm inter-continental missiles with smallpox and other pathogens (Alibek and Handelman, 1999; Henderson, 1999). A number of experts fear that following the collapse of the USSR former bio-weapon specialists might now work for terrorist groups or rough regimes (Alibek and Handelman, 1999; Henderson, 1999). This percieved threat comes at a time where no country has enough vaccinia virus stock-piled to vaccinate its population (Alibek and Handelman, 1999; Henderson, 1999). Officially, only two institutes still store variola virus stocks (Alibek and Handelman, 1999; Henderson, 1999). A moral and fierce debate exists whether these variola stocks should be destroyed to avoid a possible use by terrorists or whether they should be kept to enable scientists to work on them in order to develop a better understanding of the virus (Ellner, 1998; Henderson, 1999).

1.1.2 Life cycle of vaccinia virus

As for most viruses, the major steps in the life cycle of vaccinia virus include entry into host cells, replication and release of infectious particles. There is little knowledge about how vaccinia enters a cell but it clearly has to bind and cross the plasma membrane, suggesting that membrane fusion, eventually mediated by binding to one or more unknown receptors must play a role (Chung *et al*., 1998; Hsiao *et al*., 1998; Vanderplasschen and Smith, 1997). A role for the actin cytoskeleton in vaccinia entry has been suggested (Vanderplasschen *et al.*, 1998). In contrast to the majority of viruses, which replicate in the cell nucleus, vaccinia replicates in the cytoplasm and does not enter the nucleus (Cairns, 1960; Minnigan and Moyer, 1985). The linear double stranded DNA genome of vaccinia has been sequenced (191,000 bp) and harbours a complete transcription machinery in its 263 open reading frames (ORFs). Most vaccinia ORFs still await functional characterization (Goebel *et al*., 1990; Johnson *et al*., 1993). The name of an ORF indicates its position on the viral genome. HindIII digestion of the viral genome creates 16 fragments which are numbered alphabetically in decreasing size (DeFilippes, 1982). ORFs within such a fragment are numbered sequentially from the 5' end of the fragment and are followed by an L or R indicating the reading direction of the ORF (Rosel *et al.*, 1986). For example, A36R is the 36th ORF on the biggest HindIII fragment and is transcribed from left to right.

Three major stages of viral transcriptional activity are discernable: early, intermediate and late gene expression (Figure 1). Prior to the onset of viral DNA replication, early genes are transcribed minutes after the virus entered the host cell. Intermediate and late proteins are subsequently expressed after viral DNA replication has started (Moss, 1996). Vaccinia DNA replication takes place in huge, perinuclear, electron dense viral "factories" (Dales, 1963) (see Figure 26).

Spherical immature virions (IVs) form from viral crescents, membranes derived from the ER-to-Golgi intermediate compartment which have been loaded with viral proteins (Sodeik *et al*., 1993). After inclusion of the viral DNA and proteolytic cleavage of some viral core proteins IVs develop into brick-shaped 250 by 350 nm large intracellular mature viruses (IMVs) (Dubochet *et al.*, 1994; Moss, 1996; Roos *et al.*, 1996) (Figure 1). IMVs represent one of the two infectious forms of vaccinia virus and are released by cell lysis. IMVs can undergo a further wrapping process with membranes derived from the *trans*-Golgi network (TGN) to create intracellular enveloped virions (IEVs) (Payne and Kristenson, 1979; Schmelz *et al*., 1994) (Figures 1, 2). The number of IMVs that undergo this second wrapping varies from cell type to cell type and depends further on the viral strain. Prior to wrapping, the TGN has been loaded with IEV specific proteins, some of which (B5R and F13L) have been shown to mediate the wrapping process by interacting with another viral protein, A27L, on the IMV surface (Duncan and Smith, 1992; Rodriguez and Smith, 1990).

IEVs exhibit the striking feature of using actin-based motility to enhance cell-to-cell spread (Cudmore *et al*., 1995; Cudmore *et al*., 1997; Strauss, 1996). IEVs are propelled at a speed of 2.8 μm/min through the cytoplasm in what appears a random fashion (Cudmore *et al.*, 1995). Upon contact with the plasma membrane an IEV fuses its outermost membrane releasing a cell attached enveloped virus (CEV) (Blasco and Moss, 1992; Cudmore *et al.*, 1996). It is thought that CEVs mediate short range cell-to-cell spread, while extracellular enveloped viruses (EEVs), that have been released from the host cell facilitate long range dissemination and represent the second infectious form of vaccinia virus (Blasco and Moss, 1992; Payne, 1980). Extracellular IMVs are released after cell lysis but their efficiency in viral spread is minimal compared to CEV and EEV (Blasco and Moss, 1991; Morgan, 1976). IMVs can also be released by budding through the plasma membrane (Tsutsui, 1983; Tsutsui *et al.*, 1983). IMV budding would result in EEV-like

viruses, as budded IMVs contain the same number of membranes around the viral core as do EEVs, although the origin of the outer membrane is different. To date it has not been shown if the infectivity of EEVs is different from the infectivity of IMVs released by budding.

1.1.3 IEV specific proteins

Six vaccinia virus proteins are specifically associated with IEV: A33R (gp23-28), A34R (gp22-24), A36R (p45-50), A56R (gp89), B5R (gp42), and F13L (p37) (Duncan and Smith, 1992; Engelstad *et al.*, 1992; Hiller and Weber, 1985; Isaacs *et al.*, 1992; Parkinson and Smith, 1994; Payne and Norrby, 1976; Roper *et al.*, 1996). Of these all but A36R and F13L are glycosylated and exist consequently in multiple forms. With the exception of F13L, all known IEV specific proteins are transmembrane proteins with a single membrane spanning region. F13L is membrane associated via palmytic or oleic acids covalently bound to cysteine 185 and 186 (Grosenbach *et al*., 1997). All IEV specific proteins are localized to the Golgi apparatus consistent with the wrapping of IMVs with the TGN (see Figure 26). Their proposed membrane topologies on IEVs and their possible roles in EEV formation are depicted in figure 2 and table 1, respectively. As EEVs are derived from IEVs, these proteins are also found on EEVs albeit with reversed topology. However, there might be a difference between extracellular virions that have been released from a cell by budding of IMV and by fusion of IEV. For budded IMVs the outer-most membrane derives from the plasma membrane of the cell, while for fused IEVs the outer-most membrane derives from the TGN.

Table 1: Role of IEV specific proteins in EEV formation

IEV protein	A33R	A34R	A36R	A56R	F13L	B5R
role in EEV formation	yes	yes	yes	no	yes	yes

A role in EEV formation is derived from quantitative EEV preparations as it is hard to judge IEV formation quantitatively within cells and no pure IEV preparation is available. A 'yes' denotes that there are different amounts of EEV found in preparations derived from viruses lacking the respective IEV specific proteins than from wild type viruses.

1.2 Actin polymerization

Actin is the most abundant protein in most eukaryotic cells. Actin filaments make up the actin cytoskeleton which, together with the microtubules and intermediate filaments, maintain the shape of eukaryotic cells. Actin exists as monomeric, 42 kDa globular actin (so called G-actin) and polymerized, filamentous actin (F-actin). The two ends of an actin filament are distinct. While at the fast growing end actin monomers add to the filament with fast kinetics at the other end actin monomers are added more slowly (Pollard, 1986; Rickard and Sheterline, 1986). Studies in which actin filaments are decorated with the S1 motor domain from myosin reveals a characteristic appearance in the electron microscope, with the S1 domains pointing to the slow growing end of the filament. In analogy to an arrow the slow growing end of an actin filament is called the "pointed" end, and the fast growing the "barbed" end.

Numerous so called actin binding proteins have been identified and characterized that bind to either G-actin or F-actin, or both (Pollard and Cooper, 1986; Schafer and Cooper, 1995). *In vitro* polymerization assays with purified actin binding proteins have yielded invaluable insight into how actin polymerization can be controled *in vivo* (Machesky and Way, 1998; Pollard, 1986). *In vitro*, actin polymerizes spontaneously with characteristic kinetics and one can easily assess the effect a purified putative actin binding protein has on this process (Figure 3). How are actin filaments nucleated? The rate limiting step of actin filament nucleation is the assembly of a G-actin trimer, which is a rare event, as the reaction constants are unfavorable to its formation (Oosawa and Asakura, 1975). In a cell actin polymerization is temporally and spatially regulated. This suggests that it cannot depend on random and unlikely events but has to rely on specific proteins or protein complexes (see below).

The concentration of monomeric actin within a cell is well above the critical concentration needed for actin polymerization. A number of G-actin binding proteins sequester actin and prevent it from being added on pre-existing filaments or from spontaneously nucleating new filaments. Also, capping proteins bind to the ends of actin filaments and inhibit the addition (or release) of actin to (or from) a filament.

Profilin is a small G-actin binding protein. The association of profilin with actin is inhibited by binding of phosphatidylinositol-4,5-bis-phosphate ($PI(4,5)P_2$) to profilin. Profilin enhances the exchange of ADP-actin to ATP-actin (Sohn and Goldschmidt, 1994). Actin monomers are thought

to be only added to a filament if they have ATP bound. Once ATP-actin assembled a filament it is slowly turned over into ADP-actin. Therefore, although profilin inhibits actin polymerization by sequestering G-actin, it primes actin for fast polymerization once the profilin-actin complex is disrupted, e.g. by signalling events that lead to elevated $PI(4,5)P_2$ (Carlier *et al*., 1993). However, profilin inhibits spontaneous actin nucleation and might therefore only play a role in actin polymerization from pre-existing filaments (Machesky *et al*., 1999).

In a quiescent cell, most ends of filaments are capped. The concentration of actin filament capping proteins in a cell is about 1% of the concentration of actin (Xu *et al*., 1999). Therefore, if the average length of an actin filament is about 200 actin monomers, most filament ends are capped and not available for the addition of actin monomers. For actin polymerization to occur the capping protein has to dissociate from the end of a filament. Like for profilin, the binding of the barbed end capping protein CapZ to actin is inhibited by $PI(4,5)P_2$ (Heiss and Cooper, 1991). One can envisage a simple scenario where filaments in a cell are capped and most of the G-actin is sequestered by profilin. If the cell produces soluble $PI(4,5)P_2$ in response to an extracellular signal the actin filaments are uncapped and ATP-actin is released from profilin and can be added to a filament, thereby promoting filament growth until a regulatory mechanism would terminate it, or the free actin is used up. However, this simple picture is complicated by the existence of a myriad of other proteins that interact with actin (Kreis and Vale, 1999; Schafer and Cooper, 1995) of which I will only introduce a few.

First isolated on a profilin chromatography column the Arp2/3 complex consists of the actin related proteins 2 and 3 as well as five evolutionary conserved polypeptides of 16, 20, 21, 34 and 41 kDa mass (Machesky *et al*., 1994; Machesky *et al*., 1997; Welch *et al*., 1997a; Welch *et al*., 1997b). Actin related proteins belong to the growing family of Arps and share 20-80% sequence similarity with actin (Schroer *et al*., 1994). *In vitro* actin polymerization and electron microscopy studies show that the Arp2/3 complex binds to pointed ends and sites of actin filaments (Mullins *et al*., 1998a). This raises the possibility that Arp2/3 caps the pointed ends of filaments and allows fast polymerization at the barbed ends. This process is stopped by capping of the barbed end by a protein like CapZ (Mullins *et al*., 1998a). The nanomolar binding affinity to the pointed end and the abundance of the complex (2 μM, 1% of total cellular actin) suggest that all pointed ends in a cell are capped by Arp2/3 (Mullins *et al*., 1998a; Mullins *et al*., 1998b). This obviously raises the

question of how actin disassembly is achieved from a filament capped on both ends (Mullins and Pollard, 1999b; Zigmond, 1998). Actin disassembly can be achieved by severing (e.g. by gelsolin or ADF/cofilin, see below) or by regulated release of Arp2/3 complex from the pointed end. So far no regulatory mechanism has been found that could account for the latter.

Comparison of data from a number of actin polymerization experiments with kinetic simulations led Mullins and colleagues to suggest that Arp2/3 complex binds to preformed actin dimers (Mullins *et al*., 1998a). 98% of these bound dimers will then dissociate from the Arp2/3 complex before trimer formation (the rate limiting step in actin polymerization) sets the filament elongation on track (Mullins *et al*., 1998a; Oosawa and Asakura, 1975). The binding of Arp2/3 complex to the sides of existing actin filaments causes the formation of a 'dendritic' network of filamentous actin which is observed at the leading edge of a cell (Mullins *et al*., 1998a; Svitkina and Borisy, 1999). One can now envisage that regulated polymerization of actin from activated Arp2/3 complexes can lead to the formation of complex networks at the periphery of the cell. How this polymerization can lead to changes in the shape of the cell will be described in chapter 1.4.1.

Polymerization of actin can be stopped by capping proteins, but how are filaments recycled? There are many ways to disassemble an actin filament. Uncapping of one of the ends under conditions unfavorable for polymerization is but one. Severing, breaking the filament in two, is another. An interesting example for severing proteins are members of the actin depolymerizing factor/cofilin family of proteins, which bind both, monomeric and filamentous actin. *In vitro* ADF/cofilin leads to the acceleration of the rate of filament turnover by increasing the off-rate of actin from the pointed end of the filament while increasing the on-rate of actin monomers to the barbed ends (Carlier *et al*., 1997; Ressad *et al*., 1999).

The proteins and processes described above have lead to a picture of actin filament turnover that is depicted in figure 4. Most of the data described so far come from *in vitro* experiments. How is the situation *in vivo*?

1.3 Signalling to the actin cytoskeleton

Within an eukaryotic cell, actin polymerization is strictly regulated and can be activated by many different stimuli (reviewed by Carlier *et al*., 1999; Machesky and Insall, 1999). The tight regulation of actin polymerization within a cell is necessary for co-ordinated cell movements that occur during embryonic development, wound healing and cytokinesis to name but a few. Abnormal regulation of actin dynamics can lead to many malignancies including cancer metastasis (Janmey and Chaponnier, 1995).

How is actin polymerization regulated *in vivo* and what are the features of the resulting actin structures? Members of the Rho family of GTPases control a wide range of actin polymerization events in eukaryotic cells (Hall, 1998). Rho proteins belong to the Ras-superfamily and work as binary switches that cycle between an 'activated' GTP bound and an 'inactive' GDP bound form. In the GTP bound state the Rho family member Cdc42 promotes actin polymerization that leads to the formation of filopodia, spike like extensions that form from the surface of a cell. GTP-Cdc42 is able to activate another Rho family member, Rac, which in turn can activate Rho. Rac mediated actin polymerization leads to the formation of lamellipodia, thin actin sheets that protrude from the membrane, often spanning the filopodia, like the skin between the 'toes' of a duck. Micro-injection of Rho into cultured fibroblasts leads to the formation of thick actin cables (bundles of actin filaments) called stress fibers that connect sites of cell-matrix or cell-cell adhesion, so called focal adhesions (Hall, 1998). Focal adhesions are multi-protein complexes that link the extracellular matrix and therefore maintain cell attachment (Sarkar, 1999).

Multiple signalling events can lead to the activation of Rho family GTPases. One such example is the binding of epithelial growth factor (EGF) to the EGF-receptor. Stimulation of cells with EGF leads to the activation of Rac and the formation of membrane ruffles (Schmidt and Hall, 1998). As no Rho protein interacts directly with actin, there must be a number of other proteins involved in this signalling cascade. Which molecules are involved in the transduction of this signal? The understanding of the detailed process is obscured as EGF is a promiscuous ligand that can bind multiple subtypes of EGF-receptors (Riese and Stern, 1998). However, it is clear that upon binding of EGF the EGF-receptor undergoes autophosphorylation of tyrosine residues within the cytoplasmic domain (Riese and Stern, 1998). The adaptor proteins Grb2 and Nck are able to bind to these phosphorylated tyrosines with their respective src homology 2 (SH2) domain (Buday,

1999). Grb2 and Nck are classical adaptor proteins that consist of one SH2 domain and two (Grb2) or three (Nck) SH3 domains. While the SH2 domain is able to bind phosphorylated tyrosine residues their SH3 domains interact with poly-proline rich sequences (Buday, 1999; McCarty, 1998). This feature enables these adaptors to link phosphorylated transmembrane receptors to poly-proline rich effector molecules. Adaptors are often already complexed to other effector proteins and recruit these to activated receptors (Buday, 1999). One of the effector proteins that is able to bind both, Grb2 and Nck is WASP, the protein mutated in Wiskott-Aldrich syndrome (Rivero-Lezcano *et al.*, 1995; She *et al.*, 1997). After activation of EGF-receptors WASP can be immunoprecipitated in a complex of Nck, WASP and EGF-receptor (Li *et al.*, 1992). However, even before activation Nck is already complexed to WASP (Li *et al.*, 1992).

Wiskott-Aldrich syndrome is an X-linked primary immunodeficiency that leads among other maladies to recurrent infections and eczema (Snapper and Rosen, 1999). Patients who do not recieve bone marrow transplantation usually die from B-cell lymphomas associated with Epstein-Barr virus infections in their 30s or 40s (Snapper and Rosen, 1999). While WASP is only present in cells of hematopoietic origin, N-WASP is more ubiquitously expressed (Miki *et al.*, 1996). Both proteins share a similar domain organization with an N-terminal WASP homology domain (WH1) followed by a GTPase binding so called Cdc42 and Rac interactive binding domain (CRIB), a poly proline rich stretch and a WH2 domain with an acidic C-terminus (Figure 6) (Higgs and Pollard, 1999; Miki *et al.*, 1996). A calmodulin binding IQ-motif is found additionally in N-WASP C-terminal to the WH1 domain suggesting that N-WASP function could be controlled by changes in the concentration of intracellular calcium (Miki *et al*., 1996). Intramolecular interactions between the acidic C-terminus and a basic strech N-terminal of the CRIB have been suggested to play a role in regulating N-WASP (Miki *et al.*, 1998). Binding of Cdc42 or Rac to the CRIB domain of N-WASP could play a role in EGF induced actin polymerization and membrane ruffling as activating (opening) N-WASP would expose the adaptor binding site of the molecule (Aspenström *et al.*, 1996; Ramesh *et al.*, 1999; Symons *et al.*, 1996). The originally suggested N-terminal pleckstrin homology (PH) domain which was based on only weak sequence homology has recently been compared with the structure of the related, poly proline binding Ena/VASP homology domain that has been solved by X-ray crystallography (Fedorov *et al.*, 1999; Prehoda *et al.*, 1999). Although this domain is not identical to the $PI(4,5)P_2$ binding PH domain they are very similar. However, it

is a possibility that the WH1/PH domain of N-WASP binds poly proline rich sequences and not PI(4,5)P_2. Although binding of PI(4,5)P_2 to N-WASP has been demonstrated *in vitro*, the *in vivo* relevance of these results are a matter of debate.

Overexpression of N-WASP with activated Cdc42 causes formation of filopodia in tissue culture cells (Miki *et al*., 1998). Both WASP and Scar1, another member of the WASP family of proteins, are able to regulate the actin cytoskeleton by direct interaction with p21 of the Arp2/3 complex (Machesky and Insall, 1998). The combined WA domain (WH2 domain and acidic motifs) of Scar1 was sufficient to disrupt membrane ruffling in transfected cells and enhanced actin nucleation by Arp2/3 complex in *in vitro* essays (Machesky and Insall, 1998; Machesky *et al*., 1999). The homologous domain in N-WASP had similar effects (shortening of lag phase and increasing the elongation rate) on actin polymerization by Arp2/3 complex (Rohatgi *et al*., 1999). Interestingly, full length N-WASP was less efficient in activating Arp2/3 promoted actin nucleation. However, when Cdc42 and PI(4,5)P_2 were added to the polymerization mix, the same quantitative effects on actin polymerization were achieved, consistent with the idea that N-WASP has to be opened for activation (Rohatgi *et al*., 1999). This indicates that N-WASP has to be activated (opened) in order to achieve full stimulatory activity. In summery, WASP family members are good candidates to link extracellular stimuli to actin polymerization via the Arp2/3 complex (Carlier *et al*., 1999; Higgs and Pollard, 1999; Machesky and Insall, 1999).

1.4 Actin-based motility of intracellular pathogens

'Actin-based motility' is the term for a process in which actin polymerization drives the movement of something, for example a vesicle, a cell, a virus, or the plasma membrane. The intracellular pathogens *Listeria, Shigella, Rickettsia* and vaccinia virus have managed to subvert the cytoskeletal machinery of actin polymerization to facilitate their spread. In doing so, they assemble an actin 'comet' tail (Figures 5, 8). On the tip of this actin tail, the pathogens are propelled through the cytoplasm of an infected cell and from one cell to another (reviewed by Dramsi and Cossart, 1998). Studies of the actin-based motility of pathogens have provided interesting insights into the subversive mechanisms used by these pathogens and the regulation of actin polymerization in the cell.

1.4.1 Actin-based motility of *Listeria monocytogenes*

The Gram positive bacterium *Listeria monocytogenes* is responsible for listeriosis, a milk borne disease which can lead to severe meningo-encephalitis. Although infections are usually not life-threatening death can occur in pregnant women, unborn or young babies and immuno-compromised individuals (Tilney and Portnoy, 1989). *Listeria* thrieves as an intracellular parasite as it is capable of entering cells by a special phagocytic process refered to as the "zipper" mechanism (Dramsi and Cossart, 1998). Two bacterial surface proteins, internalin A and B are involved in binding the bacteria to the host cell. Each of these internalins is sufficient for entry into cultured cells. The receptor for internalin A is E-cadherin, while the receptor for internalin B still awaits identification. The entry but not the adhesion of bacteria can be inhibited by cytochalasin D, which disrupts the actin cytoskeleton. Entry is also inhibited by tyrosine kinase inhibitors and inhibitors of phosphoinositide PI-3 kinase, suggesting that bacterially triggered signalling events are involved in *Listeria* entry (Dramsi and Cossart, 1998). Bacteria escape the endosome or phagosome by secreting the pore forming listeriolysin O and replicate in the cytoplasm within one hour (Tilney and Portnoy, 1989). In the cytoplasm the bacteria can recruit actin, which organizes into an actin cloud that surrounds the bacterium (Figures 5, 10). After bacterial division the actin cloud is transformed into an actin tail. Bacteria are able to move on the tip of this actin tail, as the dense actin cytoskeleton is providing a rigid scaffold while actin polymerization at the bacterial pole pushes the bacteria forward (Dabiri *et al*., 1990; Mounier *et al*., 1990; Tilney and Portnoy, 1989). Upon encountering of the plasma membrane, bacteria moving intracellularly can project into adjacent cells, where phagocytic uptake and bacterial escape start the cycle over again (Figure 5). Using this direct cell-to-cell spread the bacteria manage to evade the humoral defense system of the host organism (Tilney and Portnoy, 1989).

How does *Listeria* achieve actin-based motility? Shortly after the inital reports of actin-based motility by *Listeria*, the ActA protein was identified as the bacterial surface protein responsible for actin tail formation (Domann *et al*., 1992; Kocks *et al*., 1992) (Figure 6). This identification was achieved by analysis of transposon induced mutants which were impaired in cell-to-cell spread (Kuhn *et al*., 1990; Sun *et al*., 1990). The ActA gene is under the control of the PrfA regulator gene that has been shown to regulate the expression of other virulence factors, like listeriolysin (Chakraborty *et al*., 1992; Mengaud *et al*., 1991). Although the amino acid sequence of 610

residues suggests a molecular weight of about 67 kDa, the protein runs at 90 kDa in SDS gels (Kocks *et al*., 1992). This shift to slower mobility might be explained by the presence of a seven times repeated small motif of acidic residues, phosphorylation on serine and/or threonine and an isoelectric point calculated to 4.74 (Brundage *et al*., 1993; Kocks *et al*., 1992). ActA is localized at the site of the associated actin tail on intracellular bacteria (Kocks *et al*., 1993; Niebuhr *et al*., 1993). A possible explanation for this polarity comes from the observation that intracellular bacteria assemble an actin tail only after the first division (Kocks *et al*., 1993; Tilney *et al*., 1992). When bacteria divide their cell wall has to be degraded at the site of division, which leaves the newly formed pole ActA-free (Kocks *et al*., 1993; Tilney *et al*., 1992). Similarly, a fusion of ActA to the LytA protein of *Streptococcus pneumoniae* resulted in actin-based motility only after *Streptococci* divided (Smith *et al*., 1995). Interestingly, when latex beads are coated with ActA, small beads would move in cell extracts, while bigger beads only move when coated on one site (Cameron *et al*., 1999).

The use of cell extracts, in which bacteria are able to move, has greatly advanced the field of actin-based motility, as it proved possible to immunodeplete and add back certain proteins or protein complexes (Moreau and Way, 1999). Cytoplasmic cell extracts that support motility of *Listeria* have been prepared from platelets, brain, or *Xenopus* eggs (Laurent *et al*., 1999; Theriot *et al*., 1994a). Expression of ActA on the surface of non-motile bacteria leads to actin-based motility of these micro-organisms in *Xenopus* egg extracts (Kocks *et al*., 1995). Targeting ActA to mitochondria or the plasma membrane in uninfected cells also resulted in the recruitment of actin to the site of ActA localization (Friederich *et al*., 1995; Pistor *et al*., 1994). Taken together, these experiments show that ActA is indeed the only bacterial factor needed for actin polymerization. Interestingly, proline rich repeats homologous to those in the focal adhesion and actin binding proteins vinculin and zyxin are found in ActA indicating that ActA might mimic their function in actin assembly (Domann *et al*., 1992; Golsteyn *et al*., 1997; Kocks *et al*., 1992). Micro-injection of a peptide corresponding to the proline rich repeats of ActA, vinculin and zyxin resulted in the arrest of *Listeria* movement indicating that this region of ActA might plays a role in actin tail assembly (Southwick and Purich, 1994). However, the micro-injection also resulted in massive membrane retractions raising the question if the arrest of *Listeria* is an indirect effect of the proline rich peptide or other cellular targets (Southwick and Purich, 1994).

Studies using micro-injection of fluorescently labelled actin monomers or photoactivation of fluorescence of caged actin showed that actin is incorporated into the *Listeria* tail at the bacterial surface (Sanger *et al*., 1992; Theriot *et al*., 1992). Time-lapse microscopy revealed that the rate of actin polymerization is proportional to the rate of bacterial actin-based motility (Sanger *et al*., 1992; Theriot *et al.*, 1992). These observations lead to two theoretical studies in which the "elastic brownian ratchet" model was proposed to explain the transduction of force from polymerizing actin to the propulsion of the bacterium (Mogilner and Oster, 1996; Peskin *et al.*, 1993). Briefly, brownian motion would bent not yet cross-linked filaments within the actin tail from the bacterial cell wall allowing actin monomers to be added onto these filaments. These elongated filaments would eventually flip back and, due to the rigid structure of the tail (cross-linked filaments), produce a net elastic pressure onto the cell wall of the bacterium. This would result in a movement of the bacterium away from the tail and could account for the fact that bacteria only move when the actin tail reaches a certain length, then being more restricted in its movements than the bacterium.

Does ActA bind directly to actin and initiate actin polymerization or are other host cell factors required for the actin-based motility of *Listeria*? A number of studies showed that almost every actin binding protein is localized to the actin tail (tropomyosin (Dabiri *et al.*, 1990), α-actinin (Dabiri *et al.*, 1990), vinculin (Dold *et al.*, 1994) fimbrin/plastin (Kocks and Cossart, 1993), ezrin/radixin (Temm-Grove *et al*., 1994), villin (Temm-Grove *et al*., 1994), profilin (Marchand *et al*., 1995; Theriot *et al*., 1994a), talin (Dold *et al*., 1994), CapZ, cofilin, coronin and Rac (David *et al*., 1998)). Considering the dense network of actin in the tail it is not surprising that many actin-binding proteins are also there. Therefore the localization of a protein is not enough to indicate whether the protein plays a role in actin-tail formation.

Which proteins are of functional interest? The finding that disruption of functional cross-linking by the F-actin cross-linking protein α-actinin halts actin-based motility of *Listeria* can be nicely explained by the elastic brownian ratchet model (Dold *et al.*, 1994; Mogliner and Oster, 1996). The functional implication of α-actinin in actin tail formation could also be valid for the other cross-linkers associated with the actin tail. However, it is unlikely that α-actinin interacts directly with ActA, as it is localized throughout the actin tail, while ActA is only present on the bacterium (Dabiri *et al.*, 1990; Kocks *et al.*, 1993; Niebuhr *et al.*, 1993). Also α-actinin is not localized in actin tails projecting from the cell surface (Sechi *et al*., 1997). Furthermore, *in vitro*

studies using pure components have shown that α-actinin is not needed for actin tail formation (Loisel *et al*., 1999). However, actin tails were denser in the presence of α-actinin and bacteria did not drift as much on the tip of the tails as they did in its absence, suggesting that the structural integrity of a cross-linked tail is helpful for efficient motility *in vivo* (Loisel *et al*., 1999).

A different role is suggested for the actin binding proteins actin depolymerizing factor (ADF) and cofilin (reviewed by Maciver, 1998; Theriot, 1997). Increased concentrations of ADF/cofilin in *Xenopus* extracts lead to shortened actin tails and enhanced *in vitro* motility of *Listeria.* This higher speed is possibly due to an increasing number of actin monomers available for actin polymerization at the barbed ends of filaments near the bacterial surface (Carlier *et al.*, 1997; Rosenblatt *et al*., 1997) Indeed, ADF/cofilin is a crucial factor for reconstituted actin-based motility *in vitro* (Loisel *et al*., 1999).

In contrast to the above mentioned proteins, the focal adhesion protein vasodilator-stimulated phosphoprotein (VASP) was found to bind directly to the proline rich repeats of ActA (Chakraborty *et al.*, 1995; Pistor *et al*., 1995). VASP was named after it was found to be phosphorylated by cAMP and cGMP dependent protein kinases upon application of vasodilators on platelets, which caused inhibition of platelet aggregation (Butt *et al*., 1994; Waldmann *et al*., 1987). VASP exists as a tetramer in intact cells and binds F-actin. It also binds the actin monomer binding protein profilin in a phosphorylation independent way (Reinhard *et al*., 1995). However, the affinity of VASP for F-actin is 40 fold higher when phosphorylated (Laurent *et al*., 1999). Early evidence suggested that the VASP-profilin complex provides the link between ActA and actin tail formation (Chakraborty *et al*., 1995; Niebuhr *et al*., 1997). However, it was found that deletion of the VASP binding site of ActA (the proline rich repeats) did not completely inhibit actin tail formation, although no VASP was bound to the bacteria (Pistor *et al*., 1995; Smith *et al*., 1996). A lack of VASP resulted in a strong decrease in the ability of the bacteria to nucleate a tail. Those bacteria that did move, were slower, suggesting VASP has a regulatory role in actin tail formation (reviewed by Smith and Portnoy, 1997). Indeed, the presence of VASP increases the speed of *Listeria* movement reconstituted from the minimal components, actin, ADF, Arp2/3 complex and capping protein 10-fold (Loisel *et al*., 1999). *In vivo,* the role of VASP could also be complemented by its homologues Evl and Mena, but one can only specülate if those are able to bind ActA on a site different from the proline rich stretches (Laurent *et al*., 1999).

Controversial reports on the role of profilin in actin tail assembly could be explained by the different strains of *Listeria* and the different protocols used to obtain *Xenopus* egg extracts utilized by the researchers (Marchand *et al*., 1995; Smith and Portnoy, 1997; Theriot *et al*., 1994b). Like VASP profilin is not needed for *in vitro* reconstituted motility, but does increase the speed of moving *Listeria* approximately three fold (Loisel *et al*., 1999).

A 'dendritic' network of F-actin similar to that observed at the leading edge of a cell is also found in the *Listeria* actin tail (Gouin *et al*., 1999; Mullins *et al*., 1998a; Sechi *et al*., 1997; Svitkina and Borisy, 1999). This indicates that the Arp2/3 complex might also play a role in *Listeria* actin tail formation. *In vitro* studies showed that purified Arp2/3 complex stimulated actin polymerization only weakly. However, the addition of the N-terminal domain of ActA greatly reduced the lag time prior to polymerization, indicating that ActA might stimulate polymerization of actin by interaction with the Arp2/3 complex (Welch *et al*., 1998) (Figure 3). Interestingly, however, full length ActA did not enhance actin polymerization indicating that the protein has to become activated to expose its N-terminal region (Welch *et al*., 1998). This N-terminal region of ActA is responsible for dimerization of the molecule, however, it is not clear if dimerization plays an essential part in actin-based motility (Mourrain *et al*., 1997).

Arp2/3 complex, the holy grail or just the beginning of a long quest? The Arp2/3 complex seems to be a key component of pathways leading to actin polymerization during cell motility. The Arp2/3 complex is necessary for Rho family GTPase stimulated actin polymerization (Ma *et al*., 1998; Mullins and Pollard, 1999a) as well as for the actin-based motily of *Listeria* (Loisel *et al*., 1999; May *et al*., 1999; Welch *et al*., 1997b) and *Shigella* (Egile *et al*., 1999; Loisel *et al*., 1999). It is not yet clear how Arp2/3 is stimulated by ActA. One possibility would be that binding of ActA to the complex would cause a structural change so that the Arp2 and Arp3 subunit of the complex could form a surface that mimics the barbed end of an actin filament (Mullins and Pollard, 1999b).

How does the N-terminal domain of ActA promote Arp2/3 stimulated actin polymerization? If amino acids 21-231 of ActA are deleted, no actin assembly occurs on the surface of intracellular *Listeria* (Lasa *et al*., 1997). The N-terminus contains two regions that have been shown to play two differential roles in actin tail formation. If residues 117-121 are deleted the bacteria can still assemble an actin cloud but no tail, while deletion of residues 21-97 results in the formation of discontinuous actin tails (Lasa *et al*., 1997) (Figure 6). As a synthetic peptide comprising amino

acids 33-74 could interact specifically with F-actin it has been suggested that residues 117-121 are necessary for Arp2/3 binding and/or activation leading to actin polymerization, while residues 21-97 (or 33-74) are needed to provide a link to F-actin necessary for the formation of an actin tail (Lasa *et al*., 1997). That cross-linking of actin filaments is important for actin tail formation on the bacteria has been demonstrated and discussed (Dold *et al*., 1994; Sechi *et al*., 1997). Although the authors suggested that cellular actin cross-linking proteins fulfill this job, it could be possible that cross-linking of F-actin is achieved by ActA dimers. The rate of actin-based motility of *Listeria* expressing the N-terminus of ActA fused to LacZ is three times slower than of *Listeria* expressing wild type ActA (Lasa *et al*., 1997). This shows that, although the N-terminus is sufficient for actin cloud and tail formation, the proline rich repeats of ActA are required for efficient actin-based motility (see figures 6, 7).

In summary, it is clear that Arp2/3 stimulates actin polymerization at the bacterial surface, but additional factors are required for the organisation of actin filaments into an actin tail which is required for efficient actin-based motility.

1.4.2 Actin-based motility of *Shigella flexneri*

The Gram negative bacteria of the genus *Shigella* are the cause of a bacillary dysentery called shigellosis. The disease is caused after bacteria penetrate into the intestinal mucosa of the colon and cause degeneration of the epithelium and a strong inflammatory reaction (La Brec *et al*., 1964). *Shigella* are transmitted by the fecal-oral route or - less frequently - by contaminated food (Wharton *et al*., 1990). *Shigella* infections are believed to be responsible for the death of over one million people annually, mainly children under the age of 5 in developing countries (Kotloff *et al*., 1999).

The migration of intracellular *Shigella* was first described in 1968 by Ogawa and co-workers using phase contrast microscopy (Ogawa *et al*., 1968). They observed that the bacteria moved "conspicuously independent of the movement of cellular organella" and could detect bacteria projecting within microfibrillar protusions from the cell membrane. They further demonstrated that the motile bacteria exhibit a polarity during their movement with always one end heading the movement. Finally, based on their electron microscopical observations that intracellular bacteria are not surrounded by a cellular membrane, they discuss the possibility that bacterial components on the *Shigella* surface could interact with cellular components to achieve this motility.

In 1982 Sansonetti and co-workers found a 230 kb (kilobase) so called virulence plasmid to be required for *Shigella* invasion into colonic epithelial cells (Sansonetti *et al.*, 1982). Subsequently, the 4 kb virG region on this virulence plasmid was shown to be required for cell-to-cell spread of *Shigella* (Makino *et al*., 1986). The virG region was found to encode for a 120 kDa protein named IcsA for intracellular spread A by researchers at the Pasteur Institute in Paris (Bernardini *et al*., 1989). Independently the same protein was identified at the University of Tokyo and the Walter Reed Army Institute of Research from different strains of *Shigella flexneri* (Lett *et al*., 1989) (strain YSH6000), (Pal *et al*., 1989) (strain M90T) and (Bernardini *et al*., 1989) (strain M90T)). Bernardini and co-workers could further show that the intracellular spread of *Shigella* was dependent on F-actin which accumulated and resulted in an actin tail behind the bacterium. Furthermore, the formation of actin tails was dependent on the presence of IcsA (Bernardini *et al*., 1989). This report represented the first in a series describing actin-based motility of bacterial pathogens.

How does IcsA mediate actin-based motility? Most of the motile *Shigella* were shown to be undergoing cell division explaining the oriented phenotype described by Ogawa and co-workers (Prevost *et al*., 1992). IcsA is a 1102 amino acid protein with its N-terminus (α-domain) extending from the bacterial surface and the β-domain buried in the bacterial membrane (Lett *et al*., 1989; Suzuki *et al*., 1996) (see Figure 6). There is no obvious sequence similarity between IcsA and ActA (Domann *et al*., 1992; Kocks *et al*., 1992). IcsA can be phosphorylated by protein kinase A (d'Hauteville and Sansonetti, 1992) and cleaved into a 95 kDa secreted protein (Goldberg and Sansonetti, 1993). Like ActA on *Listeria*, IcsA is localized to the bacterial pole adjacent to the actin tail (d'Hauteville *et al*., 1996). Cleavage of IcsA is mediated by the bacterial protease IcsP and deletion of IcsP from the genome leads to a 27% increase in the mean intracellular motility as well as a 23% increase in the number of *Shigella* moving (Shere *et al*., 1997). As with many experiments in the field that use different strains of bacteria, findings showing that disruption of the respective IcsA proteases or mutation of the cleavage site in IcsA leads to shorter bacterial projections from cells (Egile *et al*., 1997) or a more rapid spread through an epithelial monolayer (d'Hauteville and Sansonetti, 1992) remain controversial (Fukuda *et al*., 1995; Shere *et al*., 1997). It still remains to be established how minor differences in parameters that affect intercellular spread in tissue culture cells would contribute to the virulence of a pathogen in the wild (see below).

IcsA, like ActA, has been expressed on the surface of *E.coli* and confers actin-based motility to this bacterium (Goldberg and Theriot, 1995; Kocks *et al*., 1995). In the same experiments, however, *E.coli* expressing IcsA move approximately 25% faster than *E.coli* expressing ActA (Goldberg and Theriot, 1995; Kocks *et al*., 1995). Deletion of the N-terminal amino acids 1-507 of IcsA on *Shigella* results in the loss of actin tail formation, although the protein is localized in a polarized fashion on the bacterial pole (Suzuki *et al*., 1996). Thus, it is the N-terminal half of IcsA that recruits host factors for actin tail initiation. A polar distribution of IcsA seems to be required for actin-based motility as deletion of amino acids 508 - 729 results in a uniform distribution of the protein on the bacteria which are still able to nucleate actin, but fail to form an actin tail (Suzuki *et al*., 1996). Deletion of amino acids 421 - 482 of IcsA abrogated actin tail formation (Charles *et al*., 1999). However, competition analysis with a polypeptide corresponding to these 62 amino acids did not alter the speed of motile *Shigella* or the number of motile bacteria in *Xenopus* egg extracts (Charles *et al*., 1999). The authors of this study conclude that deletion of amino acids 421-482 leads to a disruption of the native conformation of IcsA necessary for recruiting host components required for actin tail formation (Charles *et al*., 1999). We still await a definitive study on the identification of the domain of IcsA important for recruitment of the actin nucleation machinery.

Which cellular cytoskeletal protein(s) interact with IcsA? Possible roles in the actin-based motility of *Shigella* were suggested for the F-actin cross linking proteins α-actinin, filamin, fimbrin and the focal adhesion protein vinculin (Dold *et al*., 1994; Kadurugamuwa *et al*., 1991; Prevost *et al*., 1992). Of these only vinculin has been shown to interact with IcsA (Suzuki *et al*., 1996). The 95 kDa head domain of vinculin interacts with the glycine rich repeats of the IcsA α-domain (Suzuki *et al*., 1996). A series of experiments using micro-injection of peptides and proteins into infected cells is consistent with a functional role of vinculin in the actin-based motility of *Shigella* (Laine *et al*., 1997; Zeile *et al*., 1996). First, the injection of a peptide matching the second ActA oligo-proline repeat, which has been shown to terminate intracellular movement of *Listeria*, also inhibited the intracellular motility of *Shigella*, suggesting that a cellular ActA homologue is recruited by IcsA (Zeile *et al*., 1996). Work from the same laboratory then showed that an antibody raised against this peptide inhibits *Shigella* motility when micro-injected into infected cells (Laine *et al*., 1997). The authors then show that this antibody cross-reacts with vinculin and that infection of PtK2 cells by *Shigella* strain M90T specifically generated the vinculin head domain by

proteolytic cleavage of about 7% of the cellular vinculin, while other proteins like α-actinin were not cleaved (Laine *et al*., 1997). Micro-injection of the vinculin head domain resulted in a three fold acceleration of motile *Shigella* (Laine *et al*., 1997). Interestingly, however, when vinculin-deficient cells were infected neither the rates of moving *Shigella* nor the percentage of motile bacteria per cell differed from the respective values determined in the parental, vinculin containing cells (Goldberg, 1997). Furthermore a number of laboratories were not able to see vinculin cleavage upon infection of HeLa cells with the M90T variant strain SC301 (Suzuki *et al*., 1998 and C. Egile, peronal communication). Finally, vinculin is not required for the *in vitro* reconstitution of the actin-based motility of *E. coli* expressing IcsA (Loisel *et al*., 1999).

To date, the best candidate for a downstream effector binding IcsA and mediating actin tail formation is N-WASP (Suzuki *et al.*, 1998)(Figure 6). N-WASP has been shown to bind to the glycine rich region of IcsA via the WH2 domain (Suzuki *et al*., 1998). Expression of an N-WASP construct with a deletion in the WH2 domain in infected cells decreased the ability of *Shigella* to induce actin tails (Suzuki *et al*., 1998). However, recent data suggest that N-WASP is recruited to IcsA via at least one different targetting domain which is distinct from the one reported by Suzuki and co-workers. Experiments I conducted in collaboration with Violaine Moreau show that the CRIB domain of N-WASP is recruited to *Shigella* (Moreau, Frischknecht and Way, submitted). Similar results have been obtained in the group of Philippe Sansonetti at the Pasteur Institute (C. Egile, personal communication). These findings underline the suggestion that actin tail formation of *Shigella* mimics Cdc42 mediated actin polymerization (Egile *et al.*, 1999) and are consistent with a recent report showing that GTPases of the Rho family do not play a role in the actin-based motility of *Shigella* (Mounier *et al.*, 1999).

The binding of N-WASP via its CRIB domain to IcsA therefore, seems to mimic Cdc42 mediated activation by opening up N-WASP thereby promoting actin-based motility (Egile *et al*., 1999). That this series of events seems the most likely to occur *in vivo* gains further support from *in vitro* experiments showing that *Shigella* cannot move in extracts lacking N-WASP but are motile in an Arp2/3 complex dependent fashion if N-WASP is present in the extract (Egile *et al.*, 1999; Loisel *et al.*, 1999). A model of possible protein-protein interactions required to mediate actin polymerization at the *Shigella* surface is shown in figure 7.

Interestingly, the actin-based motility of *Shigella* by itself is not enough for efficient cell-to-cell spread. Additional assistance of a number of other bacterial and host factors is required. IcsB is a 54 kDa protein which is thought to be needed for efficient lysis of the bacterial vacuole that forms after intercellular protrusions projected bacteria from infected into neighboring cells (Allaoui *et al.*, 1992). A similar lysis step is also required for *Listeria* spread (Vazquez *et al.*, 1992). Further, *Shigella* spread from cell to cell is reduced in S180 cells lacking cadherins (Sansonetti *et al.*, 1994). Ultrastructural analysis of bacterial protrusions into cells demonstrate the similarity of these projections with cell-cell junctions which could explain why certain proteins are only found in inter- but not intra-cellular actin tails (Sansonetti *et al*., 1994). Stable transfection of the cadherins L-CAM or N-cadherin in S180 cells results in a rescued cell-to-cell spread (Sansonetti *et al*., 1994). Like these adhesion molecules, the membrane associated protein ezrin is also found exclusively in actin tails projecting from the surface of cells (Table 2) (Sechi *et al*., 1997). A role for ezrin in cell invasion by *Shigella* and in cell-cell contact formation has been suggested (Hiscox and Jiang, 1999; Skoudy *et al*., 1999). These data show that the intercellular spread of intracellular pathogens is a complex process that is still not fully understood and involves the abuse of more than just one host system.

1.4.3 Actin-based motility of *Rickettsia*

Bacteria of the genus *Rickettsia* are divided into three groups, the spotted fever group, typhus group and the scrub group. *Rickettsia* are the causative agents of a variety of human diseases including Rocky Mountain spotted fever caused by *R. rickettsii*, Mediteranean spotted fever caused by *R. conorii* and typhus caused by *R. prowazekii* (endemic typhus) or *R. typhi* (murine typhus) (Winkler, 1990).

In 1957 Schaechter, Bozeman and Smadel observed motile *Rickettsia* in infected cells and fibrillar projections with the occational release of a bacterium into the culture medium using phase contrast microscopy (Schaechter *et al.*, 1957). Twenty-five years later it was recognized that *Rickettsia conorii*, *Rickettsia rickettsii* and *Rickettsia typhi* are able to nucleate actin tails similar to those of *Shigella* and *Listeria* (Heinzen *et al*., 1993; Teysseire *et al*., 1992). It was noted that only a small subset of *Rickettsia typhi* showed actin tails (0.8% of intracellular bacteria after 48 hpi compared with 53.9% of *R. conorii*) and that those formed were shorter (1.6 ± 0.8 μm compared to

3.1 ± 1.0 µm for *R. conorii* in HUVEC cells) (Teysseire *et al*., 1992). Interestingly, a comparative study of virulent and avirulent strains of the spotted fever group of *Rickettsia* showed that actin tail formation, unlike for *Listeria* and *Shigella*, was not linked to virulence, but only to the spread through a tissue culture monolayer as assayed by plaque formation (Heinzen *et al*., 1993). Other differences between the actin tails of *Rickettsia* and those observed on *Listeria* or *Shigella* are evident: occasional long actin tails could be detected in *Rickettsia* infected cells treated with 0.5 µg/µl cytochalasin D but not in cells infected with *Listeria* or *Shigella* indicating that *Rickettsia* induced actin tails are more stable (Heinzen *et al*., 1993). This concentration of cytochalasin D normally disassembles cellular actin filaments. The suggestion that *Rickettsia* induced actin tails are unusually stable is solidified by the observation that in Vero cells transiently expressing GFP-actin the average half-life of an actin tail was three times longer for *Rickettsia* induced actin tails compared to *Listeria* induced actin tails (Heinzen *et al*., 1999). Interestingly, *Rickettsia* move in these cells at a speed of 4.8 ± 0.6 µm/min compared to 12.0 ± 3.1 µm/min for *Listeria* (Heinzen *et al*., 1999). Strangely, in Vero cells not expessing GFP-actin faster motilities than those found by Heinzen and colleagues were determined for intracellular bacteria (*Rickettsia*: 8 ± 1 µm/min, *Listeria*: 22 ± 5 µm/min and *Shigella*: 26 ± 5 µm/min) (Gouin *et al*., 1999). At a similar stability the tail should be longer the faster a pathogen moves (Theriot *et al*., 1992). Therefore the actin turnover in a *Rickettsia* tail must be slower than in an actin tail induced by *Listeria*. Interestingly, *Rickettsia* were able to move in *Xenopus* egg extracts, again with lower speed as *Listeria*, while *Shigella* are not able to move under the same conditions (Gouin *et al*., 1999). Intra-nuclear actin tails were described for *Rickettsia* but not *Listeria* or *Shigella* (Heinzen *et al*., 1999). It appears that actin-based motility is driving *Rickettsia* actively from the cytoplasma into the nucleus where *Rickettsia* proliferate (Heinzen *et al*., 1999).

Interesting details of the *Rickettsia* actin tail ultrastructure are evident from stunning electron micrographs of infected cells (Gouin *et al*., 1999). The actin filaments are arranged into long, loose bundles, rather than into a dense cross-linked network as described for *Listeria* (Gouin *et al*., 1999). Finally, immunolocalization experiments show that VASP and α-actinin are found in actin tails of *Rickettsia*, while the Arp2/3 complex, cofilin and CapZ are not detected, although they are present in actin tails of *Listeria* and *Shigella* (Gouin *et al*., 1999).

Little is known about actin tail formation of *Rickettsia*. Inhibition of bacterial protein synthesis leads to the loss of actin tail formation, while inhibition of host cell protein synthesis did not (Heinzen *et al*., 1993). The rickettsial outer membrane protein OmpA has been suggested to be involved in actin tail formation as it is absent from strains that do not make actin tails (Heinzen *et al*., 1993). However, no study examining the role of OmpA in *Rickettsia* motility has yet appeared in the literature. Two discontinuous domains that are repeated 13 times in OmpA of *R. rickettsii* have a high homology (50% and 34% amino acid identity, respectively) to IcsA (amino acids 450-469 and 473-498 of the α-domain, repectively) (Charles *et al.*, 1999; Heinzen *et al.*, 1993). Deletion of these IcsA sequences did indeed abolish actin tail formation of *Shigella* (Charles *et al*., 1999). However, replacement of the IcsA sequences with the rickettsial repeat domain did not rescue actin tail formation (Charles *et al*., 1999, see also chapter 1.4.2). Taken together the current data suggest that actin tail formation of *Rickettsia* is fundamentally different from the other pathogens. Future studies on the mechanisms and molecules involved in actin tail formation of *Rickettsia* will surely provide exciting new insights into the different ways actin polymerization can be achieved by pathogens and possibly by the cell.

1.4.4 Actin pedestals of EPEC

Enteropathogenic Escherichia coli (EPEC) is the major causative agent of infantile diarrhoea in developing countries (Levine and Edelman, 1984). EPEC infects the intestinal mucosa resulting in specific so called attaching and effacing (A/E) lesions. A/E lesions are characterized by local effacement of the microvilli and intimate attachment of the bacteria to the surface of the cell (Goosney *et al*., 1999). EPEC does not enter cells but uses a tye III secretion system to translocate bacterial proteins into the mammalian cell cytoplasm (Jarvis *et al*., 1995). One of the 'injected' proteins is the translocated intimin receptor Tir. (Kenny *et al*., 1997; Kenny and Finlay, 1997). Tir is inserted into the epithelial plasma membrane and binds to the bacterial surface protein intimin (de Grado *et al*., 1999; Kenny *et al*., 1997). This interaction is thought to bind EPEC to the surface of the cell, although other bacterium-host interactions are likely (Goosney *et al.*, 1999). The cytoplasmic tail of Tir is phosphorylated on tyrosine residues (Rosenshine *et al.*, 1996). Phosphorylation of tyrosine 474 results in a striking effect: the actin cytoskeleton is rearranged into a pedestal like structure that pushes the plasma membrane with the attached bacterium up to 10 μm

from the cell (Kenny, 1999; Rosenshine *et al*., 1996). Attached EPEC can surf on these pedestals along the cell surface with speeds of 0.4 μm/min (Goosney *et al*., 1999; Sanger *et al*., 1996).

EPEC pedestals contain a number of actin cytoskeletal proteins including α-actinin, myosin light chain, ezrin, talin and villin, a protein specifically found in microvilli of epithelial cells (Goosney *et al*., 1999; Sanger *et al*., 1996). Also Arp2/3 complex and N-WASP are localized to the pedestals (Kalman *et al*., 1999). The small GTPase CHP recruits N-WASP to the pedestal by binding the CRIB domain but, unlike NWASP and Arp2/3 complex, plays no functional role in pedestal formation (Kalman *et al*., 1999). To date, it is not known which protein(s) links N-WASP with phosphorylated Tir. Interestingly, cytochalasin D for example does not depolymerize EPEC pedestals (Sanger *et al*., 1996). Future research will certainly provide some interesting details into how this pathogen regulates actin polymerization.

1.4.5 Actin-based motility of vaccinia virus

The first report showing single vaccinia virions on the tip of large microvilli-like projections dates back more than 20 years to a high-voltage electron microscopy study (Stokes, 1976). Several follow-up studies confirmed that actin as well as the actin crosslinking proteins α-actinin, fimbrin and filamin were localized to these projections (Hiller *et al*., 1981; Hiller *et al*., 1979). An active role of actin in viral spread was shown when cytochalasin D was found to prevent the release of viruses (Payne and Kristensson, 1982). Using phase contrast video-microscopy it was shown that viruses are propelled through the cytoplasm of HeLa cells with an average speed of 2.8 μm/min before they project out from the cell upon encountering the plasma membrane (Cudmore *et al*., 1995). Using a recombinant virus lacking the intracellular enveloped virus (IEV) specific protein F13L and a drug which inhibits the wrapping of the intracellular mature virus (IMV) with TGN membranes, the same authors show that IEVs but not IMVs form actin tails (Cudmore *et al*., 1995). Electron microscopy showed that actin is only associated to one of the broad sides of the virus (Cudmore *et al*., 1996). Using a permeabilized cell system it was shown that rhodamine labelled actin monomers are incorporated into the actin tail at the virus surface (Cudmore *et al*., 1996). Interestingly, rhodamine actin was not incorporated along the tail, suggesting that filaments in the tail are capped on their barbed and pointed ends (Cudmore *et al*., 1996). This suggests that monomeric actin is only recruited to the virus surface where it is assembled into the actin tail.

Decoration experiments with the S1 head domain of myosin revealed that the actin filaments in an actin tail are oriented with their barbed end to the virus (Cudmore *et al.*, 1996). The same orientation has been observed for *Listeria, Shigella* and *Rickettsia* (Gouin *et al*., 1999; Tilney and Portnoy, 1989). Interestingly, when IEVs encounter the plasma membrane and fuse, they are able to transfer their actin nucleation site to the plasma membrane causing filopodia-like outgrowth due to actin nucleation in the absence of the virus (Cudmore *et al*., 1996). The same polarity of actin filaments as in the virus and the bacteria is also observed at the leading edge of a motile cells with the barbed end being close to the plasma membrane (Figure 4).

First steps were taken to further understand the mechanism of how vaccinia achieves actin tail formation (Higley and Way, 1997; Reckmann *et al*., 1997; Zeile *et al*., 1998). The only homology found between a vaccinia ORF, F8L, and a bacterial actin tail nucleator, iActA, from *Listeria ivanovii* which also makes actin tails (Karunasagar *et al.*, 1993) was weak. Deletion of F8L resulted in a virus that formed actin tails indistinguishable from actin tails formed by the wild type virus (Higley and Way, 1997). Identification of an 11 kDa viral protein, that was previously localized to actin tails, as the product of the F17R gene showed it was not involved in actin tail formation. It appears that F17R associates with actin by virtue of its basic nature. (Hiller and Weber, 1982; Reckmann *et al*., 1997). Micro-injection of poly-proline rich peptides corresponding to the ones found in ActA and zyxin inhibited vaccinia motility (Zeile *et al.*, 1998). The authors speculate that zyxin might play an essential role in the actin-based motility. This, however, turned out not to be true (Frischknecht *et al*., 1999a).

1.5 Aim of this thesis

When I started this thesis, the actin-based motility of vaccinia virus had just been (re-)discovered in the group of my supervisor (Cudmore *et al*., 1995; Cudmore *et al*., 1996). The goal of this thesis was to identify host and viral proteins involved in the actin-based motility of vaccinia virus which were still elusive at the start of my thesis.

1.6 Brief summary of results

I started my project with a comparative immuno-localization study of actin-binding and focal adhesion proteins in cells infected with vaccinia, *Listeria* and *Shigella*, to investigate if vaccinia recruits similar cellular components as the bacterial pathogens. In this part of the study I was able to show that a tyrosine phosphorylated protein was present at the site of vaccinia actin tail formation, but was absent from tails induced by *Listeria* and *Shigella* (Frischknecht *et al*., 1999a). In collaboration with Dr. Chris Sanderson it became clear that one or more of the IEV specific proteins was responsible for recruiting the actin nucleation machinery (Sanderson *et al*., 1998a; Röttger *et al*., 1999). I could show that a number of proteins become tyrosine phosphorylated during vaccinia infection and that one of these was the IEV specific membrane protein A36R. I then showed that tyrosines 112 and 132 of A36R were phosphorylated and that this modification was essential for actin tail assembly. I was further able to show that src family kinases are involved in the actin based motility of the virus. I also showed that phosphorylation of A36R results in the recruitment of Nck, Grb2 and N-WASP to the site of actin tail formation. Subsequently Nck and N-WASP were shown to play and essential role in the actin-based motility of vaccinia, suggesting that vaccinia mimics receptor tyrosine kinase signalling to achieve actin-based motility (Frischknecht *et al*., 1999b).