Signalling of pathogens to the actin cytoskeleton

Characterisation of the N-WASP/WIP complex in the actin based motility of EPEC, *Shigella* and vaccinia virus

Markus Zettl

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London Research Institute
Cancer Research UK
44 Lincoln's Inn Fields
London WC2A 3PX

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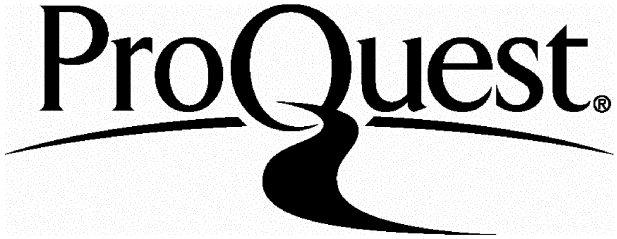

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Abstract

Surprisingly organisms like viruses and bacteria developed common mechanisms of exploiting the host machinery for actin polymerisation mostly to spread their infection. A complex of N-WASP and WASP interacting protein (WIP) plays an important role in actin-based motility of vaccinia virus and is as well recruited to the surface of the *gram*-negative bacterium *Shigella flexneri*. Both processes are dependent on actin polymerisation, which is nucleated by the Arp2/3 complex. In contrast to many other N-WASP binding proteins, WIP does not stimulate the ability of N-WASP to activate the Arp2/3 complex *in vitro*. Although the WASP homology 1 (WH1) domain of N-WASP interacts directly with WIP information is lacking concerning the nature of its binding site, which could help to understand the role of WIP *in vivo*.

This work reports the identification of the N-WASP WH1 binding motif in WIP, which turns out to be conserved in WIP homologues. To confirm our biochemical results *in vivo* we examined the effects of expressing WIP mutants deficient in N-WASP binding on actin based motility of *Shigella* and vaccinia. Expressions of these mutants led to a loss of recruitment of WIP to both pathogens and abrogated the inhibitory effects of the WASP binding domain (WBD) on vaccinia actin tail formation.

Enteropathogenic *E.coli* (EPEC) like vaccinia remodels the host actin cytoskeleton and uses WASP and the Arp2/3 complex to form actin rich pedestal depending on tyrosine phosphorylation of pathogen surface membrane proteins. WIP was found to localise to the tip of actin pedestals and to be functionally involved in EPEC induced pedestal formation. Like vaccinia virus EPEC recruits WIP through its proline rich and WASP binding domain. The proline rich domain of WIP binds to the SH2/SH3 adaptor protein Nck, which is essential for EPEC actin pedestals and vaccinia actin tail formation. Deletion of the proline rich region of WIP but not N-WASP was essential for WIP recruitment to EPEC pedestals. Furthermore in absence of N-WASP neither WIP nor Nck were recruited to EPEC. Taken together data in this thesis suggest that EPEC like vaccinia recruit a complex of Nck, WIP and N-WASP. A comparison of vaccinia and EPEC however shows that the complex is recruited in differently and that in contrast to vaccinia actin tail, EPEC pedestal formation is independent of Src kinases.

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Chapter 1

1 Introduction

Cell migration is important throughout our life as humans and can even contribute to our death. Migratory phenomena play an important role in embryonic development in processes such as gastrulation, during which large groups of cells migrate together in a sheet to form the precursor of the gut (Ip and Gridley, 2002). Similar migrations can be observed in the adult. In the renewal of skin fresh epithelial cells migrate up from the basal layer to replace dead epithelial cells (Alonso and Fuchs, 2003). Migration is also a prominent component of wound healing and immune surveillance, in which leukocytes from the circulation migrate into the surrounding tissue to destroy invading micro organisms (Fenteany *et al.,* 2000; Muller, 2003).

Migration contributes to several important pathological processes, including, chronic inflammatory diseases such as rheumatoid arthritis and multiple sclerosis, cancer, and mental retardation (De Strooper and Annaert, 2000; Frangiskakis *et al.,* 1996; Hood and Cheresh, 2002; Markovic-Plese and McFarland, 2001). Thus, understanding the fundamental mechanisms underlying cell migration could lead to the identification of potential drug targets thus allowing therapeutic approaches for treating disease.

In order to understand the basic principles and signalling processes underlying cell migration, motile tissue culture cells together with pathogens have a long-standing tradition as model systems. The work in this thesis, concerns signalling processes pathogens exploit to become motile to facilitate their cell to cell spread. By way of introduction a general description of cell motility, relevant signalling pathways and the pathogens used in this study follows.

1.1 Basic principle of cell motility

The migration of cells in culture, which was described in detail by Abercrombie more than 30 years ago, can be broadly broken down into a number of individual steps (Abercrombie and Heaysman, 1976) (Figure 1.1). The initial response of a cell to a motility-promoting agent is to polarise and extend protrusions in the direction of movement. These protrusions are broad, thin actin-rich structures, known as lamellipodia (Small *et al.*, 2002). The extension of the lamellipodium is largely driven by actin filament polymerisation (Figure 1.3) (Krause *et al.*, 2003; Pollard and Borisy, 2003) and can either retract or establish contact with the extra cellular matrix at sites called focal complexes (Geiger and Bershadsky, 2001). These initial sites of cell adhesion can later mature into larger structures called focal adhesions, which provide contact with the substratum. These adhesions serve as traction sites for migration as the cell moves forward over them. After establishing new adhesions at the leading edge, myosin II based contraction of the actin cytoskeleton pulls the main body of the cell forward (Figure 1.1) (Verkhovsky *et al.*, 1999). Myosin II is an actin filament binding motor protein, which moves oppositely, oriented actin filaments past each other (Korn and Hammer, 1988). The force needed for traction is generated by the interaction of myosin II with actin filaments that attach to focal adhesions (Beningo *et al.*, 2001). The activity of myosin II is activated by phosphorylation of its myosin light chain (MLC) (Bresnick, 1999). Phosphorylation of MLC by myosin light chain kinase (MLCK) promotes the interactions of myosin with actin filaments to drive contraction (Tan *et al.*, 1992). Adhesion disassembly is an important feature for cell migration and is observed at the leading edge where it coincides with the formation of protrusion as well as the cell rear where it is responsible for tail retraction. At the base of a protrusion new adhesions disassemble and form at the leading edge as the cell moves forward (Webb *et al.*, 2002). The last step in migration involves the release of the rear of the cell where adhesions must be disassembled. High tension exerted on the rear adhesions is thought to be the major factor contributing to detachment of the tail (Lauffenburger and Horwitz, 1996). *Dictyostelium* cells lacking myosin II show impaired tail retraction suggesting that the tension generated by myosin II plays an important role in disassembly of rear adhesions in a migrating cell (Chung *et al.*, 2001).

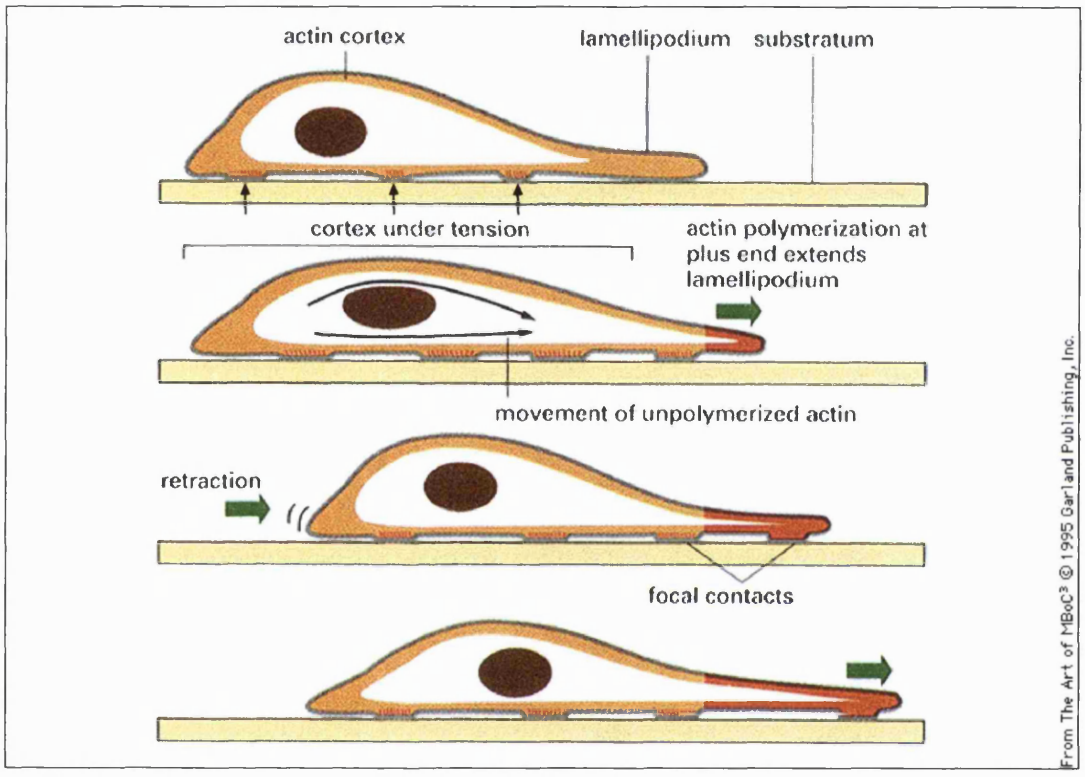


Figure 1.1. Model of cell migration.
An illustration of a cell moving along a two-dimensional substrate is shown. The cell is connected to the substratum via focal contacts (black arrows). It extends a thin actin rich protrusion, called a lamellipodium, in the directon of movement. Moving in the indicated direction (green arrow), by assembling new actin (red), the cell forms new focal contacts beneath the extending lamellipodium. Old focal contacts at the rear are dissolved to allow retraction, thereby allowing forward movement. In a moving cell the same cycle is repeated over and over again moving a cell forward. (Figure taken from Alberts *et al.*, Molecular Biology of the Cell, Third Edition)

1.2 Actin drives cell migration

Actin exists as globular 42kDa protein (G-actin), which can be assembled into a double helical filament (F-actin). In an actin filament G-actin subunits are arranged head to tail giving the filament a molecular polarity (Figure 1.2C). Based on the arrowhead pattern observed by electron microscopy when actin filaments are labelled with the S1 motor domain of myosin, one end is called the pointed end and the other the barbed end (Figure 1.2C).

Looking at lamellipodia by electron microscopy it becomes evident that actin filaments are the most dominant structural component (Svitkina and Borisy, 1999). In motile cells the barbed ends are always oriented towards the leading edge of the lamellipodium (Small and Celis, 1978). Remarkably, directional motility seems to be an autonomous property of the leading edge as isolated lamellipodia of keratocytes, lacking nuclei, centrosomes, microtubules and most organelles still retain the capacity of directional movement (Euteneuer and Schliwa, 1984; Verkhovsky *et al.*, 1999).

The force driving protrusion is generated at points where the growing barbed ends of actin filaments push the cell membrane (Forscher *et al.*, 1992; Mogilner and Oster, 2003). This mechanism is thought to rely on a ratchet mechanism generated as actin monomers are added to the growing filaments at the plasma membrane (Mogilner and Oster, 2003). According to this model, the bending of filaments due to thermal fluctuations makes room for actin monomers to polymerise onto their barbed ends (Mogilner and Oster, 1996). This allows the actin filaments to act like a molecular "spring" and exert a pushing force on the membrane.

Consistent with the notion that actin is the driving force for cell motility, microinjection of fluorescent G-actin show that lamellipodia are the primary sites of actin incorporation (Glacy, 1983). Subsequent work demonstrated that labelled actin binds to the fast growing barbed ends of actin filaments (Mogilner and Oster, 1996; Theriot and Mitchison, 1991). However, photo-bleaching experiments show that the actin cytoskeleton in fast moving cells stays stationary compared to the cell body (Theriot and Mitchison, 1991). Both observations together showed that the rate of actin

polymerisation at the barbed ends directly correlates with protrusion rate of a motile cell.

Direct evidence that actin polymerisation is involved in cell motility comes from observations that cell motility is strongly inhibited by treatment with drugs that inhibit F-actin polymerisation such as cytocalasin D (CD) (Cooper, 1987). CD used at low concentrations inhibits the association of G-actin monomers with the fast growing barbed ends of F-actin filaments thereby stopping filament elongation *in vitro* (Sampath and Pollard, 1991). Used on living cells CD treatment showed that lamelliopdial protrusion in motile cells is inhibited suggesting that G-actin monomer addition to barbed ends is essential for cell motility (Forscher and Smith, 1988; Yahara *et al.*, 1982). Thus understanding how G-actin forms actin filaments is fundamental to understand cell motility.

1.3 Actin polymerisation *in vitro.*

The rate limiting step of actin filament nucleation *in vitro* is the formation of a G-actin trimer, which is a relatively rare event since the interactions between G-actin subunits are stabilised by multiple interactions between adjacent subunits (Figure 1.2A). Actin can be labelled with a fluorescent dye such as pyrene. The fluorescence of pyrene-actin *in vitro* increases up to 20 fold when actin polymerises, which can be quantified by measuring the increase of fluorescence (Cooper *et al.*, 1983) (Figure 1.2B). The observed lag phase in these actin polymerisation assays is due to the rate-limiting step of actin trimer formation, which is concentration dependent. However, when this initial nucleation event is overcome "newborn" filaments grow as G-actin monomers add to the exposed ends of the actin polymer. The growth rate is dependent on how often actin monomers collide with barbed ends. Thus the filament growth rate is directly proportional to the concentration of G-actin monomers. However, G-actin subunits will leave the filament at constant rate independently of the concentration of free actin monomers. The concentration for which G-actin addition to the filament equals G-actin loss is called the critical concentration. At the critical concentration the actin filament grows and shrinks with the same rate so the filament stays the same length. If the G-actin concentration is above the critical concentration the actin filament will grow and shrink when the G-actin concentration is below the critical concentration. It follows

that to initiate growth the G-actin concentration has to be above the critical concentration.

Additional polarity of an actin filament is brought about by the ATP turnover of ATP-G-actin subunits as the actin filament ages (Figure 1.2C). As a result the critical concentration is lower at the barbed end (0.1 μM) and higher at the pointed end (0.7 μM). If both filament ends are exposed, actin filament growth will occur until the concentration of free G-actin monomer will reach a value above the critical concentration for the barbed end and below for the pointed end. At this steady state, G-actin monomers assemble at the barbed end and disassemble at the pointed end with the same kinetics. This property of actin filaments leads to a net flux of G-actin subunits through the filament, a process known as treadmilling. Early observation using photobleaching techniques suggested that treadmilling provides a possible mechanism for cell motility *in vivo* (Wang, 1985). However additional regulatory mechanisms have to be postulated in order to explain the complex phenomenon of cell motility.

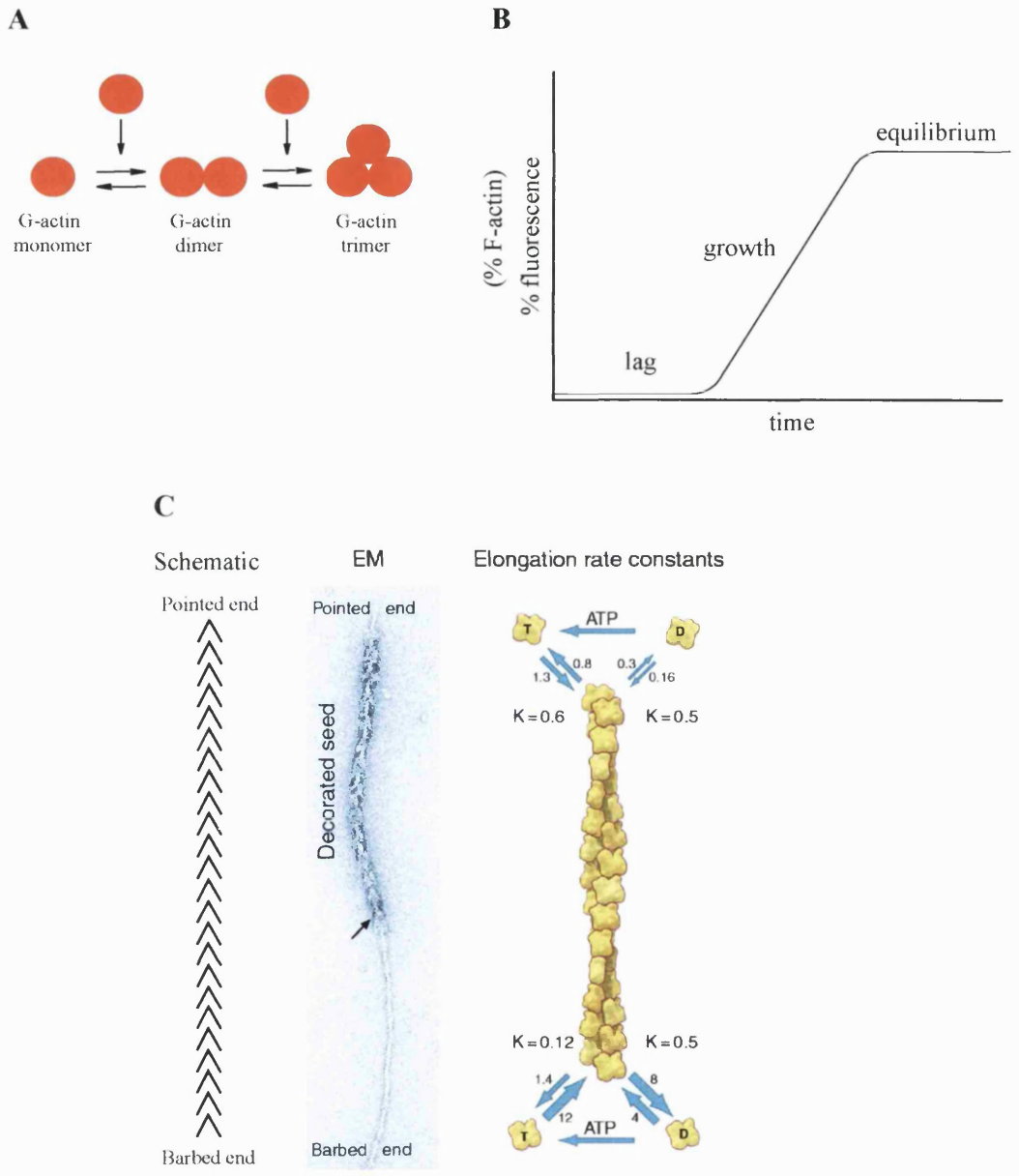


Figure 1.2. Actin filament nucleation, polymerisation and molecular polarity. **(A)** A G-actin (red) dimer is not stable since contacts between multiple adjacent subunits stabilise G-actin interactions. Thus the addition of a third G-actin monomer results in the formation of a more stable G-actin trimer. Further addition to a stable actin trimer takes place to form an actin filament. Hence the G-actin trimer acts as a filament nucleation site. **(B)** The formation of a nucleation site explains the lag phase observed in a typical *in vitro* actin growth curve. The amount of actin polymer (quantified as a % increase in fluorescence, see section 1.3 for further explanation) increases over time as G-actin monomers add to the exposed ends of actin filaments. The equilibrium phase is reached when G-actin monomer loss equals G-actin monomer addition. **(C)** EM shows and the schematic illustrates an actin filament seed decorated with S1 motor domain of myosin heads (arrow), which was elongated with ATP-actin (bright region in the direction of barbed ends). Cartoon shows an actin filament forming a helical structure. The ratio of the dissociation rate constant to the association rate constant gives K. Note that the equilibrium constants (K) for ATP-actin (T) differ at the two ends, giving rise to slow steady state treadmilling. (Figure C modified from Pollard and Borisy, 2003)

1.4 Actin polymerisation *in vivo.*

In vitro measurements of actin polymerisation suggest that actin monomers are added to a filament more efficiently only when bound to ATP and leave the filament in an ADP bound state. Thus the cell has to have mechanisms in place to regenerate the pool of ATP bound to G-actin monomers to form actin filaments. Profilin binds to G-actin and enhances the exchange of ADP-actin to ATP-actin (Sohn and Goldschmidt-Clermont, 1994). In the majority of cell types the actin concentration is way above the critical concentration, determined *in vitro* with pure actin. Actin monomers are therefore buffered, by being bound to proteins such as profilin or thymosin-β4 thus preventing spontaneous actin polymerisation *in vivo* (Pollard and Borisy, 2003). Profilin also binds to phosphatidyl 4,5-bisphosphate (PIP_2) (Sohn *et al.,* 1995), which prevents the association of G-actin with profilin and thus serves as a potential mechanism to disrupt the ATP-actin/profilin complex (Carlier *et al.,* 1993). It is thought that PIP_2 mediated release of ATP-actin from profilin provides the pool of actin monomers for polymerisation where PIP_2 is generated (e.g. membranes).

Cells must be equipped with a mechanism to prevent already existing actin filaments from growing as well as to allow for fine regulation of the amount of filament growth. Capping proteins bind the ends of actin filaments and inhibit the addition of actin monomers to a filament as well as blocking the release of actin subunits in the filament (Cooper and Schafer, 2000). Like for profilin, the binding of the barbed end capping protein CapZ to the actin filament is inhibited by PIP_2 (Heiss and Cooper, 1991). Thus the production of PIP_2 in response to an extra cellular stimulus could lead to uncapping of pre-existing filaments as well as an increase of ATP-actin monomers released from profilin. In such a way filament growth would be promoted until the available actin is used up or additional regulatory mechanisms terminate filament elongation.

The actin dynamics that underlie cell motility require the assembly of actin filaments as well as their disassembly (Cramer, 1999). Uncapping of the ends of actin filaments under conditions unfavourable for polymerisation is one potential mechanism for filament disassembly. To break the filament in two pieces, a process called severing, is another option. Once ATP-actin is assembled into a filament, it is slowly hydrolysed into ADP-actin. Actin depolymerising factor (ADF)/cofilin family proteins accelerate

the off-rate of ADP-actin from filament ends or severe ADP-actin filaments (Carlier *et al.,* 1997; Chan *et al.,* 2000; Ressad *et al.,* 1999).

Can the properties of actin *in vitro* account for cell migration *in vivo*? Growth at the barbed end in steady state *in vitro* is limited by the dissociation of ADP-actin at the pointed end which would translate into filament growth rate at the barbed end of 0.04 μm/min (Pollard and Borisy, 2003). However, keratocytes can move at speeds up to 10 μm/min (Pollard and Borisy, 2003). One therefore has to postulate regulatory mechanisms that accelerate actin polymerisation rates *in vivo*. Although the availability monomeric actin and stabilisation of existing actin filaments might contribute to cellular protrusion, the regulation of actin driven protrusion is largely controlled by the availability of free barbed ends given that there are free actin monomers available (Pollard and Borisy, 2003). One can envisage three potential mechanisms to generate free barbed ends. Severing of existing filaments, uncapping of barbed ends and *de novo* actin nucleation might increase the number of polymerisation competent filament endings (Zigmond, 1996).

1.4.1 Severing by cofilin generates barbed ends *in vivo*.

Pointed ends are capped *in vivo* by specialised proteins (e.g. the Arp2/3 complex, see below) (Mullins *et al.,* 1998) probably to allow fast growth from barbed ends. Since cofilin in vitro increases the off rate of actin monomers from the pointed end, severing actin filaments is probably the physiological relevant activity of cofilin (Blanchoin *et al.,* 2000). The severing activity of cofilin is inhibited by phosphorylation mediated by LIM kinase *in vitro* (Moriyama *et al.,* 1996). It has been demonstrated that phosphorylation of cofilin by LIM kinase *in vivo* inhibits the appearance of barbed ends and consequently lamellipod extension (Zebda *et al.,* 2000). Thus cofilin seems to be the major factor responsible for severing of actin filaments *in vivo*, which contributes to the generation of free barbed ends that are able to grow and drive cell motility (Dawe *et al.,* 2003).

1.4.2 Uncapping barbed ends

New free barbed ends are also generated by regulated dissociation of barbed-end-capping proteins (e.g. CapZ) from filaments resulting in rapid actin assembly both *in*

vitro and in permeablised cells (Hartwig *et al.,* 1995; Pantaloni and Carlier, 1993). Gelsolin is a high affinity barbed-end-capping protein (Sun *et al.,* 1999). Uncapping, as monitored by gelsolin dissociation from actin, occurs in protruding lamellae and on rocketing vesicles, with the correct spatio-temporal properties to provide sites of actin filament polymerisation during protrusion (Allen, 2003). These observations are consistent with models where uncapping of existing actin filaments provides free barbed ends for filament elongation. However, the mechanism by which the dissociation of gelsolin from barbed ends is regulated *in vivo* remains to be elucidated as well as the relative contribution it provides to cell motility.

1.4.3 *De novo* actin polymerisation and the discovery of the Arp2/3 complex.

De novo stimulated actin filament nucleation is generally thought to be the most important factor promoting actin polymerisation at the leading edge of migrating cells (Pollard and Borisy, 2003). However, the problem with this theory was that no cellular factors were known to have such activity. The discovery that the Arp2/3 complex stimulates *de novo* actin polymerisation focused cytoskeletal researches almost too much on this multiprotein complex. More recently however, formins were found to be able to initiate new actin filaments *in vivo* (Pruyne *et al.,* 2002). However, in contrast to the Arp2/3 complex, which nucleates branched actin network at the leading edge, formins induce polymerisation of unbranched actin filaments such as those found in structures like filopodia and the actomyosin ring (Glotzer, 2001; Small *et al.,* 1978).

1.5 The Arp2/3 complex

Actin related proteins (Arps) belong to a growing family of proteins that share 20-80% sequence similarity to actin (Schroer *et al.,* 1994). Arps participate in a diverse array of cellular processes (Schafer and Schroer, 1999). They contribute to microtubule-based motility driven by dynein, serve as integral components of large protein complexes required for chromatin remodelling and modulate assembly of conventional actin (Schafer and Schroer, 1999).

The Arp2/3 complex was originally identified in 1994 in attempts to purify binding partners for profilin in Acanthamoeba (Machesky *et al.,* 1994). It consists of two actin-related proteins (Arp2 and Arp3) and five unique polypeptides (ARPC1–5) (Machesky

et al., 1994). After its discovery the Arp2/3 complex was identified and purified from several other organisms including human and other vertebrates as well as the yeast *Saccharomyces cerevisiae* (Ma *et al.,* 1998; Rohatgi *et al.,* 1999; Welch *et al.,* 1997; Winter *et al.,* 1997). Its subunit composition is conserved in all of these organisms, suggesting the complex arose early in the evolution of eukaryotic cells.

Atomic models of Arp2 and Arp3 based on their sequences and the structure of actin provided some potential clues about their function. Both Arps were predicted to bind ATP and a divalent cation (Kelleher *et al.,* 1995). However, neither Arp2 nor Arp3 has the residues required to co-polymerise with actin, but it was speculated that an Arp heterodimer present in the profilin-binding complex might serve as a pointed end nucleus for actin polymerisation. Consistent with this hypothesis both *Acanthamoeba* Arps are localised in the cortex of *Acanthamoeba* (Kelleher *et al.,* 1995). The activity and subunit composition, prompted Welch et. *al.,* 1997, to propose that the Arp2/3 complex forms a nucleation point for actin polymerisation.

Genetic experiments have clearly pointed to an essential role for the Arp2/3 complex in the regulation of the actin cytoskeletal. In *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, deletion of Arp2/3 complex subunits results in severe growth defects or lethality (McCollum *et al.,* 1996; Winter *et al.,* 1997). Viable mutant strains exhibit gross disruptions in cortical actin cytoskeletal organisation and in actin-dependent processes such as endocytosis (Shaw *et al.,* 2001).

Several experiments have also suggested a critical role for Arp2/3 in generating and organising cellular actin arrays in mammalian tissue culture cells. Microinjection of an antibody, directed against the ARPC2 subunit, prevents lamellipodia extension (Bailly *et al.,* 1999). Overexpression studies using dominant negative constructs of N-WASP interfere with the function of the Arp2/3 complex inhibits lamellipodia protrusion (Machesky and Insall, 1998).

If the actin concentration is above the critical concentration actin filaments polymerise spontaneously. Using *in vitro* actin polymerisation assays the purified Arp2/3 complex was found to accelerate spontaneous actin polymerisation in a concentration dependent manner and to cap actin filaments at their pointed ends (Mullins *et al.,* 1998). Electron

microscopy studies confirmed that the Arp2/3 complex interacts with pointed ends and the sides of actin filaments (Mullins *et al.,* 1998). Furthermore the Arp2/3 complex generates a branched network with filament ends attached to the sides of other filaments at fixed angle of 70° *in vitro* (Mullins *et al.,* 1998). Electron micrographs of the leading edge of motile fish keratocytes showed that actin filaments are branched in the same way than *in vitro* and that the Arp2/3 complex is localised at filament branch points (Svitkina and Borisy, 1999; Svitkina *et al.,* 1997). The capacity of the Arp2/3 complex to generate branched actin networks and to promote actin filament elongation only at their barbed ends makes it an ideal candidate to control the actin network organisation at the leading edge of a motile cell.

1.6 Model for protrusion of the leading edge

Rho familiy GTPases couple signal transduction pathways to changes in the actin cytoskeleton (Hall, 1998). Extracellular stimuli can result in activation of Rho GTPases such as Cdc42 and/or the production of signalling intermediates such as PIP_2. PIP_2 directly regulates the actin cytoskeleton *in vivo* by modulating the activity and targeting of actin regulatory proteins as discussed earlier (Yin and Janmey, 2003). Cdc42 as well as PIP_2 are well known for their capacity to stimulate the activity of WASP family proteins (Higgs and Pollard, 2000; Rohatgi *et al.,* 2000). WASP proteins are signalling molecules that integrate multiple inputs leading to the activation of the Arp2/3 complex. The Arp2/3 complex gives rise to new filaments and creates a branched network of actin filaments, seen in electron microscopy images of the leading edge of a motile cell (Blanchoin *et al.,* 2000) (see 1.4). ATP-actin bound to profilin provides the source for rapid filament growth, which push the membrane forward. Each filament grows until a capping protein binds to the end of the filament and inhibit further actin monomer addition. Hydrolysis of ATP and dissociation of the θ-phosphate appear to be a molecular clock that indicates the age of a filament as well as promotes processes that disassemble actin filaments in cells. ATP hydrolysis into ADP-Pi is fast and occurs with a half time of about 2 s (Blanchoin and Pollard, 2002). It is more the relatively slow dissociation of the θ-phosphate that initiates dissasembly of actin filaments since all properties of ADP-Pi and ATP-actin filaments are the same (Pollard and Borisy, 2003). The bonds in actin filaments between ADP-actin subunits are weaker than those between ATP-actin or ADP-Pi-actin subunits. Thus ADP-actin is preferentially severed

and depolymerised by cofilin (Bamburg and Wiggan, 2002). ADP actin binds to profilin, which triggers exchange of ADP for ATP, thereby regenerating the ATP-actin, which is ready for another round of actin polymerisation (Figure 1.3).

In vivo actin filaments are produced in a branched array at leading edge of a migrating cell in a way that their pointed ends are bound at the contact with the mother filament explaining why filament growth from barbed ends are the major driving force for cell motility. Barbed ends are capped in order to focus the direction of filament growth to maintain the direction of cell migration and to keep the concentration of unpolymerised actin high. Thus it is more the actin filament array that treadmills rather than a single filament, branching and growing at the front and disassembling as the filaments grow older.

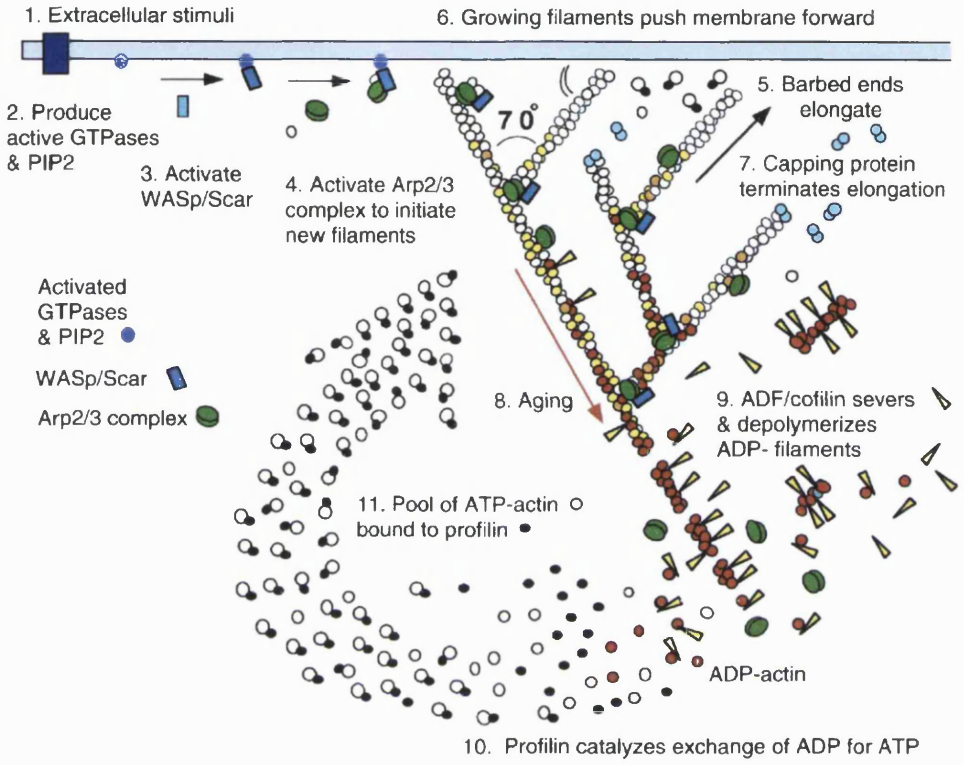


Figure 1.3. Dendritic Nucleation/Array Treadmilling Model for Protrusion of the Leading Edge

(1) Extracellular stimuli activate receptors. (2) The induced signal transduction pathways produce active Rho-family GTPases and PIP2 that (3) activate WASp/Scar proteins. (4) WASp/Scar proteins bring together Arp2/3 complex and an actin monomer on the side of a preexisting filament to form a branch. (5) Rapid actin incorporation at the barbed end of the new branch (6) pushes the membrane forward. (7) Capping protein terminates growth. (8) Filaments age by hydrolysis of ATP bound to each actin subunit (white subunits turn yellow) followed by dissociation of the γ–phosphate (subunits turn red). (9) ADF/cofilin promotes phosphate dissociation, severs ADP-actin filaments and promotes dissociation of ADP-actin from filament ends. (10) Profilin catalyzes the exchange of ADP for ATP (turning the subunits white), returning subunits to (11) the pool of ATP-actin bound to profilin, ready to elongate barbed ends as they become available. (Figure taken from Pollard and Borisy 2003).

1.7 Arp2/3 complex branching activity

Currently there are three theories as to how the Arp2/3 complex branches and generates new actin filaments. There is evidence that the Arp2/3 complex binds the side of an already pre-existing actin filament (Amann and Pollard, 2001; Bailly *et al.,* 1999). In support of this, live video light microscopy using actin filaments locked in various nucleotide states (ATP, ADP+Pi and ADP) *in vitro* suggests that the Arp2/3 complex binds preferentially to sides of filaments containing ADP- or ADP+Pi actin (Blanchoin *et al.,* 2000). This provides indirect evidence that the Arp2/3 complex does not bind to the barbed end of an uncapped filament thereby generating two new filaments.

Growth from the side of a pre-existing actin filament would predict that, overall actin polymerisation is faster when actin filaments are added to a polymerisation reaction compared to pure G-actin. Consistent with this actin filament seeds potentiate nucleation by the Arp2/3 complex (Machesky *et al.,* 1999). However, the kinetics of actin polymerisation correlated strongly with concentration of barbed ends and not with that of the F-actin seeds suggesting that filament growth is initiated by barbed ends (Pantaloni *et al.,* 2000). This suggests a model in which Arp2/3 complex branches actin filaments by binding barbed ends (Pantaloni *et al.,* 2000). In support of this model Pantaloni *et al.,* 2000 find that filament lengths following the branching point from the original "mother" compared to the newly formed "daughter" filament are similar. If branching would occur randomly along the side of the "mother" filament one would predict great differences in lengths of the mother compared to daughter filaments.

More recent data suggest a compromise between barbed end branching and side branching activity of the Arp2/3 complex. In contrast to previous observations (Blanchoin *et al.,* 2000) the branching activity of the Arp2/3 complex is higher on ATP-actin filaments than filaments polymerised with ADP-actin or ADP+Pi-actin (Ichetovkin *et al.,* 2002). Real time microscopy on growing actin filaments *in vitro* shows that branches tend to form near the fast growing barbed ends of actin filaments (Ichetovkin *et al.,* 2002). This observation has lead to the latest hypothesis that Arp2/3 complex branching occurs preferentially on sides of ATP-actin filaments rather than ADP or ADP+Pi-actin filaments (Ichetovkin *et al.,* 2002). These data does not directly rule out an involvement of the barbed end in actin filament branching, due to limits of

resolution of a light microscope. However it offers an interesting hypothesis, as how to create a branched network on ATP rich regions of a growing filament. This mechanism would bias the Arp2/3 generated branch pattern in direction of new growing filaments at the leading edge and thus be a very efficient pushing mechanism *in vivo*.

1.8 The structure of the Arp2/3 complex – implication for actin nucleation

Important insights into the actin nucleation activity of the Arp2/3 complex have come from structural studies using electron microscopy and X-ray analysis. Electron cryomicroscopy on actin filament branches shows that the complex binds the side of the mother filament suggesting that Arp2 and Arp3 are the first two subunits of the daughter filament (Volkmann *et al.*, 2001). This suggests that nucleation occurs because Arp2 and Arp3 mimic the subunits at the barbed end of an actin filament. The crystal structure of bovine Arp2/3 complex at 2.0 Å resolution reveals the structure of each subunit as well as their relationships to each other in the complex (Robinson *et al.*, 2001). From this structure it is evident that the Arp2 and the Arp3 subunit do not touch each other. Thus in this state Arp2 and Arp3 do not appear to mimic a G-actin dimer, which could serve as a nucleation point for actin filament nucleation (Robinson *et al.*, 2001). Thus activation of the Arp2/3 complex probably involves a large conformational change.

1.9 Activation of the Arp2/3 complex

Biochemically, the Arp2/3 complex nucleates actin filaments *in vitro* (Mullins *et al.*, 1998). However, as purification of the Arp2/3 complex improved, its actin nucleation activity *in vitro* decreased. To date it is generally accepted that the Arp2/3 complex on its own is inactive and unable to stimulate actin polymerisation on its own (Weaver *et al.*, 2003). Activation of the Arp2/3 complex actin nucleation activity initially requires the so-called nucleation promoting factors. However, once activated its activity is greatly stimulated by barbed ends of actin filaments (Higgs and Pollard, 2001). This mechanism creates a positive feed back loop, in which an actin filament, once its nucleated by the Arp2/3 complex additionally stimulates the Arp2/3 complex.

The first identified activator of the Arp2/3 complex was ActA (Welch *et al.,* 1998). ActA is a protein found on the surface of *Listeria* necessary to hijack the host cell actin cytoskeleton in order to induce an actin comet tail on the surface of the bacterium (Smith *et al.,* 1995) (see 1.11.4.1).

The first mammalian activators of the Arp2/3 complex were found by yeast two hybrid studies and identified as WASP protein family members (Machesky and Insall, 1998). Subsequent work established that a C-terminal acidic domain of WASP proteins is sufficient to bind and activate the Arp2/3 complex (Machesky *et al.,* 1999; Rohatgi *et al.,* 1999) (see 1.11.2).

Cortactin is an 80-kDa Src substrate, which binds actin filaments and localises to lamellipodia (Kaksonen *et al.,* 2000a; Weed *et al.,* 2000; Wu *et al.,* 1991). Cortactin binds the Arp2/3 complex (Weed *et al.,* 2000). It contains an N-terminal acidic motif (A), resembling those in other Arp2/3 complex activators (see 1.11.1), which is required for the stimulation of the actin nucleating activity of the Arp2/3 complex (Uruno *et al.,* 2001). Furthermore cortactin stabilises branches formed by Arp2/3 complex, suggesting that cortactin might serve to prolong the lifetime of dendritic networks (Weaver *et al.,* 2001).

In yeast, Abp1p (actin binding protein 1) localises to cortical actin patches, and its overexpression causes severe defects in cellular actin organisation (Drubin *et al.,* 1988). Abp1p binds and activates the yeast Arp2/3 complex (Goode *et al.,* 2001). Abp1p contains two acidic sequences similar to those found in WAVE/WASP proteins (see 1.11.1), which are responsible for Arp2/3 complex activation (Goode *et al.,* 2001). Several studies suggest that Abp1 plays a role in endocytosis which depends the Arp2/3 complex and thus Abp1p might provide a link between Arp2/3 complex-mediated actin polymerisation and endocytosis (Moreau *et al.,* 1997; Qualmann *et al.,* 2000; Wesp *et al.,* 1997).

Type I myosins are highly conserved actin-based molecular motors that localise to the actin-rich cortex of crawling cells (e.g. *Dictyoselium*) and participate in functions such as endocytosis, polarised morphogenesis, and cell migration (Pollard *et al.,* 1991). Myosin-Is (Myo3p and Myo5p) from budding as well as fission yeast contain a WASP-

like acidic domain at the COOH terminus, which is important for binding to the Arp2/3 complex (Evangelista *et al.,* 2000; Lechler *et al.,* 2000; Lee *et al.,* 2000). However, only the fission yeast Myo1p tail, containing the acidic region, was demonstrated to stimulate nucleation activity of Arp2/3 complex (Lee *et al.,* 2000).

PAN1 is an essential gene required for the internalisation step of endocytosis in yeast (Wendland and Emr, 1998). Pan1p contains an acidic stretch similar to motifs found WAVE/WASP proteins and also activates the Arp2/3 complex (Duncan *et al.,* 2001). The acidic motif of Pan1p is important in order to stimulate the actin nucleation activity of the Arp2/3 complex (Duncan *et al.,* 2001).

It appears that that all these Arp2/3 interacting proteins have developed a common or related strategy to activate the Arp2/3 complex. WAVE/WASP proteins are particular interesting because their multidomain structure allows them to interact with a variety of signalling molecules (Higgs and Pollard, 1999) linking extracellular stimuli to actin polymerisation and cell motility.

1.10 WASP family protein members

Wiskott Aldrich syndrome (WAS) gave its name to a family of proteins that are involved in the transduction of signals to the actin cytoskeleton (Carlier *et al.,* 1999; Higgs and Pollard, 2001). WAS is a rare (approximate incidence 1 in 250,000 individuals in the European population) X-linked recessive disease that involves immune dysregulation (immunodeficiency, eczema and autoimmunity) and micro-thrombocytopenia (a decreased number of small platelets) (Derry *et al.,* 1994). As a consequence WAS patients suffer from recurrent infections and complications that are related to the platelet defect, which vary from minor bruising to life-threatening haemorrhages (Imai *et al.,* 2004). Conventional therapy for WAS includes the use of antibiotics and immunoglobulin for the prevention of infections (Conley *et al.,* 2003). Splenectomy is usually effective at increasing platelet numbers and reducing bleeding complications. At present, the only curative therapy is stem-cell transplantation, although recent advances in gene-transfer technology might provide an effective alternative (Strom *et al.,* 2003).

The gene encoding Wiskott-Aldrich syndrome protein (WASP) was found to be mutated in patients suffering from WAS (Derry *et al.,* 1994). Only haematopetic cells appear to have WASP, which accounts for the restricted defects in WAS. WASP was the founding member of a family of proteins that include neural WASP (N-WASP) (Miki *et al.,* 1996), Scar (suppressor of G-protein-coupled cyclic-AMP receptor; originally isolated from *Dictyostelium* (Bear *et al.,* 1998)) and three human Scar homologues (SCAR1, SCAR2 and SCAR3), otherwise known as WASP-family verprolin-homologous proteins (WAVE1–WAVE3) (Miki *et al.,* 1998b). The family of WASP proteins fall into two groups, WASP/N-WASP and WAVE1-3, according to their domain organisation (Figure 1.4). The two subgroups share a conserved binding site for Arp2/3 complex and actin monomers at their C-termini but differ in their binding sites for regulatory proteins (Figure 1.4). The ability to bind to a different set of proteins but exert the same basic function broadens the range of potential signals that can lead to Arp2/3 complex mediated actin polymerisation.

1.10.1 Domain organisation of WASP and WAVE proteins

The N-terminus of N-WASP was first proposed to display weak sequence conservation to pleckstrin homology (PH) domains and to bind to PIP_2 (Miki *et al.,* 1996). Since the N-termini of N-WASP and WASP are highly identical (50%) they are sometimes referred to as PH domains (Miki *et al.,* 1996). However, the N-terminus of N-WASP shares a higher degree of identity to the EVH1 domain of VASP/MENA than to PH domains (Prehoda *et al.,* 1999). VASP/MENA function is implicated in cell motility and their conserved EVH1 domains bind short peptide ligands containing a characteristic FPPPP motif (Bear *et al.,* 2000; Bear *et al.,* 2002; Carl *et al.,* 1999). The WH1 and EVH1 domains have the same fold but are surprisingly different in the way they interact with their respective ligands (Fedorov *et al.,* 1999; Prehoda *et al.,* 1999; Volkman *et al.,* 2002). In contrast to previous findings the WH1 domain of N-WASP does not bind to PIP_2 (Volkman *et al.,* 2002). The only known binding partners for the WH1 domain are the WASP interacting proteins (WIP) and WIP family proteins CR16 and WICH/WIRE (WIP- and CR16-homologous protein) (Aspenstrom, 2002; Ho *et al.,* 2001; Kato *et al.,* 2002; Ramesh *et al.,* 1997) (Figure 1.4). The corresponding region on Scar proteins (the Scar homology domain, SHD) differs completely in sequence from the WH1 domains but is highly homologous between WAVE1-3 (Miki and Takenawa, 2003). Currently the function of the SHD domain is unknown. C-terminal

to the WH1/SHD domains both subgroups share a basic stretch of amino acids (Figure 1.4). For N-WASP this region has been shown to interact with PIP_2, which induces WASP and N-WASP to stimulate the Arp2/3 complex mediated actin nucleation (Higgs and Pollard, 2000; Prehoda *et al.,* 2000). However, no binding partners have been assigned in WAVE proteins so far. WASP/N-WASP contain a Cdc42 Rac interactive binding motif (CRIB), which binds with high affinity to the Rho GTPase Cdc42 and very weakly to Rac if at all relevant *in vivo* (Abdul-Manan *et al.,* 1999; Aspenström *et al.,* 1996). The corresponding region in WAVE is not homologous to the CRIB domain and varies considerably between WAVE 1-3 suggesting that these proteins are regulated differently and thus cover different functions which is reflected in their tissue specific expression patterns (Higgs and Pollard, 2001; Sossey-Alaoui *et al.,* 2003). The second common domain between WASP and WAVE proteins is the central proline rich domain (PolyPro). The PolyPro region of WASP/N-WASP has been reported to bind to SH2/SH3 containing adaptor proteins such Grb2 and Nck as well as profilin and the WASP interacting SH3 protein (WISH) (Carlier *et al.,* 2000; Fukuoka *et al.,* 2001; Higgs and Pollard, 1999; Rivero-Lezcano *et al.,* 1995). Several SH3 domain containing proteins including Abl, IRSp53 and WAVE-associated RacGAP protein (WRP) have been shown to associate with the proline rich region of WAVE proteins (Miki *et al.,* 2000; Soderling *et al.,* 2002; Westphal *et al.,* 2000). WASP and WAVE proteins share the C-terminal VCA or WA domain, which is responsible for binding and activating the Arp2/3 complex (Higgs and Pollard, 1999) (Figure 1.4). This region can be subdivided into two WASP homology 2 (WH2) motifs, a central region (C) and an acidic region (A) at the very C-terminus (Figure 4). The WH2 motif binds actin monomers, the acidic region interacts with the Arp2/3 complex and the C-region is important for the activation of the Arp2/3 complex (Machesky and Insall, 1998; Marchand *et al.,* 2000; Miki and Takenawa, 1998; Panchal *et al.,* 2003) (Figure 1.4).

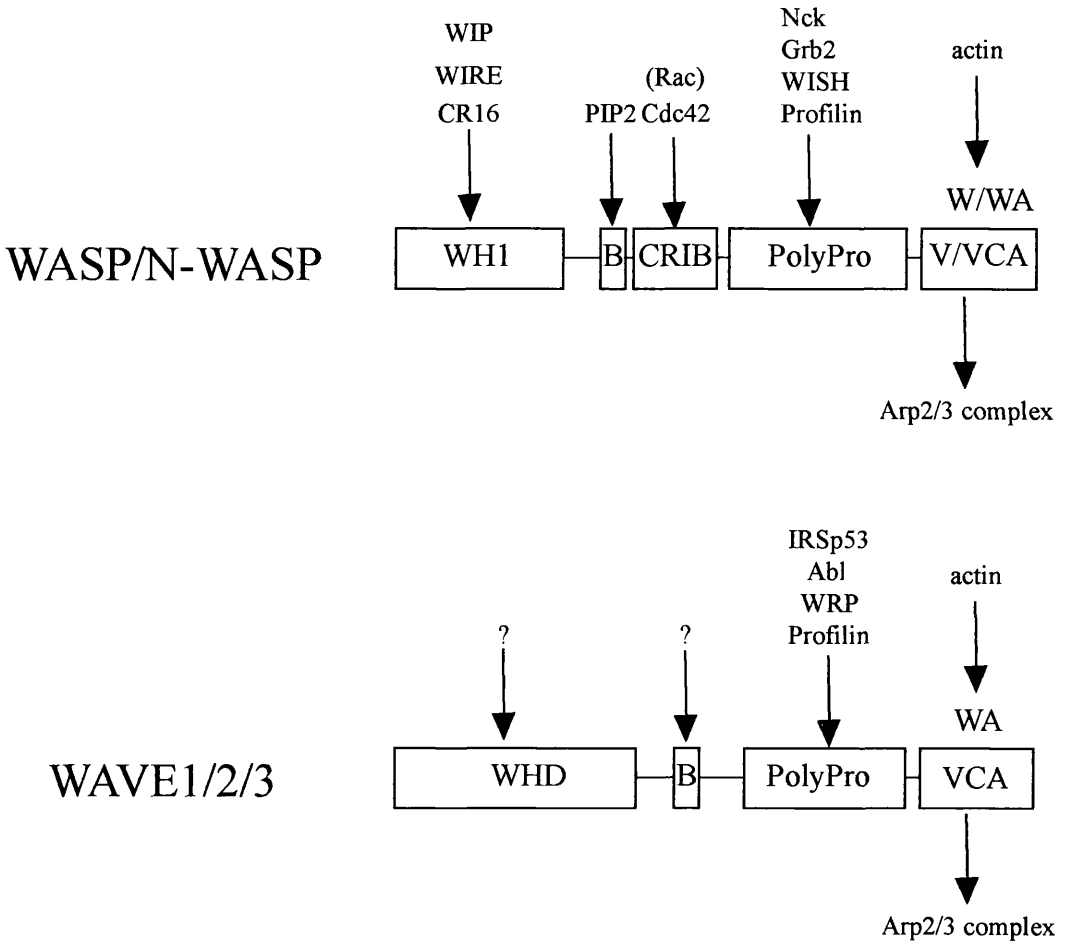


Figure 1.4. Domain organisation of WASP family proteins and known binding partners.

The rectangles show domains, which include the Wiskott–Aldrich homology 1 (WH1) domain, WAVE homology domain (WHD), a highly basic region (B), the Cd42/Rac interactive binding (CRIB) domain, a proline-rich region (PolyPro), and the verprolin homology region (V), the cofilin homolgy region (C) and the acidic region (A). The C-terminus of WASP/N-WASP and WAVE proteins is collectively called the VCA also known as WA (WASP homology 2 acidic) domain. Note that N-WASP contains an additional V region compared to WASP. Binding partner proteins are also indicated where interactions have been established.

1.10.2 Activation of the Arp2/3 complex by WASP/WAVE proteins

Based on the structure of the Arp2/3 complex it has been proposed that a big conformational change, which brings Arp2 and Arp3 in close proximity must occur in order to interact with an actin monomer forming a nucleation point (see 1.8). One hypothesis as to how the WA domain activates the Arp2/3 complex is that it stabilises the interaction of an actin monomer with the Arp2/3 complex on the side of an actin filament, thereby forming a trimolecular complex, which serves as a nucleation point for filament growth. One can imagine that the formation of this complex might induce the conformational changes needed to bring Arp2 and Arp3 in close proximity. Consistent with this actin filaments have been shown to enhance the nucleation efficiency of the Arp2/3 complex (Machesky *et al.,* 1999; Pantaloni *et al.,* 2000). Thus the actin nucleating activity of filament bound Arp2/3 complex is favoured over that of the free Arp2/3 complex. This links actin filament branching directly to nucleation, which could account for the appearance of a branched actin filament network at the leading edge of motile cells.

The WA domains of WASP/WAVE proteins are sufficient to activate the Arp2/3 complex (Machesky *et al.,* 1999; Rohatgi *et al.,* 1999; Yarar *et al.,* 1999). One can distinguish three functions of WA domains. First the WA domain binds actin monomers (Prehoda *et al.,* 2000). Second the WA interacts with the Arp2/3 complex and thirdly the activation of the Arp2/3 complex, which could be a result of the formation of an actin and Arp2/3 complex. However mutations in the WA domain that alter nucleation by the Arp2/3 complex without affecting affinity for actin or the Arp2/3 complex were identified suggesting that binding of the WA domain and actin to the Arp2/3 complex is not sufficient to activate the actin nucleating activity of the Arp2/3 complex (Marchand *et al.,* 2000). Conversely Marchand *et al.,* 2000 generated mutants in the WA domain that show significant reduction in their ability to activate the Arp2/3 complex but are unaffected in actin or Arp2/3 complex interactions. This suggested that there might be an additional activation step in the Arp2/3 complex mediated actin nucleation. Thus the process responsible for the activation of the Arp2/3 complex still remains to be elucidated. However, it has been shown that ATP binds to both Arp2 and Arp3 and that hydrolysis of ATP is required for full actin nucleation activity (Dayel *et al.,* 2001; Le Clainche *et al.,* 2001). It is not unreasonable to think that the hydrolysis of bound ATP is responsible at least in part to induce the conformational changes needed

to bring Arp2 and Arp3 in close proximity in order to be able to interact with an actin monomer and nucleate a new actin filament.

1.10.3 Regulation of WASP/WAVE proteins

The WA domains of WASP/WAVE proteins constitutively activate the Arp2/3 complex *in vitro* (Machesky *et al.,* 1999; Rohatgi *et al.,* 1999; Yarar *et al.,* 1999). In contrast to the WA domain, full length N-WASP does not activate the Arp2/3 complex *in vitro* (Higgs and Pollard, 2000). This contributed an important step in strengthening the proposal that the WA domain is masked in full length N-WASP and that N-WASP therefore exists in an auto inhibited conformation (Miki *et al.,* 1998a). In contrast to N-WASP, purified WAVE1 activates the actin nucleation activity of the Arp2/3 complex (Machesky *et al.,* 1999). Thus in a cell WAVE1 activity is likely to be regulated differently than N-WASP.

1.10.3.1 WAVE1 regulation

The active GTP bound form of Rho GTPase Rac induces actin polymerisation driven formation of membrane ruffles, lamellipodia and neurite extensions structures, which are all involved in cell motility (Small *et al.,* 2002). Upon Rac activation WAVE is translocated from the cytoplasm to membrane ruffles (Miki *et al.,* 1998b). Dominant negative WAVE constructs harbouring deletion in the actin binding domain of the WA domain of WAVE inhibit Rac dependent formation of lamellipodia and neurite extensions (Miki *et al.,* 1998b). However, no direct interaction between Rac and WAVE1 has been detected. Biochemical analysis using gel filtration on soluble bovine brain extracts reveals that WAVE1, which is predicted to be 60kDa, elutes at a size of 500 kDa. Mass spec analysis shows that WAVE1 is part of a complex containing PIR121 (p53-induceable messenger RNA), Nap125 (Nck-associated protein) and HSPC 300 (Eden *et al.,* 2002; Kitamura *et al.,* 1996; Saller *et al.,* 1999). In contrast to WAVE1 the purified WAVE-complex does not stimulate the actin nucleation activity of the Arp2/3 complex *in vitro* (Eden *et al.,* 2002). Rather than stimulating WAVE 1 directly, Rac and Nck appear to relieve the inhibitory effect of PIR121 and Nap125 in the WAVE complex on the Arp2/3 complex (Blagg *et al.,* 2003; Eden *et al.,* 2002). Addition of either Rac or Nck leads to the dissociation of the WAVE complex into two sub-complexes WAVE/HSPC300 and PIR121/Nap125 (Eden *et al.,* 2002). The molecular connections between WAVE and these inhibitory proteins as well as Nck and

Rac are unknown. However, generation of PIR121 deficient *Dictyostelium* revealed that these cells have severe defects in movement and chemotaxis as cells contain increased amounts of polymerised actin (Blagg *et al.,* 2003). Comparing *Dictyostelium* lacking PIR121 to wave null mutants, shows behaviour that is broadly consistent with over activation of WAVE. *Dictyostelium* lacking PIR121 and WAVE does not show any additional changes when compared to wave null cells suggesting that PIR121 mainly acts through WAVE *in vivo* (Blagg *et al.,* 2003). Thus consistent with *in vitro* results PIR121 appears to inhibit the activity of WAVE *in vivo*. Furthermore in cells lacking PIR121 very little WAVE is present suggesting that protein turnover provides a mechanism by which active WAVE is down regulated to negatively control actin polymerisation (Blagg *et al.,* 2003). Supporting this theory *Drosophila* PIR121 (Sra1) and Nap125 (Kette) protect WAVE from proteasome mediated degradation and are critical for WAVE localisation to Arp2/3-dependent protrusions (Kunda *et al.,* 2003).

1.10.3.2 Regulation of WASP family proteins

The WA domain of N-WASP and WASP can bind internally to their respective CRIB domains (Kim *et al.,* 2000; Miki *et al.,* 1998a). The interaction of the WA domain of N-WASP and its CRIB domain can be competed by the addition of Cdc42 *in vitro* (Miki *et al.,* 1998a). Cdc42 stimulates the N-WASP dependent Arp2/3 complex mediated actin nucleation via binding to the CRIB domain of N-WASP (Miki *et al.,* 1998a). Structural studies reveal that amino acid residues of the WA domain necessary for activation of the Arp2/3 complex are bound to the CRIB domain (Kim *et al.,* 2000). Kim *et al.,* 2000 have also provided structural evidence, that the interaction of the CRIB domain with the WA domain is released upon Cdc42 binding (Kim *et al.,* 2000). After its release, the WA domain is free to interact with and activate the Arp2/3 complex. In contrast to early observations that map the PIP_2 binding site in N-WASP to the WH1 domain (Miki *et al.,* 1996), PIP_2 interacts with the basic region of N–WASP (Prehoda *et al.,* 2000) (Figure 1.4). Full activation of N-WASP is repressed by the addition of recombinant protein containing amino acids corresponding to the CRIB domain including the basic region of N-WASP (Prehoda *et al.,* 2000). However, the CRIB domain without the basic region does not fully inhibit the activity of N-WASP to activate Arp2/3 complex mediated actin polymerisation although it is able to bind the WA domain (Prehoda *et al.,* 2000). The basic region of N-WASP is important for the direct interaction with the Arp2/3 complex (Prehoda *et al.,* 2000). Taken together these

observations suggest that direct contacts of the basic region with the Arp2/3 complex repress the WA mediated activation of the Arp2/3 complex (Prehoda *et al.,* 2000) (Figure 1.5). Prehoda *et al.,* 2000 suggest a model that full length N-WASP in its autoinhibited conformation binds directly to the Arp2/3 complex via the WA (the binding region to the CRIB domain does not overlap with the Arp2/3 binding motif) domain and the basic region. The authors suggest that the repressive effect of the basic region prevents the conformational changes induced in the Arp2/3 complex when stimulated by the WA domain alone. Thus in summary the autoinhibition is achieved by an intramolecular interaction between the CRIB domain and the WA domain as well as by an inhibitory interaction of the basic region with the Arp2/3 complex (Figure 1.5).

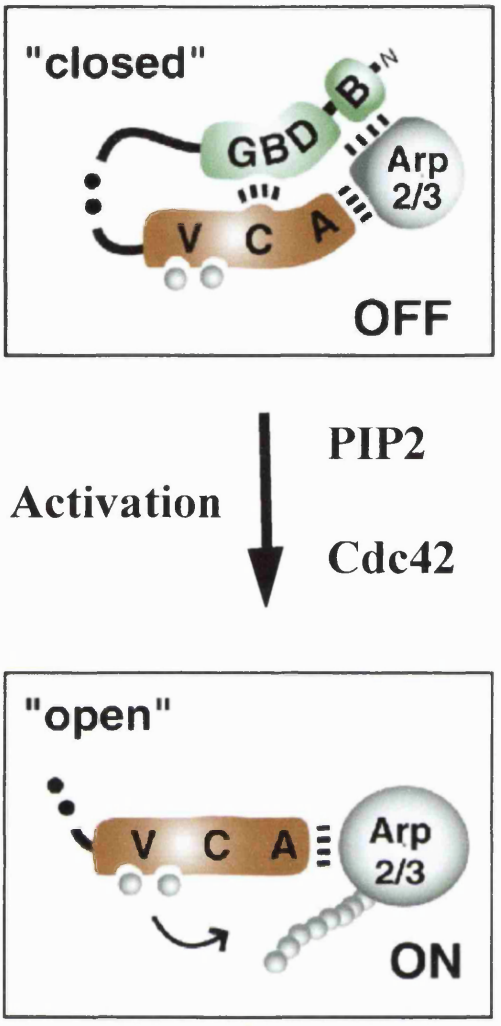


Figure 1.5. Model of WASP/N-WASP activation.
In the inactive form of the WASP molecule the CRIB (or GBD) region is bound to the WA (VCA) domain. In addition a basic region (B) of WASP binds directly to the Arp2/3 complex inhibiting the WA domain-mediated activation of the Arp2/3 complex. As a result there is no actin polymerisation by the Arp2/3 complex. Upon activation by Cdc42 and/or PIP2 the inhibitory interactions of the GBD and the B domain (green) are released and the VCA domain can stimulate the actin nucleation activity of the Arp2/3 complex leading to filament assembly (small grey circles).
(Figure adapted from Prehoda *et al.*, 2000)

1.10.3.3 Synergistic activation of WASP family proteins

Like actin polymerisation WASP proteins are regulated in time and space. The autoinhibition of WASP proteins seems to rely on a dual mechanism (Figure 1.5). Thus maximum activation relies on input of at least two different stimuli (Prehoda *et al.*, 2000). In addition WASP family proteins consist of several modular domains, which bind to a variety of signalling factors (Figure 1.4). Most of these factors appear to have some regulatory effect on WASP proteins either by inhibiting or activating its ability to stimulate the Arp2/3 complex or serving as adaptor proteins that localise N-WASP to correct cellular location where it can stimulate the Arp2/3 complex. Taken together this allows integration of multiple incoming signals with higher resolution and sensitivity.

The Rho GTPase Cdc 42 interacts with WASP and N-WASP by directly binding to the CRIB motif (Abdul-Manan *et al.*, 1999). The isolated recombinant CRIB domain of WASP/N-WASP competes directly with the Arp2/3 complex for binding WA and inhibits the nucleation promoting activity of the WA domain (Higgs and Pollard, 2000; Rohatgi *et al.*, 2000). Cdc42 and the WA domain compete for the same binding sites in the CRIB domain (Higgs and Pollard, 2000). These overlapping binding sites preclude the CRIB domain from binding simultaneously to Cdc42 and WA. Thus Cdc 42 activates full length WASP/N-WASP by competing for the same residues in the CRIB domain that are the docking site for the WA domain in the inactive conformation (Abdul-Manan *et al.*, 1999; Higgs and Pollard, 2000; Kim *et al.*, 2000). Maximum activation of WASP/N-WASP *in vitro* is achieved by simultaneous activation with Cdc42 and PIP_2 (Higgs and Pollard, 2000; Rohatgi *et al.*, 2000). However, the effects of Cdc42 and PIP_2 on full-length N-WASP are opposite to their effects on WASP (Higgs and Pollard, 2000; Rohatgi *et al.*, 1999). GTP bound Cdc42 alone releases the nucleation promoting activity of full length N-WASP, whereas PIP_2 alone does not (Higgs and Pollard, 2000; Rohatgi *et al.*, 1999). PIP_2 does however, enhance stimulation by GTP bound Cdc42 for WASP and N-WASP (Higgs and Pollard, 2000; Prehoda *et al.*, 2000; Rohatgi *et al.*, 2000). This is consistent with a model that the basic region as well as the CRIB domain of N-WASP have inhibitory functions. PIP_2 is produced at membranes and activated GTP bound Cdc42 is localised at the plasma membrane as well. This would be an efficient way to locally activate N-WASP in response to receptor signalling that leads to the production of PIP_2 as well as to activation of Cdc42 at the same time. Furthermore synergistic stimulation of WASP/N-

WASP offers the possibility of maximal activation at low concentrations of PIP_2/Cdc42 at the same time. This mechanism ensures that WASP/N-WASP and ultimatively actin polymerisation is tightly regulated at membranes.

1.10.3.4 Adaptors as activators of WASP family proteins

Many adaptor proteins serve to bridge activated cell surface receptors to various intracellular signal transduction pathways. Nck and Grb2 belong to a class of adaptor proteins entirely consisting of one src homology 2 (SH2) and several (three and two respectively) src homology 3 (SH3) domains (Lehmann *et al.,* 1990; Takenawa *et al.,* 1996; Watanabe *et al.,* 1995). SH2 and SH3 domains typically bind to tyrosine phosphorylated and proline rich peptide sequences respectively (Lim *et al.,* 1994; Songyang *et al.,* 1993). Nck and Grb2 have been reported to bind to the proline rich domain of WASP and N-WASP via their SH3 domains (Anton *et al.,* 1998; Carlier *et al.,* 2000). The SH3 domains of Nck alone are capable of stimulating an increase in the rate of nucleation of actin filaments by N-WASP in the presence of the Arp2/3 complex *in vitro* (Rohatgi *et al.,* 2001). All three SH3 domains of Nck are required to fully activate the N-WASP mediated activation of the Arp2/3 complex. This activity is further enhanced by the addition of PIP_2 but not by active Cdc42 (Rohatgi *et al.,* 2001). These results suggest that Cdc42 and Nck, although binding to different regions in N-WASP, activate N-WASP in a similar way. An interesting implication of this observation is the possible existence of a Nck dependent but Cdc42 independent mechanism to induce N-WASP activation at tyrosine phosphorylated Nck binding sites such as receptor tyrosine kinases. Indeed, the actin based motility of vaccinia virus (see 1.1.1.4) is independent of Cdc42 but strictly depends on Nck and N-WASP (Frischknecht *et al.,* 1999b; Scaplehorn *et al.,* 2002; Shibata *et al.,* 2002; Snapper *et al.,* 2001).

Grb2 has been shown to be another activator of N-WASP (Carlier *et al.,* 2000). Like Nck, Grb2 interacts with the proline rich region of N-WASP (Carlier *et al.,* 2000; Scaplehorn *et al.,* 2002). The two SH3 domains of Grb2 are located at the N- and the C-terminus of the protein. The interaction of the C-terminal SH3 domain of Grb2 with N-WASP results in stimulation of the N-WASP-Arp2/3 complex mediated actin polymerisation more efficiently when compared to the N-terminal SH3 domain (Carlier *et al.,* 2000). Furthermore, Cdc42 and Grb2 can bind to N-WASP at the same time to

act synergistically to activate N-WASP (Carlier *et al.*, 2000). It still has not been tested whether Grb2 and Nck or Grb2 and PIP_2 can stimulate N-WASP in co-operative manner. In contrast to Grb2, Nck does not cooperate with Cdc42 suggesting that Grb2 and Nck stimulate N-WASP differently. Indeed it has been suggested that Nck and Grb2 act in a cooperative manner to stabilise and/or activate the vaccinia actin-nucleating complex including N-WASP (Scaplehorn *et al.*, 2002). Since adaptor proteins like Grb2 and Nck can locally activate N-WASP as well as target it to sites of tyrosine phosphorylation in response to extracellular stimuli, they are ideal modules for achieving the temporal spatial regulation required for actin polymerisation during cell migration.

1.10.3.5 Phosphorylation activates WASP and N-WASP mediated actin polymerisation

Phosphorylation events play an important role in many signalling cascades including events taking place at the leading edge of a migratory cell. Mass spec analysis of radioactively labelled immunoprecipitates of WASP reveals that endogenous WASP is phosphorylated on serines and threonines (She *et al.*, 1997). Recent studies have shown that Ser 483 and Ser484 in the WA domain of WASP are phosphorylated (Cory *et al.*, 2003). Furthermore biochemical assays reveal that phosphorylation of these residues increases the affinity of the WA domain for the Arp2/3 complex. Phosphorylation of full length N-WASP is required for efficient *in vitro* actin polymerisation mediated by the Arp2/3 complex suggesting that N-WASP could potentially be activated by phosphorylation of its WA domain *in vitro* (Cory *et al.*, 2003). Thus Cory *et al.*, 2003 propose that the phosphorylation of the WA domain is required for optimal stimulation of the Arp2/3 complex by WASP. However, the physiological relevance Ser phosphorylation of WASP remains to be elucidated.

To date there is no evidence for threonine phosphorylation of WASP or N-WASP. However, WASP is also tyrosine phosphorylated following antigen receptor stimulation of B cells (Baba *et al.*, 1999). Co-expression of the Src family kinase Hck with WASP results in the phosphorylation of Tyr291 of WASP (Cory *et al.*, 2002). Subsequently it has been confirmed that the equivalent residue Tyr253 in N-WASP is also phosphorylated by overexpression of Src family kinases (Suetsugu *et al.*, 2002). Glutamine mutants in Tyr291 and Tyr253 in WASP or N-WASP respectively

mimicking the negative charge introduced by phosphorylation, increase the ability to stimulate actin polymerisation *in vitro* and induce filopodia or promote neurite extensions *in vivo* (Cory *et al.,* 2002; Suetsugu *et al.,* 2002). Tyrosines 291 and 253 in WASP/N-WASP lie in the CRIB domain of their respective protein. Phosphorylation of Tyr291 and Tyr256 provides an additional route to regulate the activity of WASP or N-WASP presumably by affecting the interaction of the CRIB domain with the WA domain. Based on NMR data Tyr291 is not accessible for phosphorylation as it is buried when the CRIB domain is bound to the WA domain (Torres and Rosen, 2003). It is envisaged that phosphorylation of Tyr291 decreases the binding affinity of the CRIB domain to the WA domain thereby activating N-WASP (Torres and Rosen, 2003). In addition Torres *et al.,* 2003 showed that binding of GTP-Cdc42 is necessary for efficient phosphorylation and dephosphorylation of WASP on Tyr291. When Cdc42 is not bound to the CRIB domain Tyr291 can not be phosphorylated. Thus according to their model N-WASP is not phosphorylated unless Cdc42 and the kinase act simultaneously. *In vitro* phosphorylation studies on the CRIB domain demonstrate that once phosphorylated, Tyr291 is highly protected form tyrosine phosphatases (Torres and Rosen, 2003). Once WASP is phosphorylated the protein will have a basal activity towards Arp2/3 complex but be protected against dephosphorylation *in vitro.* In that way WASP might be kept in a conformational state, which can be quickly fully activated by additional interactions of SH2 domains for instance (Torres and Rosen, 2003). Hence the structural properties of WASP provide a sensing mechanism for simultaneous GTPase and kinase signals which will result in maximum activity. Furthermore the protection against phosphatases once the Tyr291 is phosphorylated might function as a memory device maintaining activity after initiation.

These observations are interesting but do not answer the question what is the physiological relevant function of tyrosine phosphorylation of WASP/N-WASP and where it occurs in a cell. N-WASP as well as WASP localise to the nucleus (Miki *et al.,* 1996; Vetterkind *et al.,* 2002). When Tyr253 is mutated to Phe N-WASP is primarily found in the nucleus (Suetsugu and Takenawa, 2003). In contrast when phosphorylation is mimicked by the introduction of a negative charge at Tyr253, the majority of N-WASP is found in the cytoplasm (Suetsugu and Takenawa, 2003). Phosphorylation of N-WASP by co-expression of active Fyn kinase drastically increased the cytoplasmic fraction of N-WASP, whereas a dominant negative form of

Fyn had the opposite effect (Suetsugu and Takenawa, 2003). Thus the nuclear localisation of N-WASP is dependent on its phosphorylation status (Suetsugu and Takenawa, 2003). Furthermore N-WASP might have some functions in the nucleus, which are only exerted when the protein is not phosphorylated. Microarray experiments and western blot analysis of selected targets in cells stably expressing nuclear and cytoplasmic versions of N-WASP suggested that the nuclear form of N-WASP may modulate gene expression (Suetsugu and Takenawa, 2003). However, the authors base all their experiments analysing gene expression levels and localisation of N-WASP on overexpression of N-WASP. Given these overexpression phenotypes a more thorough analysis of the function of N-WASP in the nucleus is required especially since convincing evidence showing a clear localisation of endogenous N-WASP in the nucleus is still lacking.

The proteasome is a multiprotein complex responsible to degrade protein that are not needed anymore. The inhibition of protein degradation via the proteasome pathway induces cell migration events such as neurite extensions (Fenteany *et al.,* 1995). Based on western blot analysis tyrosine phosphorylated N-WASP is more stable than unphosphorylated form when neurons are stimulated to form neurite extensions (Suetsugu *et al.,* 2002). The fact that levels of tyrosine phosphorylated N-WASP were increased after inhibition of the proteasome lead Suetsugu *et al.,* 2002 to speculate that tyrosine phosphorylation of N-WASP is a mechanism in order to protect N-WASP from being degraded.

1.10.4 N-WASP activation by pathogens

Evolutionary distinct pathogens including bacteria and viruses have developed the ability to exploit the host actin cytoskeleton in order to propagate and facilitate their infections (Frischknecht and Way, 2001; Goldberg, 2001). Over the last decade studies with pathogens have yielded valuable insights into how the machinery responsible for actin polymerisation is regulated. Like cell migration the actin-based motility of many pathogens is also dependent on the Arp2/3 complex (Goldberg, 2001; Machesky and Insall, 1998). Moreover, different pathogens have developed alternative strategies to recruit and activate the Arp2/3 complex on the surfaces to polymerise actin required for their mobility.

1.10.4.1 The actin based motility of *Listeria monocytogenes*

Listeria monocytogenes is a *gram*-positive bacterium, which causes food- borne disease, severe meningo-encephalitis and death in immunosurpressed individuals (Cossart and Lecuit, 1998). *Listeria* induces its own uptake via inducing phagocytic processes of the host cell (Cossart and Lecuit, 1998) (Figure 1.6A). *Listeria* induced phagocytosis is mediated by the surface proteins internalin A and B, which interact with E-cadherin and the ubiquitously expressed receptor gC1q-R respectively (Cossart *et al.,* 2003). Once inside the cell *Listeria* lyses the phagocytic vacuole using acid-activatable pore-forming proteins to mediate escape from acidic phagosomes into the host cell cytosol (Geoffroy *et al.,* 1987; Tilney and Portnoy, 1989). *Listeria monocytogenes* induces polymerisation of actin into structures known as an actin tails (Figure 1.6B). These actin tails propel the bacterium within the host cytoplasm (Cossart, 2000). Driven by actin polymerisation *Listeria* reaches the plasma membrane and induces the formation of a protrusion containing the bacterium at its tip. This bacterial tipped protrusion commits the neighbouring cell to phagocytosis thereby spreading the bacterial infections from cell to cell. This gives rise to a two-membrane vacuole that in turn is lysed to release the bacterium, thereby infecting the cytoplasm of the second cell (Figure 1.6A). *Listeria* lacking the ability to form actin tails are three log units less virulent in a mouse model of infection suggesting that the process of actin tail formation is important in the pathogenicity of the bacterium (Kocks *et al.,* 1992).

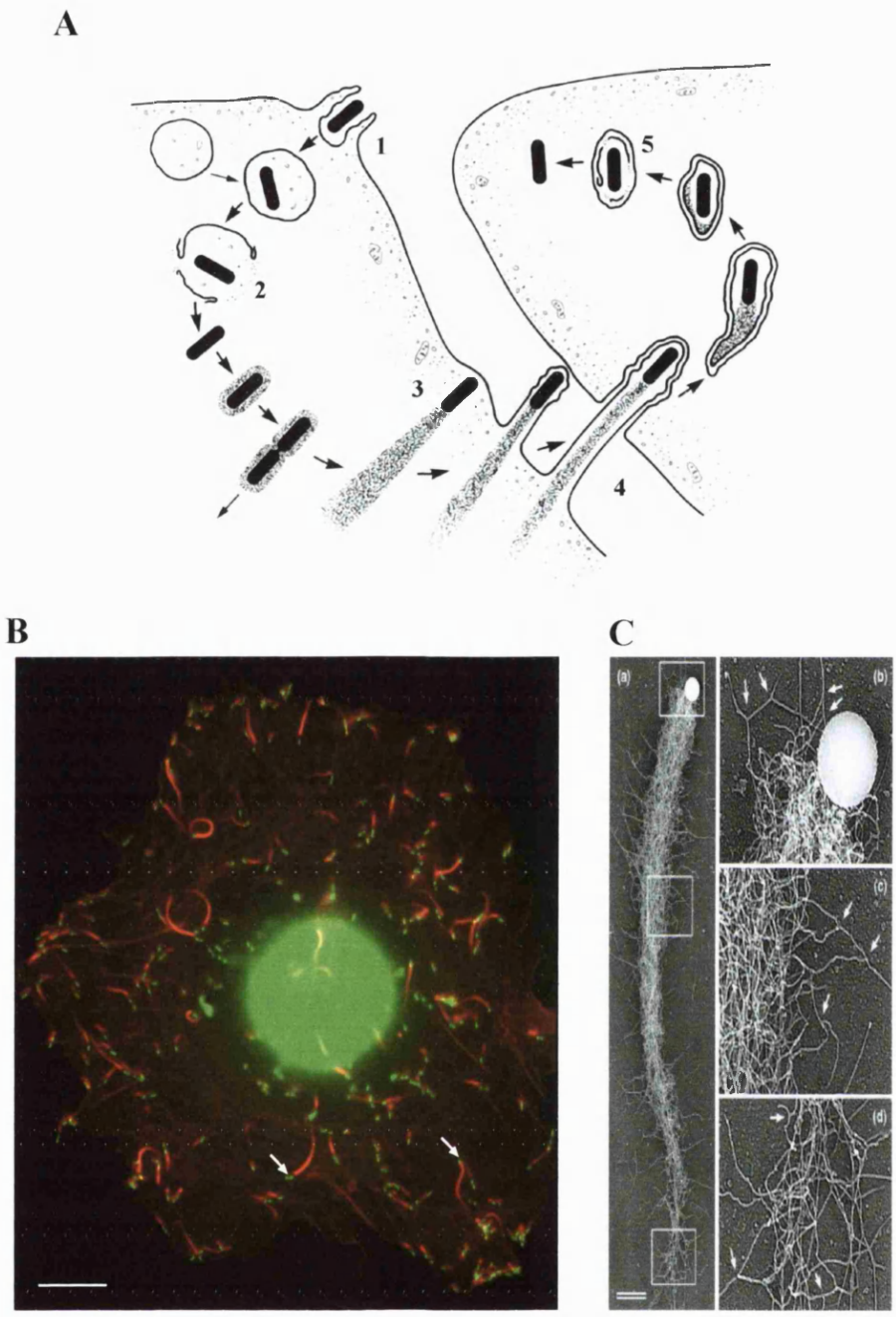


Figure 1.6. (A) Life cycle of *Listeria* monocytogenes. Entry of *Listeria* into the host cell (1) is followed by lysis of the phagocytic vacuole (2) and ActA dependent actin based motiltiy of *Listeria* (3). *Listeria* spreads its infection from cell to cell (4) after being engulfed by a neighbouring cell and escapes the vacuole, which is surrounded by a double membrane again (5). (Figure adapted from Tilney *et al.*, 1989). **(B)** Immunofluorescence analysis of a *Listeria* infected HeLa cell demonstrates that upon infection *Listeria* (white arrows) polymerises actin, visualised with phalloidin (red), on one pole of its surface. DAPI staining reveals the bacteria and the nucleus in green. Scale bar represens 10 µm. (**C**) Electron micrograph of an actin tail formed by ActA (residues 1-234) coated beads in *Xenopus* egg extract. (a) An overview shows the very dense array of branched actin filaments in the tail. (b-d) Enlargements of the boxed regions show Y-junctions (arrows) in different parts of the tail. Filaments have the dendritic appearance of actin filaments near the leading edge of lamellipodia. (Figure taken from Cameron *et al.* 1999).

How does *Listeria* nucleate an actin tail? *L. monocytogenes* strains that carry a gene disruption in ActA are unable to assemble actin tails or spread from cell to cell in a tissue culture monolayer and are less virulent (Brundage *et al.,* 1993; Kocks *et al.,* 1992). Expression of ActA in *L. innocua*, a *Listeria* species that does not normally polymerise actin, enables this organism to assemble actin tails (Kocks *et al.,* 1995). Purified ActA that is linked to the surfaces of beads enables beads to form actin tails (Cameron *et al.,* 1999) (Figure 7C). Thus ActA is necessary and sufficient to mediate actin-based motility of *Listeria.* ActA was the first protein described to stimulate the actin nucleation activity of the Arp2/3 complex *in vitro* (Welch *et al.,* 1998). Reconstitution of *Listeria* motility using purified cytoskeletal proteins requires the Arp2/3 complex (Loisel *et al.,* 1999). Consistent with this, the Arp2/3 complex has been shown to be essential for *Listeria* motility *in vivo* (Skoble *et al.,* 2000; Welch *et al.,* 1998).

How does ActA promote Arp2/3-stimulated actin polymerisation? Welch *et al.,* 1998 showed that the N-terminus of ActA (residues 29–263) reduces the lag phase of Arp2/3-stimulated actin polymerisation *in vitro.* Deletion of amino acids 50-260 of ActA leads to loss of the ability to assemble actin tails in *Listeria* infected cells (Lasa *et al.,* 1995). A fragment of ActA consisting of amino acids 30-263 is sufficient to induce motility in cytoplasmic extracts when bound to the bacterial surface (Lasa *et al.,* 1997). Sequence comparison reveals that the N-terminus of ActA has high degree of sequence homology with the WA domains in WASP/WAVE proteins (Skoble *et al.,* 2000; Zalevsky *et al.,* 2001). This sequence conservation is particular evident in the WH2 motif, the C-region and the acidic region of WASP/WAVE proteins, which explains why ActA activates the Arp2/3 complex mediated actin polymerisation (Figure 1.7).

The actin-monomer-binding region of ActA, residues 60–101 and 121-138, are only required for actin nucleation *in vitro* and are dispensable *in vivo* (Skoble *et al.,* 2000; Zalevsky *et al.,* 2001). The region around residues 146–150 of ActA, which contains a cluster of basic residues, has sequence similarity to the C-terminal WA domain of WASP/WAVE proteins (Skoble *et al.,* 2000). Deletion analysis reveals that residues 146–150 of ActA are required for actin tail formation being crucial to activate the Arp2/3 complex *in vitro* possibly due to a loss of interaction with the Arp2/3 complex

(Lasa *et al.*, 1997). Point mutants in ActA demonstrate that changing Arg148Lys and Arg149Ser in the basic cluster are required for activation of the Arp2/3 complex but not for its binding, as although actin based motility is abolished the Arp2/3 complex is still recruited to the bacteria (Pistor *et al.*, 2000). These data suggest that the region between 146-150 serves perhaps a double function in binding as well as activating the Arp2/3 complex.

Deletion of amino acids 136-165 in the context of the full-length molecule does not abolish the interaction with the Arp2/3 complex *in vitro* (Skoble *et al.*, 2000). This suggests the existence of second Arp2/3 binding site in ActA similar to N-WASP. The acidic stretch of ActA, residues 33–46 aligns with the acidic region of WASP/WAVE proteins found at the extreme C-terminus (Skoble *et al.*, 2000). In WASP protein this acidic region is implicated in the interaction with the Arp2/3 complex (Marchand *et al.*, 2000). The acidic residues in ActA increase the efficiency of Arp2/3 actin nucleation *in vitro* and act to enhance the rate and frequency of *Listeria* motility *in vivo* (Lasa *et al.*, 1997). However deletion of residues 32-42 does not greatly affect the Arp2/3 complex activation (Skoble *et al.*, 2000). Taken together these observations suggest that the acidic region in ActA may represent a second region with which ActA binds the Arp2/3 complex. Thus *Listeria* mimics the mechanism by which WASP/WAVE family proteins activate the Arp2/3 complex (Figure 1.7).

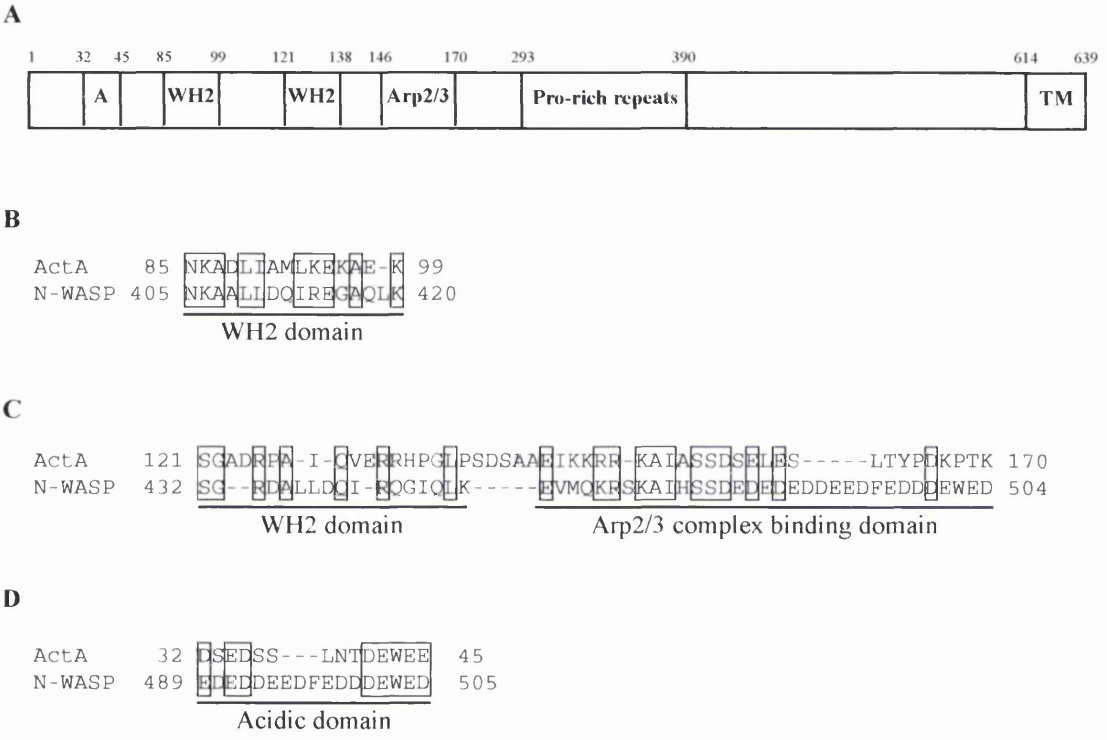


Figure 1.7. Domain organisation and alignment of functional regions of *Listeria* ActA.

(**A**) Schematic of ActA. WASP acidic domain (A), WASP homology 2 domain (WH2), Arp2/3 complex interacting and activating domain (Arp2/3), proline-rich repeats (Pro-rich repeats) and transmembrane domain (TM) are shown. (**B**) Alignment of ActA amino acids 85 to 99 with the first of two WH2 domains of N-WASP. Boxed residues in (B), (C) and (D) indicate similar amino acids according to groupings see Figure 3.2. (**C**) Alignment of ActA amino acids 121 to 170 with the second WH2 domain and the Arp2/3 complex binding and activation domain of N-WASP. (**D**) Alignment of ActA amino acids 32 to 45 with the acidic domain of N-WASP.

The focal adhesion protein VASP binds to the central proline rich repeat region of ActA and acts to enhance the rate of *Listeria* motility (Lasa *et al.,* 1995; Niebuhr *et al.,* 1997; Smith *et al.,* 1996). Furthermore, VASP is known to bind to the actin-monomer-binding protein profilin (Reinhard *et al.,* 1995). *Listeria* is able to move in *Xenopus* cell extract, which was inhibited upon immunodepletion of profilin (Theriot *et al.,* 1994) suggesting that profilin plays a crucial role in *Listeria* motility. However, the actin based motility of *Listeria* can be reconstituted without profilin (Loisel *et al.,* 1999). A more recent report showed that cross-linking of profilin with actin impaired *Listeria* actin tail formation in vitro and *in vivo* (Grenklo *et al.,* 2003). The fact that profilin covalently cross-linked with actin does not inhibit the initiation of Listeria actin tails indicates that profilin-actin complex is only required for elongation of actin filaments at the bacterial surface (Figure 1.8).

However, Arp2/3 activation alone is not sufficient to promote actin-based motility of *Listeria in vitro* (Loisel *et al.,* 1999). An actin-filament capping protein, either CapZ or gelsolin, and the filament-severing protein ADF/cofilin, are also required to achieve motility (Loisel *et al.,* 1999). It is thought that CapZ or gelsolin ensure that the actin polymerisation driving *Listeria* motility is limited to uncapped filament ends closely opposed to the bacterium, whereas ADF/cofilin ensures that capped actin filaments are turned over in the bulk of the tail (Carlier *et al.,* 1997; Pantaloni *et al.,* 2000). *Listeria* will not, however, provide us with an answer as to how WASP family members are recruited to and activated at sites of actin polymerisation as its motility is independent of N-WASP (Snapper *et al.,* 2001).

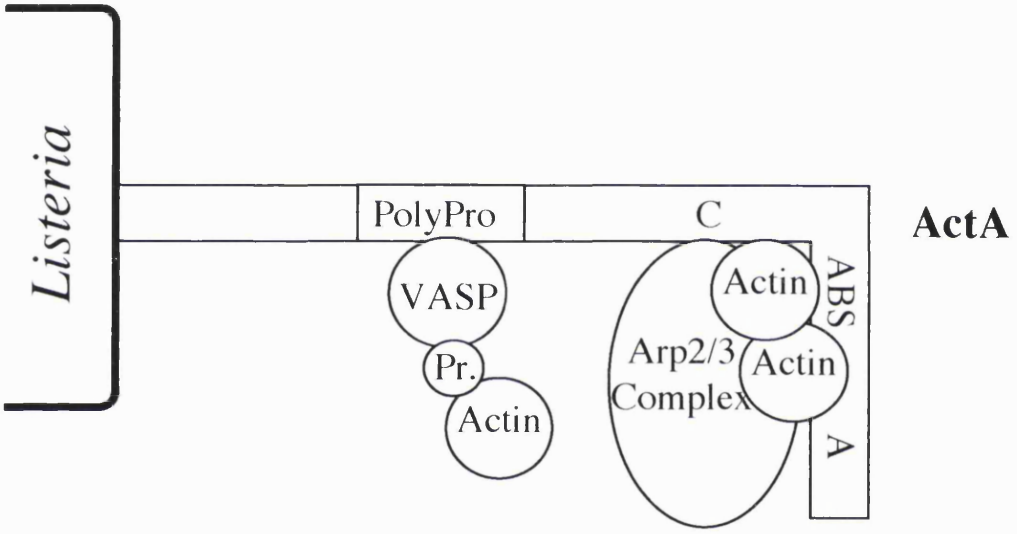


Figure 1.8. Schematic model of proteins essential for *Listeria* actin based motility. *Listeria* uses ActA to directly recruit and activate the Arp2/3 complex. ActA shares high sequence identity with N-WASP in the so called connecting region (C), WH2 domains and the acidic region (A). Similar to N-WASP ActA interacts with the Arp2/3 complex via the C region and potentially also the A motif and uses the WH2 domain to bind to monomeric actin. The proline rich repeat region (PolyPro) binds to VASP, which recruits profilin (Pr.) bound to an actin monomer (Actin). (for detailed information refer to the text 1.11.4.1).

1.10.4.2 The actin based motility of *Shigella flexneri.*

Invasion of *gram*-negative bacteria of the genus *Shigella* into the intestinal mucosa of the colon induces a degeneration of the epithelium and a strong inflammatory reaction that results in bacillary dysentery or shigellosis (Tran Van Nhieu *et al.,* 2000; Tran Van Nhieu and Sansonetti, 1999). Upon contact with the host cell, *Shigella* secretes a complex of proteins that induces signalling cascades that activate Src, Abl kinases and Rho family GTPases, resulting in the actin-mediated phagocytosis of the bacterium (Burton *et al.,* 2003; Tran Van Nhieu *et al.,* 2000; Tran Van Nhieu and Sansonetti, 1999) (Figure 1.9). After inducing lysis of the phagosome, *Shigella* induces actin polymerisation on its surface in form of so called actin tails ((Figure 10B) (Cossart, 2000). Mutants of *Shigella* that do not assemble actin are greatly attenuated in human volunteers as well as in monkey and mouse experimental models (Bernardini *et al.,* 1989; Lett *et al.,* 1989; Makino *et al.,* 1986; Sansonetti, 1991) indicating that actin-based motility is essential in the pathogenesis of the disease (Figure 9A).

The ability of *Shigella flexneri* to induce actin polymerisation is encoded by a large (230kbp) virulence plasmid (Sansonetti *et al.,* 1982). Random transposon integration into the virulence plasmid of *Shigella flexneri* lead to identification of the virulence G (*virG*) region, which turned out be important for the actin based motility of *Shigella* (Lett *et al.,* 1989). Deletion of the gene product IcsA (also called VirG) leads to a loss of *Shigella* mediated actin tail assembly and drastic reduction in cell to cell spread of the bacterium (Bernardini *et al.,* 1989; Makino *et al.,* 1986). Expression of IcsA in *E.coli* that does not normally induce actin tails is sufficient to restore actin based motility (Goldberg and Theriot, 1995; Kocks *et al.,* 1995). Taken together, these data indicate that IcsA is both necessary and sufficient for *Shigella* induced actin tail formation.

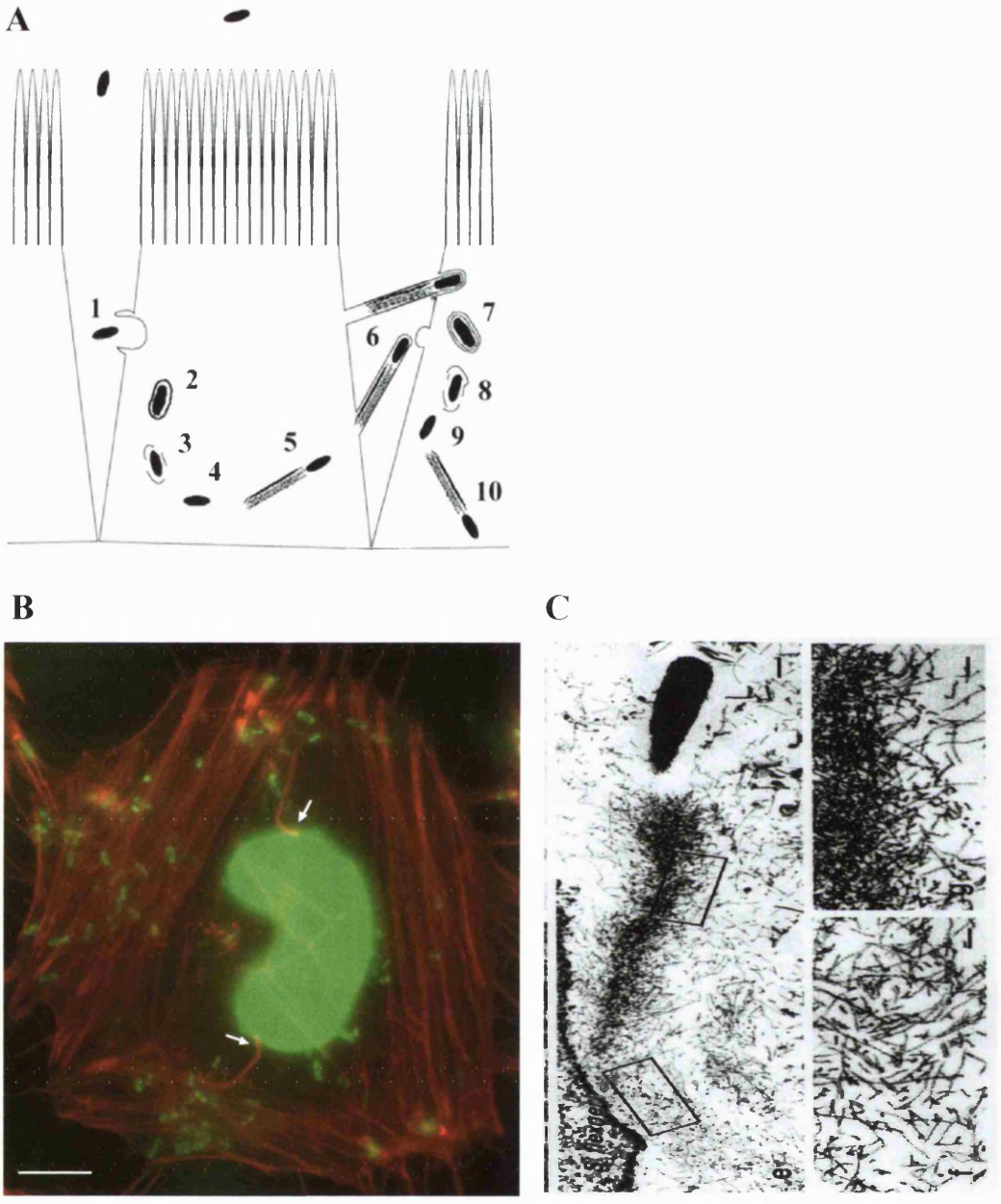


Figure 1.9. (A) Life cycle of *Shigella* flexneri. *Shigella* (solid ellipses) enter the mammalian host cell by inducing phagocytosis (1). The bacterium within a phagocytic vacuole (2), escapes the vacuole by lysing it (3). Thereby *Shigella* is released into the cytoplasm of the host cell (4) followed by the assembly of an actin tail on one pole (5). Actin tails propel *Shigella* through the cell cytoplasm and enables the bacterium to form protrusions from the cell surface (6). *Shigella* is then within a double -membrane vacuole, which it lyses, thereby releasing it into the cytoplasm of the adjacent cell (7-9). The process of actin tail assembly is repeated in the infected cell (10). (Figure taken from Goldberg 2001). **(B)** Immunofluorescence analysis of a *Shigella* infected HeLa cell demonstrates that the bacterium upon infection induces actin tails (arrows), visualised with phalloidin (red). DAPI staining reveals the bacteria and the nucleus shown in green. Scale bar represents 10 μm. **(C)** Electron microscopy of myosin S1 decorated actin tail of *Shigella*. Magnification of the two boxes are shown on the right and reveal a branched network of actin filaments similar to *Listeria* and lamellipodia of migrating cells (Figure taken from Gouin *et al.*, 1999).

IcsA is a large 1,102-amino-acid protein with a predicted molecular weight of 110-kDa that is anchored in the bacterial outer membrane by a carboxy-terminal domain (the θ-domain)(Suzuki *et al.,* 1995). Approximately 750 amino acids of the mature amino terminus (the θ-domain) are exposed on the bacterial surface, which contain repetitive glycine rich sequences (Suzuki *et al.,* 1996). The θ-domain of IcsA is exposed on the bacterial surface and contains all regions that are required for actin assembly (Egile *et al.,* 1999) (see Figure 1.10).

As with the actin based motility of *Listeria*, the Arp2/3 complex is essential for *Shigella* induced actin tails (Egile *et al.,* 1999) (Figure 1.9). However, in contrast to ActA, IcsA has no obvious homology to WASP family members. This suggests that additional proteins are required for the recruitment of the Arp2/3 complex to *Shigella*. First insights into how this might be achieved, came from the observation that endogenous N-WASP was localised to the surface of *Shigella* (Egile *et al.,* 1999). Subsequently it has been shown that IcsA interacts directly with N-WASP (Egile *et al.,* 1999; Suzuki *et al.,* 1998; Suzuki *et al.,* 2002). Expression of dominant negative constructs of N-WASP, immunodepletion experiments in *Xenopus* extracts and genetic evidence show that N-WASP is essential for actin based motility of *Shigella* (Moreau *et al.,* 2000; Snapper *et al.,* 2001; Suzuki *et al.,* 1998). Thus *Shigella* induced actin tails are dependent on IcsA mediated recruitment of N-WASP, which activates the Arp2/3 complex (Egile *et al.,* 1999; Frischknecht and Way, 2001; Goldberg, 2001). The WASP interacting protein (WIP) and the adaptor protein Nck are recruited to the surface of *Shigella* (Figure 1.11). However, expression of dominant negative constructs of WIP and Nck have no effect on the actin tail formation of *Shigella* (Moreau *et al.,* 2000). Thus no function in *Shigella* motility for WIP or Nck has been discovered yet.

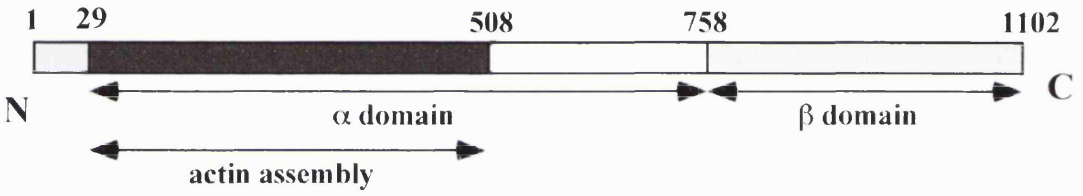


Figure 1.10. Domain organisation of IcsA.
IcsA can be divided into two domains. The α-domain (residues 1-508) is exposed on the surface of *Shigella* and is sufficient to polymerise actin *in vitro* (see 1.11.4.2). The β-domain (residues 508-1102) anchors IcsA into the outer membrane of *Shigella*. Amino acids 29-508 are sufficient to mediate actin polymerisation in *Xenopus* extracts. (Figure taken from Goldberg 2001).

How does IcsA recruit N-WASP? The N-WASP-binding site has been mapped to the IcsA glycine-rich repeats located in the θ-domain (Suzuki *et al.,* 1998). Residues 156–273 of N-WASP, which contain the CRIB motif, are sufficient for recruitment to *Shigella in vivo* in N-WASP deficient cells (Lommel *et al.,* 2001). This domain of N-WASP also acts as a dominant negative to block *Shigella* actin tail formation (Moreau *et al.,* 2000). As mentioned in 1.10.3.2 the interaction of the activated GTP-bound form of the Rho-family GTPase Cdc42 with the CRIB motif plays an important role in regulating the ability of N-WASP to stimulate the actin-nucleating activity of the Arp2/3 complex (Kim *et al.,* 2000; Rohatgi *et al.,* 2000). Thus, *Shigella* appears to use IcsA to mimic the activation of N-WASP by Cdc42 and therefore potentially provides an excellent system to study this form of N-WASP activation (Egile *et al.,* 1999; Frischknecht and Way, 2001).

Interestingly, there are no obvious sequence homologies between IcsA and Cdc42 to suggest that they will compete for the same binding site in N-WASP. Furthermore, an N-WASP H208D mutant, which is deficient in Cdc42 binding, is still recruited to *Shigella* (Suzuki *et al.,* 2000). Furthermore, a N-WASP construct lacking amino acids 160-225 (CRIB domain) rescues *Shigella* actin tail formation in the absence of N-WASP (Lommel *et al.,* 2001), suggesting that IcsA and Cdc42 bind to different regions in N-WASP. Consistent with this hypothesis, it is possible to form a ternary complex of Cdc42, IcsA and N-WASP (Suzuki *et al.,* 2000). Furthermore, this complex is more efficient at stimulating Arp2/3-induced actin polymerisation than a complex of IcsA and N-WASP (Suzuki *et al.,* 2000). Taken together this evidence strongly suggests that IcsA and Cdc42 do not activate N-WASP in the same way. Moreover using cell lines which are deficient in Cdc42 it has been demonstrated that the actin based motility of *Shigella* is unaffected (Shibata *et al.,* 2002). Thus the exact role of the CRIB domain of N-WASP in the actin based motility of *Shigella* remains to be elucidated.

Although WASP and N-WASP share the same domain organisation only N-WASP will support *Shigella* actin tail formation when expressed in infected N-WASP null cells (Snapper *et al.,* 2001). Consistent with this IcsA only interacts with N-WASP and not WASP or WAVE (Suzuki *et al.,* 2002). Using a series of hybrid chimeras obtained by swapping N-WASP and WASP domains, it has been demonstrated that the specificity of the interaction of IcsA with N-WASP lies in the N-terminal WH1 domain of N-

WASP (Suzuki *et al.,* 2002). Furthermore the WH1 domain on its own is recruited to the surface of *Shigella* in N-WASP -/- cells (Lommel *et al.,* 2001). It is still an open question whether the WH1 domain can directly interact with IcsA and whether such an interaction can induce the conformational changes required to activate N-WASP.

WIP-like molecules are the only known confirmed binding partners of the WH1 domain of N-WASP. This interaction is maintained by the WASP binding domain if WIP (WIP-WBD), which is recruited to the surface of *Shigella* depending on its ability to interact with N-WASP (Moreau *et al.,* 2000; Zettl and Way, 2002). WIP also reduces N-WASP/WASP stimulated Arp2/3 complex mediated actin polymerisation activity *in vitro* and *in vivo* (Martinez-Quiles *et al.,* 2001; Sasahara *et al.,* 2002). Thus IcsA could interfere with this inhibition thereby locally activating N-WASP on the bacterial surface. *In vitro* analyses of IcsA stimulated actin polymerisation would give insights whether IcsA can reverse the WIP inhibition or not. Ultimatively structural analysis of IcsA in complex with N-WASP will be required to provide information into the mechanism by which N-WASP is activated by *Shigella.*

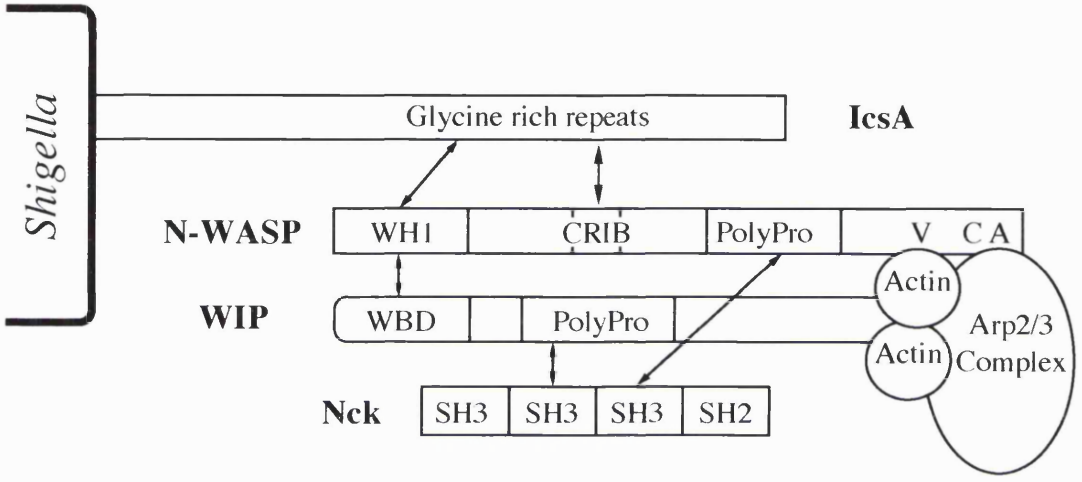


Figure 1.11. Schematic model of proteins recruited to *Shigella*.
In contrast to *Listeria*, *Shigella*, achieves Arp2/3 complex activation by recruiting N-WASP. *Shigella* recruits N-WASP by direct interaction of the glycine-rich repeats of IcsA with N-WASP. N-WASP recruitment depends on its WH1 and CRIB domain. Currently it is not clear whether N-WASP binds to IcsA directly via the WH1 or CRIB domain or both. WASP interacting protein (WIP) and Nck are not essential for *Shigella* actin tail formation but localise to the bacterium. Arrows or overlapping proteins indicate where interactions have been demonstrated experimentally. Abbreviations for domains in the respective proteins are as follows: PolyPro, proline-rich regions; A, acidic region; V, verprolin homology; C, cofilin homolgy region; WH1, Wiskott–Aldrich homology I domain; CRIB, Cd42/Rac interactive binding motif; WBD, WASP-binding domain; SH2, Src-homology 2 domain; SH3, Src-homology 3 domain.

1.10.4.3 The actin based motility of EPEC

Enteropathogenic *Escherichia coli* (EPEC), a *gram* negative bacterium, is the major causative agent of infantile diarrhoea in developing countries and leads to the loss of one million lives each year (Goosney *et al.,* 1999). EPEC infects the intestinal mucosa, inducing the formation of unique structures called 'attaching' and 'effacing' lesions (A/E) (Goosney *et al.,* 2000b). A/E lesions are characterised by the loss of microvilli on the intestinal epithelial surface, intimate attachment of the bacteria and the generation of so called "actin pedestals" beneath the adherent bacteria (Goosney *et al.,* 1999; Vallance and Finlay, 2000) (Figure 1.12). The formation of actin pedestals by EPEC is recapitulated on cultured mammalian cells, and the ability to form pedestals correlates with the ability of EPEC to cause A/E lesions in mammalian hosts (Donnenberg *et al.,* 1993). Currently the physiological role of actin pedestals during EPEC infections is not clear, however in tissue culture they push the bacterium up to 10 μm away from the plasma membrane (Rosenshine *et al.,* 1996). As a consequence of actin polymerisation on the inner side of the plasma membrane EPEC is moved along the cell surface at speeds up to 0.4 μm min^{-1} (Sanger *et al.,* 1996). Pedestal formation crucially depends on the secretion of specific virulence proteins located on the Locus of Enterocyte Effacement (LEE), a 35 kbp chromosomal pathogenicity island (Elliott *et al.,* 1998; McDaniel and Kaper, 1997).

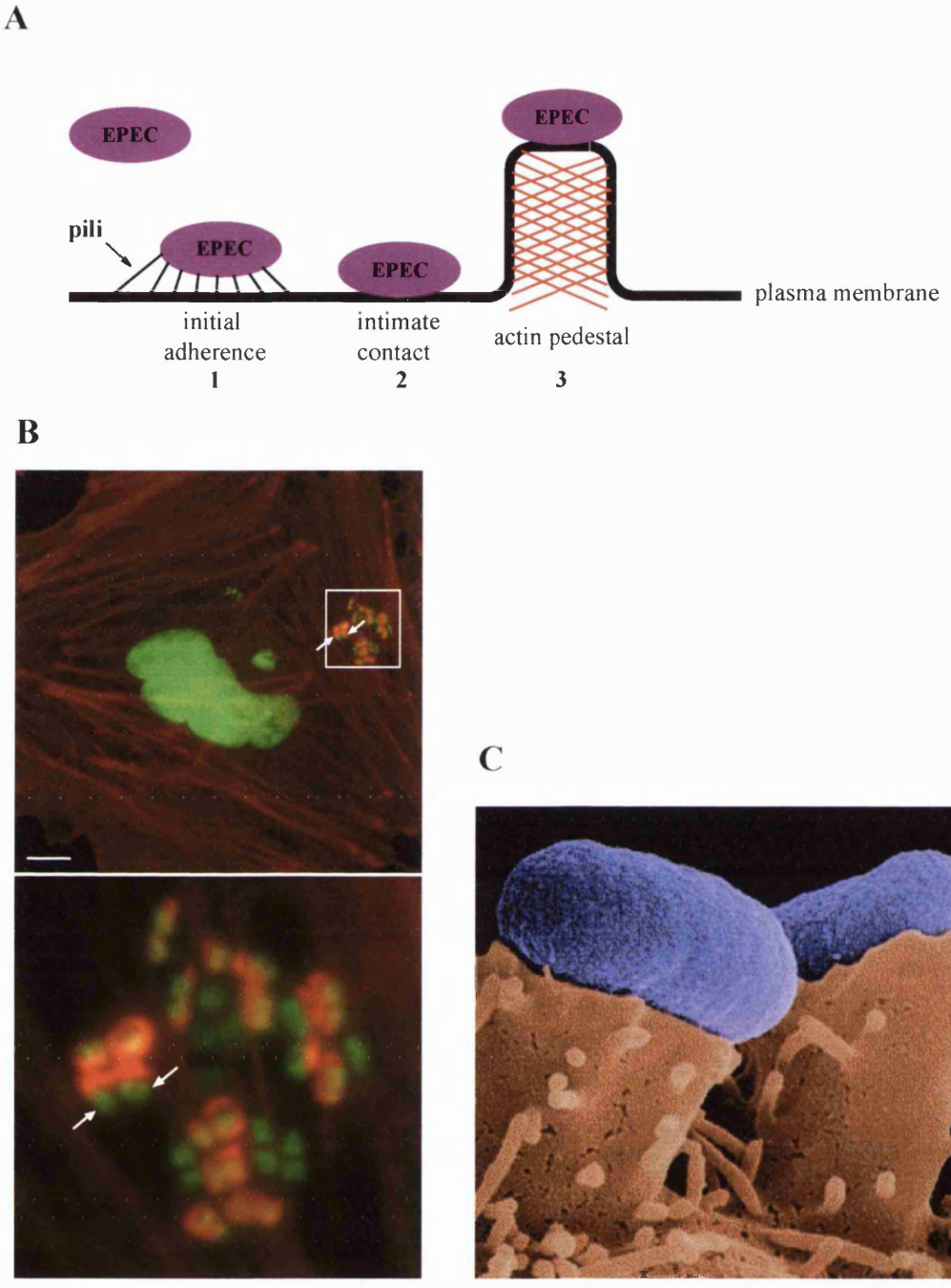


Figure 1.12. Life cycle of Enterophatogenic E.coli
(A) EPEC uses pili to make the inititial adherence with the host cell plasma membrane (1). After establishing intimate contact with the host cell (2), EPEC induces actin pedestals structures under the plasma membrane (3). **(B)** Immunofluorescence analysis of a EPEC infected HeLa cell demonstrates that upon infection EPEC induces pedestal like structrues called actin pedestals (red). The host actin cytoskelton is visualised with phalloidin (red) and DAPI staining reveals the bacteria (arrows) and the nucleus shown in green. The boxed area is enlarged on the right. **(C)** Scanning electron microscope image of EPEC (purple) sitting on top of induced pedestals (orange). (Image taken from Rosenshine *et al.*, 1992).

Secretion of virulence factors

The LEE encodes virulence genes for pedestal formation such as the components of a type III secretion system (Elliott *et al.,* 1998). Many bacterial pathogens employ a type III secretion system to deliver virulence proteins across the bacterial cell into the host cell (Hueck, 1998). One can view them as molecular syringes that inject bacterial virulence factors directly into the host cell. Type III secretion systems of *gram* negative bacteria are multimeric protein complexes (~20 proteins) that form channels spanning the inner and the outer bacterial membrane which are responsible for the subsequent secretion of so called effector molecules (Figure 1.13). Up to twelve EPEC secreted proteins (Esp) are present in the supernatant of EPEC grown in tissue culture medium and the identity many has been determined by amino-terminal sequencing (Kenny and Finlay, 1995). Secreted proteins that have been identified so far are EspA, EspB, EspD, EspF and EspG. EspA is transported to the outer surface of the bacterium where it forms a hollow filamentous structure to contact the host cell (Knutton *et al.,* 1998) (Figure 1.13). EspB and EspD are predicted to possess transmembrane domains and have been found associated with infected host cell membranes (Kresse *et al.,* 1999; Wolff *et al.,* 1998). EspB and EspD are thought to transit through the EspA filament, via an unknown mechanism, to form a pore in the host plasma membrane to allow delivery of other virulence factors into the cell (Ide *et al.,* 2001). Some of these translocated proteins are effector molecules, which subvert/inhibit cellular processes that might be disadvantageous to the bacterium or might benefit the pathogen in order to allow their survival and growth. So far three LEE effector molecules have been identified. EspF disrupts intestinal barrier function and induces host cell death by an unknown mechanism (Crane *et al.,* 2001; McNamara *et al.,* 2001). Preliminary studies show that a second effector Map (Mitochondrial-associated protein), is targeted to mitochondria where it has membrane disrupting activity (Kenny and Jepson, 2000). Tir (Translocated intimin receptor) is essential for pedestal formation and is inserted into the host cell plasma membrane (Kenny *et al.,* 1997) (Figure 1.13).

The role of Tir in EPEC in pedestal formation

Tir spans the host cell membrane twice leaving N and C-termini in the host cytoplasm (Luo *et al.,* 2000)(Figure 1.13, 1.14). Once inserted into the host-cell plasma membrane by an unknown mechanism, the extracellular loop of Tir binds directly to the bacterial outer membrane adhesin, intimin (Batchelor *et al.,* 2000). This interaction is required to establish an intimate contact between the bacterium and the host-cell (Kenny *et al.,* 1997) (Figure 1.13). EPEC lacking intimin can not to form pedestals, although they still translocate Tir into the host cell plasma membrane (Liu *et al.,* 1999). Addition of recombinant intimin to cells preinfected with an EPEC strain lacking intimin results in pedestal formation (Liu *et al.,* 1999). Thus Tir in addition to serving as bacterial adhesion receptor also induces actin polymerisation upon interaction with the bacterial outer membrane protein intimin. Tir is essential for pedestal formation, as deletion of the *tir* gene inhibits EPEC induced pedestal formation (Kenny *et al.,* 1997).

Biochemical interaction studies show that the cytoplasmic N-terminus of Tir interacts with the focal adhesion proteins θ-actinin, talin and vinculin (Freeman *et al.,* 2000; Goosney *et al.,* 2000a). Furthermore an EPEC strain containing a TIR molecule carrying a mutation that does not support actin pedestal formation is still able to recruit θ-actinin (Goosney *et al.,* 2000a). This shows, that the recruitment of θ-actinin is independent of EPEC ability to form actin pedestals and that the N-terminus might not be required for EPEC induced pedestal formation. These interactions are thought to anchor the bacterium to the host-cell cytoskeleton.

EPEC induces tyrosine phosphorylation of proteins beneath the site of adherence, which is crucial for actin pedestal formation (Rosenshine *et al.,* 1992). Western blot analysis with an antibody raised against EPEC was used to identify Tir as the tyrosine phosphorylated protein sitting in the host cell plasma membrane (Rosenshine *et al.,* 1992). Interestingly Enterohaemorrhagic *E.coli* (EHEC), which like EPEC promotes the reorganisation of actin into pedestals, does not sequester tyrosine phosphorylated proteins to pedestals based on phosphotyorsine antibody labelling of EHEC infected cells (Ismaili *et al.,* 1995). This suggested that EHEC developed a tyrosine independent mechanism to induce pedestals. Interestingly EHEC Tir can not functionally replace EPEC Tir and is not tyrosine phosphorylated (Kenny, 2001). This observation provided

an opportunity to identify sequences in EPEC Tir critical for phosphotyrosine dependent pedestal formation. EPEC Tir contains six tyrosine residues in the C-terminal half of the protein that may serve as substrates for phosphorylation (Kenny, 2001). Sequence analysis of the EHEC Tir revealed five C-terminally located tyrosine residues with a serine at the position corresponding to fourth EPEC tyrosine (residue 474) (Kenny, 1999). Generating an EHEC/EPEC Tir chimera in which 12 amino acids from EPEC Tir (EHI**Y**DEVAADP), surrounding the Tyr474, were used to replace the corresponding residues in EHEC Tir (TSN**S**NTSVQNMG) resulted in tyrosine phosphorylation of Tir as well as actin pedestal formation (Campellone *et al.,* 2002). Swapping the same 12 amino acid peptide sequence derived from EPEC Tir containing Tyr474Phe substitution into EHEC Tir did not allow for EHEC induced pedestal formation (Campellone *et al.,* 2002). EHEC induced pedestal formation is also not possible when only a 7 amino acid sequence of EPEC Tir (EEHIYDE) encompassing Tyr474 is exchanged for the corresponding region in EHEC Tir (Campellone *et al.,* 2002). Substitution of EPEC Tyr474 for serine did not affect the translocation of Tir but resulted in a loss of the tyrosine phosphorylation of Tir and actin pedestal formation (Kenny, 1999). More importantly EPEC Tir carrying Tyr474Ser mutation was not able to rescue pedestal formation when cells were infected with Tir deletion EPEC strain transformed with a Tir construct harbouring Tyr474Ser mutation (Kenny, 1999). From the series of experiments outlined above it is clear that tyrosine phosphorylation of Tyr474 of Tir is essential for EPEC induced actin polymerisation although it remains to be established how EHEC induces actin pedestals.

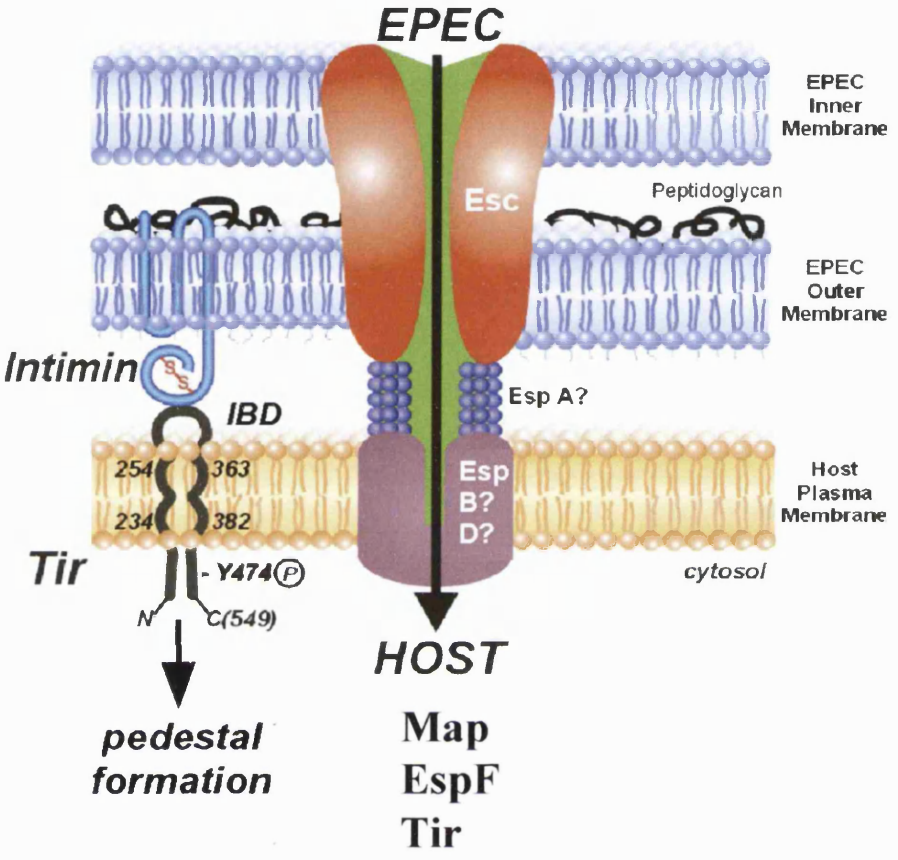


Figure 1.13. Model of EPEC infection and type III translocation apparatus and Tir-intimin binding.

LEE-encoded type III secretion components (Esc proteins) span both bacterial membranes. EspA-containing surface appendages are thought to form a pore for the translocation of bacterial effectors into the host cell cytosol. Translocated EspB and EspD form a pore in the cell plasma membrane enabling translocation of bacterial effectors. Among them are, Map, EspF and Tir. Tir is inserted into the host cell plasma membrane and subsequently phosphorylated on tyrosine-474. It serves as a receptor for the outer membrane adhesin, intimin, through its extracellular intimin-binding domain (IBD). Tir-intimin interaction mediates intimate attachment of EPEC to the host cell and triggers pedestal formation. (Figure courtesy of Brett Finlay, taken from Celli *et al.*, 2000).

The EPEC actin signalling cascade

The cytoplasmic C-terminus of Tir contains a sequence around Tyr474, which represents a potential Nck SH2 binding site (pYDEV) (Kenny, 1999). It was shown that Nck localised to EPEC induced pedestals (Gruenheid *et al.,* 2001). Changing Tyr474 to phenylalanine abolishes the ability of EPEC to recruit Nck and to induce actin pedestal (Gruenheid *et al.,* 2001; Kenny, 1999). Furthermore cell lines derived from Nck deficient mice were unable to form pedestals when infected with EPEC but not when infected with EHEC (Gruenheid *et al.,* 2001). Lastly a phosphorylated peptide encompassing the region around Tyr474 was shown to bind directly to Nck indicating that Nck is the most upstream component in the EPEC signalling cascade (Gruenheid *et al.,* 2001). Taken together this data indicates that Nck is recruited to EPEC in a phosphorylation dependent manner and is essential for actin pedestal formation.

How does Nck recruitment lead to actin pedestal formation? The Arp2/3 complex has been demonstrated to localise to EPEC actin pedestals (Kalman *et al.,* 1999). Furthermore it has been shown that WASP localises to EPEC induced pedestals and that overexpression of dominant negatives forms of WASP block recruiting of the Arp2/3 complex to EPEC induced pedestals (Kalman *et al.,* 1999). EPEC infection of N-WASP deficient cell lines does not result in pedestal formation, showing that N-WASP is an essential component for this process (Lommel *et al.,* 2001). Thus Tir appears to mimic a tyrosine kinase receptor signalling to the actin cytoskeleton via Nck, WASP and the Arp2/3 complex.

In summary the current model (Figure 1.14) shows that EPEC adheres to the outside of the host cell and inserts the virulence factor Tir into the host cell plasma membrane. The phosphorylation of Tyr 474 of Tir leads to the recruitment of the adaptor protein Nck. It is currently not known how N-WASP is recruited to EPEC. However N-WASP recruits and activates the Arp2/3 complex on the surface of EPEC (Kalman *et al.,* 1999). The net result is local actin polymerisation, which drives the formation of pedestals.

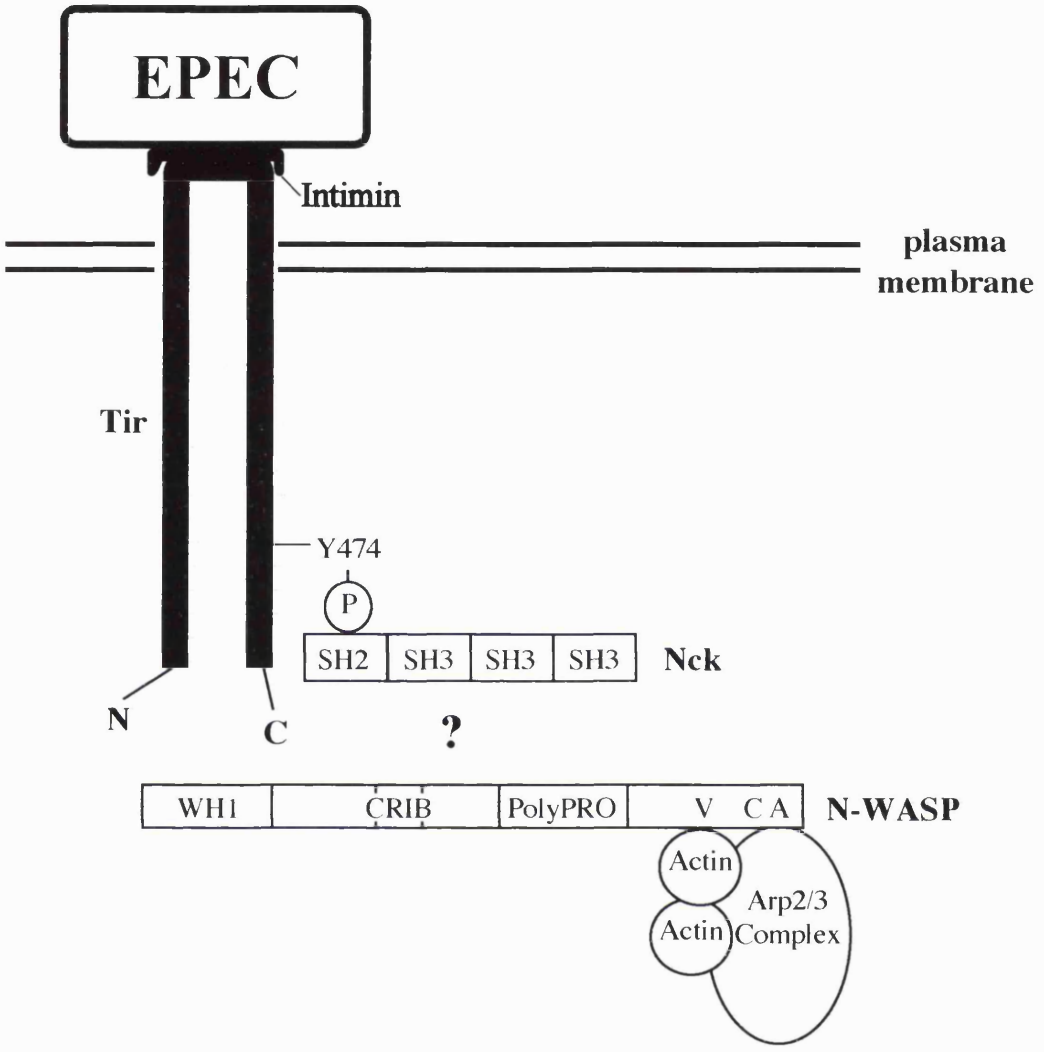


Figure 1.14. Schematic model of proteins recruited to EPEC peestals.
After binding to intimin, the EPEC surface protein Tir needs to be phosphorylated on Tyr 474 in order to directly recruit Nck. EPEC pedestal formation is dependent on WASP/N-WASP and the Arp2/3 complex. However, the mechanism by which WASP/N-WASP are recruited to EPEC pedestals is not clear.
(Abreviations like Figure 11).

1.10.4.4 The actin based motility of vaccinia

Vaccinia belongs to the orthopoxvirus family of large enveloped double-stranded DNA viruses, which includes variola virus, the causative agent of smallpox (Oldstone, 1998). Vaccinia virus was used as the vaccine in the World HeLath Organisation program that eradicated smallpox in 1974 (Fenner, 1996; Moss, 1996). Vaccinia virus is the first ever virus visualised by light microscopy due to it large size measuring 350 by 250nm (Roos *et al.,* 1996). The complete linear genome of the Copenhagen strain of vaccinia virus has been sequenced (191.000 bp) and contains 263 open reading frames (ORFs) larger than 50 amino acids. The genome includes its own transcription machinery (Goebel *et al.,* 1990; Johnson *et al.,* 1993). ORFs nomenclature is derived in the following way. Digestion of the vaccinia genome with HindIII yields 16 fragments, which are numbered alphabetically in decreasing size (DeFilippes, 1982). ORFs on such a fragment are numbered sequentially from the 5' end followed by L or R indicating the reading direction of the ORF. For example A36R is the 36th ORF on the biggest HindIII fragment transcribed from left to right.

Life cycle of vaccinia virus

The major steps in the vaccinia virus life cycle, as for most viruses, involve entry into the host cell, replication and release of infectious particles. The mechanism of vaccinia entry into cells remains to be firmly established, but it has to bind and cross the plasma membrane of the host cell. This suggests that membrane fusion and receptor binding might play role in the vaccinia virus entry into host cells (Chung *et al.,* 1998; Hsiao *et al.,* 1998; Vanderpasschen and Smith, 1997). However, a receptor still remains to be identified.

Studies on entry of vaccinia virus are further complicated by the fact that the virus produces two different infectious forms the intracellular mature virus (IMV) and the extracellular enveloped virus (EEV). Binding studies suggest that IMV and EEV bind to different receptors consistent with the different proteins on their surfaces, but the nature of these receptors remains unknown (Vanderpasschen and Smith, 1997). The binding of IMV and EEV particles to the host cell affect the cell differently consistent with the notion of different receptors for entry. Binding of IMV induces signalling cascades and the production of actin-containing cell surface projections, whereas the

binding of EEV does not (Krijnse-Locker *et al.,* 2000). Thus EEV enters in an actin- and signalling-independent fashion, whereas the IMV requires the Rho GTPase Rac, tyrosine phosphorylation and actin polymerisation (Krijnse-Locker *et al.,* 2000). During vaccinia virus entry only cores containing viral DNA are released into the cytoplasm (Krijnse-Locker *et al.,* 2000). Since IMV and EEV particles are surrounded by a different set of membranes, the mechanism by which they cross the host cell plasma membrane and release the viral cores are most likely different (Vanderpasschen *et al.,* 1998). Evidence for virus-plasma membrane fusion being involved in IMV entry is provided by electron microscopy and by the dispersal of fluorescent dye in pre-labelled IMV particles after binding and entry (Chang and Metz, 1976; Doms *et al.,* 1990). It is currently not clear how many membranes wrap the IMV particle (one, two or several have been suggested) (Griffiths *et al.,* 2001; Hollinshead *et al.,* 1999). However, what is clear is that EEVs always contain one more membrane bilayer than IMV particles (see below). Thus to release virus cores into the cytoplasm EEVs need to get rid of one more set of membranes. Working on the assumption that IMV particles contain one set of membranes EEV entry was proposed to involve an endocytosis step, during which the outermost membrane of EEVs would be dissolved by low pH resulting in IMV particles trapped in acidic endocytotic vesicles. Followed by the fusion of IMV with endosomal membranes, viral cores could be released into the cytoplasm (Ichihashi, 1996). However, this route of entry has never been observed in electron microscopy.

In contrast to most viruses, vaccinia replicates in the cytoplasm of the host cell in specialised structures, so called viral factories that are located close to the nucleus (Figure 15). After cores have entered the cytosol, they move inward to sites where virus factories develop. Confocal microscopy on fixed samples showed that cores and microtubules colocalised (Mallardo *et al.,* 2001). Furthermore viral cores have been shown to bind directly to microtubules (Ploubidou *et al.,* 2000). Recent live video microscopy shows that GFP labelled vaccinia virus cores, derived from IMVs, move inward towards the nucleus (Carter *et al.,* 2003). The process was inhibited reversibly by nocodazole but not cytochlasin D and the velocity of core movement is consistent with movement on microtubules (Carter *et al.,* 2003).

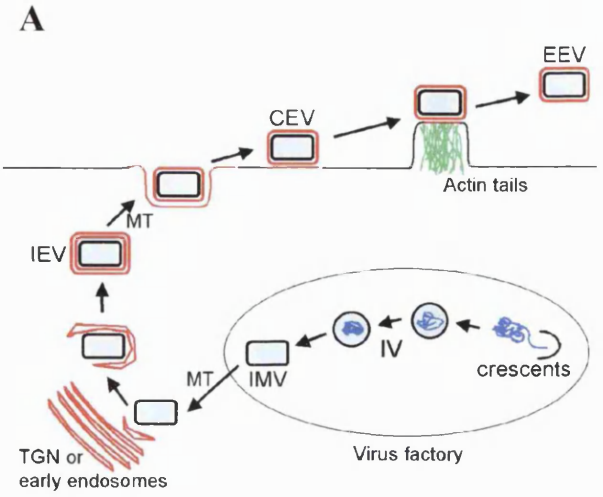
Vaccinia virus DNA replication takes place in a perinuclear area that is surrounded by membrane derived from endoplasmic reticulum (ER), resembling nuclear envelope

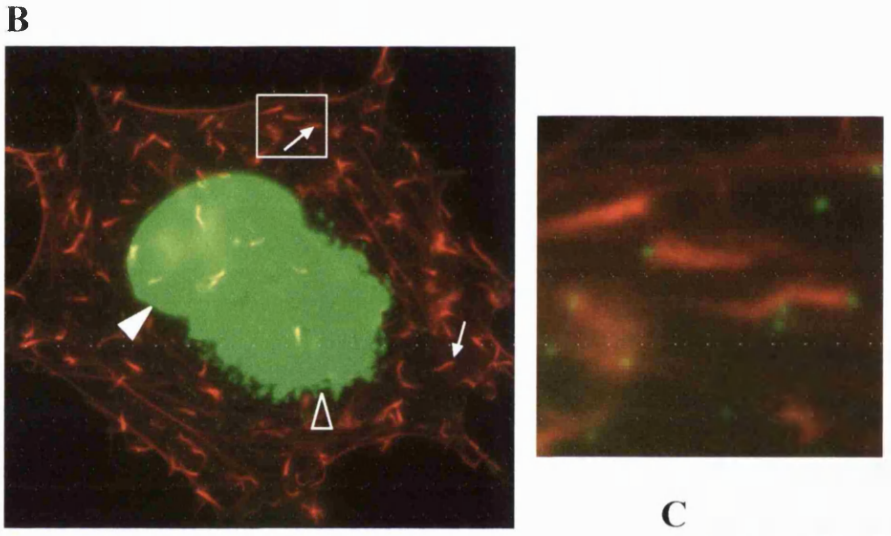
assembly in late anaphase/telophase of the cell cycle called the viral factory (Tolonen *et al.,* 2001). Following DNA replication spherical virions (IV immature virions) are made from viral crescents, which are membranes derived from the ER-to-Golgi intermediate compartment loaded with viral proteins (Sodeik *et al.,* 1993). IVs mature into IMV, when the proteolytic cleavage of several capsid proteins and the inclusion of viral DNA occurs (Moss, 1996; Roos *et al.,* 1996).

Most IMV particles remain intracellular and are released only upon cell lysis. However a 5-15% depending on cell type and virus strain of IMVs move from virus factories to the *trans*-Golgi network (TGN) (Sanderson *et al.,* 2000). This movement requires microtubules as treatment of vaccinia virus infected cells with nocodazole or colchicine, prevented the dispersal of IMV from virus factories (Sanderson *et al.,* 2000). Subsequently IMV particles get wrapped with TGN derived membranes, which results in the formation of the intracellular enveloped virus (IEV) (Schmelz *et al.,* 1994).

The IEV uses microtubules to reach the plasma membrane where it fuses in order to release extracellular virus particles (Geada *et al.,* 2001; Hollinshead *et al.,* 2001; Rietdorf *et al.,* 2001; Ward and Moss, 2001). Live video microscopy shows that GFP labelled IEV virus particles move along microtubules towards the plasma membrane (Rietdorf *et al.,* 2001). Overexpression of dominant negative construct of the microtubule plus end directed motor kinesin blocked outward movement of IEV particles (Rietdorf *et al.,* 2001). Two IEV specific proteins F12L and A36R have been implicated in mictrobule based motility of IEV particles (Rietdorf *et al.,* 2001; van Eijl *et al.,* 2002). However the molecular details as to how IEV particles are linked to kinesin motor proteins remain to be established. Viruses that stay attached to the membrane are called cell-associated enveloped viruses (CEV) while those released into the surrounding media are referred to as extracellular enveloped virus (EEV) (Figure 15A). CEV particles upon membrane fusion are thought to leave the outermost IEV membrane behind as part of the plasma membrane as the integral membrane protein A36R and membrane associated protein F12L are absent in the CEV but not IEV particles (Krauss *et al.,* 2002; van Eijl *et al.,* 2002; van Eijl *et al.,* 2000). CEV, which are attached to the outside of the plasma membrane continue to extend on cellular projections that are driven by actin polymerisation on the inner surface of the plasma membrane (Figure 15B, C), until they fall off and become EEVs (Blasco and Moss,

1991; Blasco and Moss, 1992; Cudmore *et al.,* 1995; Cudmore *et al.,* 1996; Rietdorf *et al.,* 2001) (Figure 15A). Vaccinia strains that cannot form actin tails have a small-plaque phenotype on confluent cell monolayers, suggesting that actin-based motility of the virus is required for efficient cell-to-cell spread (Sanderson *et al.,* 1998; Wolffe *et al.,* 1997; Wolffe *et al.,* 1998).





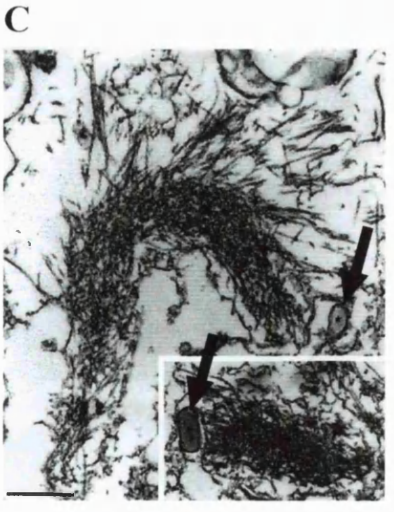


Figure 1.15. (A) Schematic of the vaccinia virus life cycle. After entry in the host cell IMV (intracellular mature virus) are made in the virus factory and move on microtubules (MT) to the *trans*-Golgi network where they are wrapped and acquire a double membrane to form IEV (intracellular enveloped virus). IEV move to the plasma membrane on MT. At the cell surface the IEV membrane fuses with the plasma membrane and form CEV (cell-associated extracellular virus) which induce actin tails that are used to infect neighbouring cells. CEV can also be released to form EEV (extracellular enveloped virus). (Figure taken from Smith *et al.*, 2002). **(B)** Immunofluorescence analysis of a vaccinia infected HeLa cell demonstrates that upon infection vaccinia virus induces actin structures, called actin tails (red, arrows) beneath CEV on the inner side of the plasmamembrane. DAPI staining reveals virus particles (green) on top of actin tails (inset left), nucleus (green, arrow head) and viral factory (green, open arrow head). **(C)** Electron microscopy of myosin S1 decorated actin tail of vaccinia. The arrows point to virus particles. Similar to lamellipodia of migrating cells vaccina virus induces a branched network of actin tails on its surface. (Figure taken from Cudmore *et al.*, 1996).

The vaccinia virus actin signalling cascade

The actin based motility of vaccinia is dependent on a type Ib integral membrane protein called A36R, which has an exposed domain of ~195 residues on the surface of the IEV that has no obvious homology to any cellular protein (Frischknecht *et al.,* 1999b; Röttger *et al.,* 1999). The A36R protein is situated just underneath the CEV with the majority of its amino acids on the cytosolic side of the plasma membrane (van Eijl *et al.,* 2000). Although required it still remains to be shown whether A36R is sufficient for vaccinia actin tail assembly. Vaccinia actin tail formation requires tyrosine phosphorylation of residues 112 or 132 of A36R, although Tyr112 plays the more important role (Frischknecht *et al.,* 1999b). Subsequent work using recombinant viruses carrying point mutations in Tyr112 and Tyr132 to Phe showed however, that while Tyr112 is essential Tyr132 plays an auxiliary role to make vaccinia actin tail formation more efficiently (Scaplehorn *et al.,* 2002). The sequence around Tyr112 and Tyr 132 are predicted substrates for c-Src (Songyang *et al.,* 1993). Consistent with this prediction the Src-family kinase inhibitor PP1 blocks vaccinia actin tail formation, as does overexpression of dominant-negative c-Src (Frischknecht *et al.,* 1999b). It is thought Src mediated tyrosine phosphorylation of A36R induces a cascade of events that results in the recruitment of Nck, WASP-interacting protein (WIP) and N-WASP beneath the CEV at the plasma membrane (Frischknecht *et al.,* 1999b; Moreau *et al.,* 2000). Recruitment of Nck and Grb2 depend on the on the phosphorylation of Tyr112 and Grb2 is recruited via phosphorylated Tyr132 to vaccinia virus (Scaplehorn *et al.,* 2002). *In vitro* experiments have shown that Nck and Grb2 bind directly to phosphorylated peptides containing residue 112 and residue 132 of A36R respectively (Frischknecht *et al.,* 1999b; Scaplehorn *et al.,* 2002). The recruitment of Grb2 to vaccinia virus also depends on the proline rich domain of N-WASP and increases the efficiency vaccinia actin tail formation (Scaplehorn *et al.,* 2002). Experiments overexpressing dominant-negative constructs in infected cells have demonstrated that Nck, WIP and N-WASP are all required for actin-based motility of vaccinia (Frischknecht *et al.,* 1999b; Moreau *et al.,* 2000). Using cells lacking N-WASP confirmed that vaccinia actin tail formation is strictly dependent on N-WASP (Snapper *et al.,* 2001). In addition Snapper *et al.,* 2001 confirmed that in N-WASP null cells WIP and Nck are not recruited to virus particles suggesting that Nck/WIP and N-WASP are recruited as a complex (Snapper *et al.,* 2001). Taken together, it appears that

vaccinia achieves actin-based motility by mimicking and hijacking components of a tyrosine kinase receptor signal-transduction pathway (Figure 1.16).

The molecular basis how vaccinia manages to induce actin tails is not clear. However the observation that vaccinia actin tail formation is unaffected by ToxB (an inhibitor of Rho GTPases) treatment or by overexpression of a dominant negative form of Cdc42 suggests that vaccinia uses alternative ways in order to stimulate N-WASP locally. Although a GFP-Cdc42 constructs localises to virus particles no endogenous Cdc42 could be detected on the virus particles (Moreau *et al.,* 2000). The involvement of N-WASP activators like PIP_2 or tyrosine phosphorylation in vaccinia induced N-WASP activation has not been thoroughly investigated yet. However, vaccinia recruits Nck and Grb2, both of which have been shown to stimulate N-WASP *in vitro* (Carlier *et al.,* 2000; Rohatgi *et al.,* 2001). Efficient formation of vaccinia actin tails requires Nck and Grb2 suggesting that they might act co-operatively to optimally stimulate N-WASP (Scaplehorn *et al.,* 2002). However Nck appears to be sufficient to induce vaccinia virus actin tails.

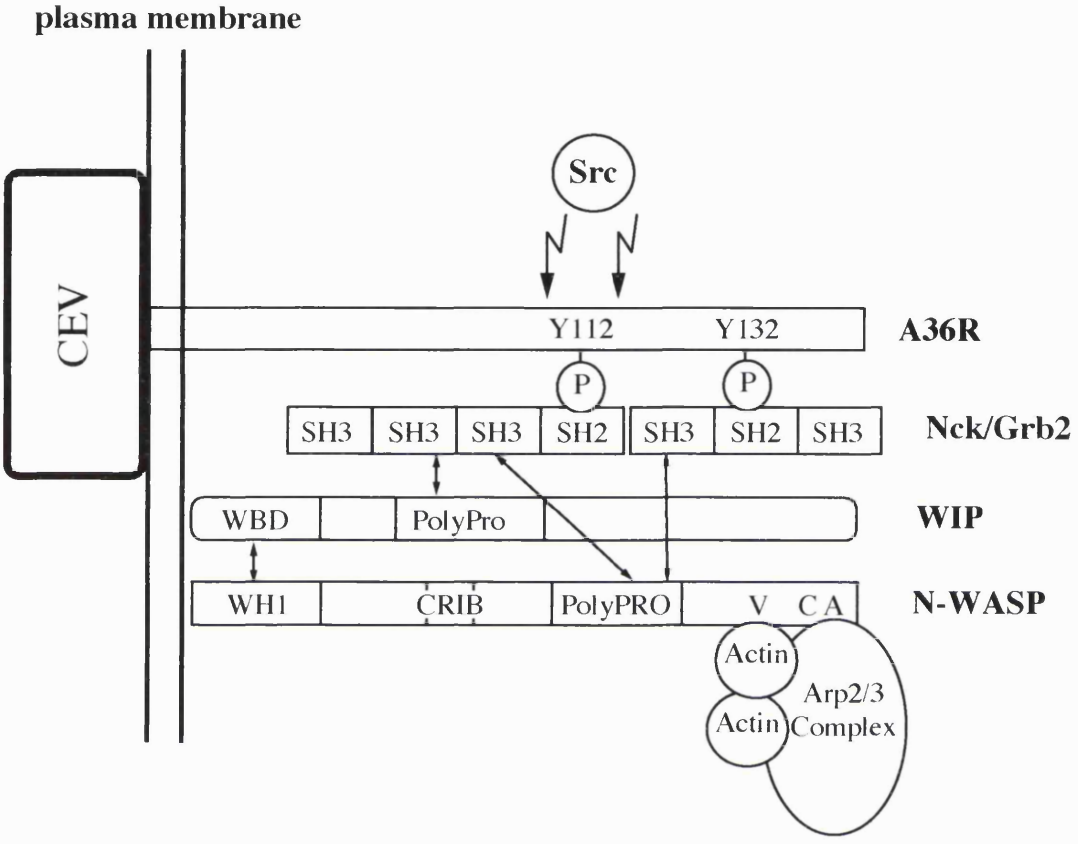


Figure 1.16. Schematic model of proteins involved in vaccinia virus actin tail formation.

A36R is tyrosine phosphorylated presumeably by Src kinases at Tyr112 and Tyr132 after fusion with the plasma membrane. Phosphorylation of A36R on Tyr112 or Tyr132 is required for recruitment of Nck and Grb2 respectively. N-WASP is recruited to vaccinia virus indirectly via Nck, Grb2 and WIP (WASP interacting protein). N-WASP activates the Arp2/3 complex locally beneath the CEV to stimulate actin polymerisation. Arrows or overlapping proteins indicate where interactions have been demonstrated experimentally. (Abbreviations see Figure 1.11).

1.10.4.5 N-WASP/WIP complexes in pathogens

Pathogens like *Shigella flexneri*, *Listeria* monocytogenes, EPEC and vaccinia virus are widely used as model systems to study the molecular components and signalling events leading to actin polymerisation. All four pathogens recruit the Arp2/3 complex in order to polymerise actin on their surface or beneath their surface at the plasma membrane. However, they have all developed different strategies to recruit and activate the Arp2/3. *Listeria*, for instance, uses its bacterial surface protein ActA to bind and activate the Arp2/3 complex directly (Figure 1.17). *Shigella* recruits N-WASP to its surface in order to bind and activate the Arp2/3 complex. It has been shown that WIP and Nck are also recruited via N-WASP to *Shigella* but they do not appear to play a functional role in the actin based motility of *Shigella* (Moreau *et al.*, 2000). EPEC actin pedestal formation is dependent on Nck and N-WASP but it is not clear whether WIP is also involved. Furthermore it is not known how N-WASP is recruited to EPEC, although a ToxB resistant Rho GTPase and the WH1 domain of N-WASP have been proposed to play a role in N-WASP recruitment to the bacterium (Kalman *et al.*, 1999) (Lommel *et al.*, 2001). Considering the molecular components involved in vaccinia actin tail and EPEC pedestal formation, which both occur at the plasma membrane it appears that two evolutionary distinct pathogens have developed a similar mechanism to exploit the host cell actin cytoskeleton.

This work has sought to understand signalling events leading to actin polymerisation of various pathogens such as vaccinia virus, *Shigella* and EPEC. In particular the molecular basis of the N-WASP/WIP complex is investigated using vaccinia and *Shigella* as model systems. Since vaccinia virus and EPEC induce actin polymerisation from the outside of the host cell I have also investigated these two pathogens comparatively specifically addressing the questions whether Src and WIP are required for EPEC induced pedestal formation and how N-WASP and WIP are recruited to EPEC.

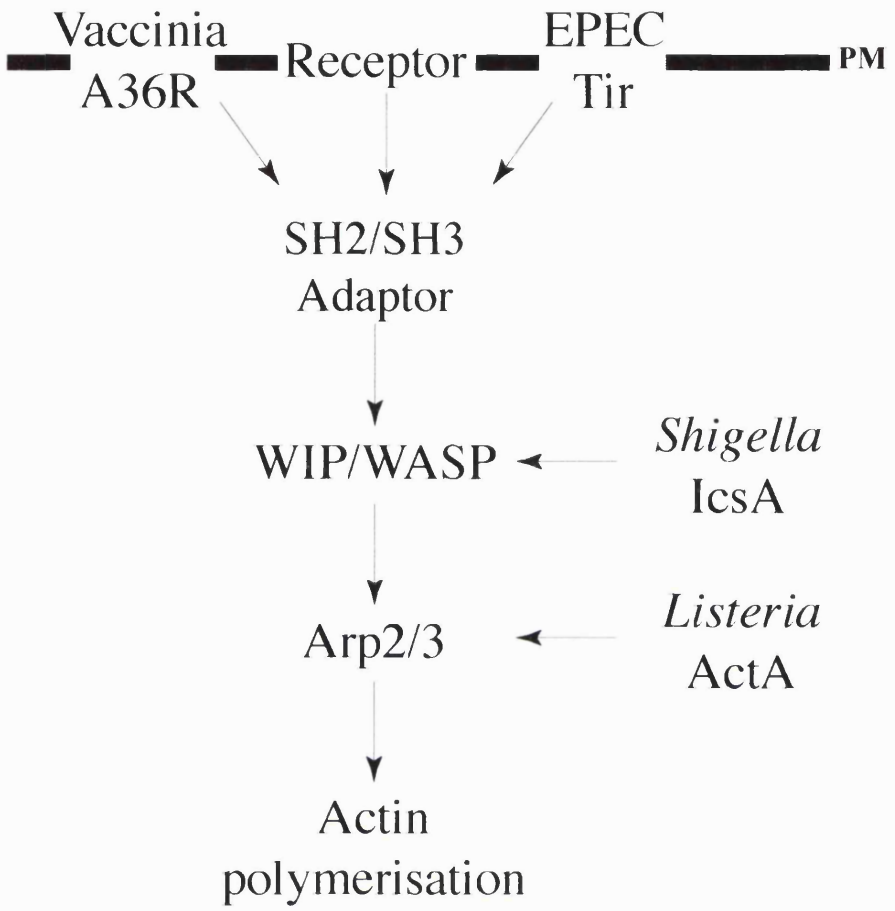


Figure 1.17. Intracellular pathogens hijack receptor tyrosine kinase signalling cascades at different levels.
Vaccinia and EPEC induce actin polymerisation sitting on the outside of the cell plasma membrane (PM) and are dependent on tyrosine phosphorylation of their respective surface proteins A36R/Tir. This leads to the recruitment of SH2/SH3 domain containing adaptor proteins. On the other hand, *Shigella* and *Listeria* induce actin tails in the cytoplasm of the host cell. IcsA, a surface protein of *Shigella* recruits the WIP/N-WASP complex. N-WASP activates the Arp2/3 complex on the surface of *Shigella*. The surface protein ActA of *Listeria* directly binds and activates the Arp2/3 complex on its surface, initiating actin tail formation.

Chapter 2

2 Materials and Methods

2.1 Cell Types

HeLa cells were obtained form Dr. Sally Cudmore (EMBL, Heidelberg, Germany). Parental wild type (N-WASP WT) and N-WASP -/- primary mouse embryonic fibroblasts (MEF) cells were provided by Dr. Snapper (Harvard Medical School, Boston, USA) (Snapper *et al.,* 2001). WT (SYF+/+) and MEF lacking the ubiquitously expressed Src kinases Src, Fyn and Yes (SYF-/-) were provided by Dr. Soriano (Fred Hutchinson Cancer Research Centre, Washington, USA) (Klinghoffer *et al.,* 1999).

2.2 Buffers, media and solutions

Luria-Bertani (LB), Brain-heart-infusion (BHI) medium, Phosphate Buffered Saline (PBS), Tris Buffered saline (TBS), trypsin, Glutamine, EDTA, minimal essential medium (MEM), Dulbecco's MEM (DMEM) were made by CR-UK research services.

Luria-Bertani (LB)

10 g/l tryptone
5 g/l yeast extract
10 g/l NaCl
adjusted to pH 7.0 using NaOH

Brain-heart-infusion (BHI) medium

37 g/l Brain heart infusion broth powder

Phosphate Buffered Saline (PBS)

8 g NaCl
0.25 g KCl
1.43 g Na_2HPO_4
0.25 g KH_2PO_4

Dissolve in distilled water and check pH is 7.2 and make up to 1 L.

Tris Buffered Saline (TBS)

8 g NaCl

3 g Trizma Base

Dissolve in distilled water and add HCl to pH =7.7 and make up to 1 L.

Trypsin solution

8 g NaCl

0.1 g Na_2HPO_4

1 g D-glucose

3 g Trizma Base

2 ml 19% (w/v) KCL solution

1.5 ml 1% (w/v) phenol red solution

Dissolve in 200 ml distilled water and add HCl to pH 7.7. Then add 0.06 g penicillin and 0.1 g streptomycin. Dissolve Trypsin (Difco 1:250) in 200 ml distilled water and assist dissolving by bubbling air through the solution. Add dissolved Trypsin to the TBS solution. Make up to 1 l with distilled water, Check pH =7.7. The solution is sterilised by filtration using 0.22 μm filters and stored at –20°C.

EDTA solution (Versene)

8 g NaCl

0.2 g KCl

1.15 g Na2HPO4

0.2 g KH2PO4

0.2 g ethyldiaminotetraacetic acid disodium (EDTA) salt

1.5 ml 1 % (w/v) phenol red solution

Dissolved in 1L distilled H_2O and the pH was adjusted to pH7.2 before the solution was autoclaved.

Glutamine solution

A 10x stock solution (0.24 M) was prepared by dissolving 35.05 g L-Glutamine in distilled water. The solution was sterilised by filtration using 0.22 μm filters and stored at –20°C.

Minimal essential medium (MEM)

The MEM powder used in the following formulations is supplied by ICN FLOW Cat. No. 10-121-24.

9.68 g MEM powder

3.7 g Bicarbonat ($NaHCO_3$)

0.06 g Penicillin

0.1 g Streptomycin

Adjust pH 7.0 with CO_2.

Dulbecco's MEM (DMEM)

DMEM containing 4500 g/l glucose was purchased from Sigma Cat. No. D6546. DMEM was supplemented with glutamine.

2.3 DNA constructs

In this thesis I used vectors belonging to three categories. For protein expression in bacteria I used an *E.coli* T7 based expression vector, pMW172-His which includes six histidines at the N-terminus derived form the original pMW172 (Way *et al.,* 1990). To drive protein expression in tissue culture cells I relied on the CMV driven expression vector CB6 (Reckmann *et al.,* 1997). To express proteins in vaccinia virus infected cells I used a vector containing a constitutive early/late viral promotor (pE/L) (Frischknecht *et al.,* 1999b). All following DNA constructs have tags such as six histidines (His), glutathione-S-transferase (GST) and enhanced GFP (eGFP) fused to their N-terminus to allow multiple detection and binding studies. All vectors with affinity tags allow N-terminal fusion with the respective proteins by cloning into Not1 and EcoR1 restriction sites. All N-WASP constructs used in this study are PCR derived from rat cDNA. Nck 1, WIP, VASP-EVH1 domain and WIRE (WICH) constructs originate from a PCR on human cDNA. The numbers in brackets with each DNA construct indicate the corresponding amino acid sequence.

Table 1: pMW *E.coli* expression constructs

insert	Used in Figure	Source
His-WH1 (1-145)	3.1	(Moreau *et al.*, 2000)
His-Nck1 (1-373)	4.22	(Moreau *et al.*, 2000)
His-WH1 W54A (1-145)	3.3	this study (see 2.2.1.4.3.1)
His-EVH1 (1-115)	3.4	this study (see 2.2.1.4.3.2)

Table 2: CB6 DNA constructs

insert	Used in Figure	Source
GFP-N-WASP (1-501)	4.5	(Moreau *et al.*, 2000)
GFP-CRIB (148-273)	4.5	(Moreau *et al.*, 2000)
GFP-N-WASPΔPolyPro (Δ274-385)	4.5	Dr. Violaine Moreau
GFP-N-WASPΔWH1 (148-501)	4.5	(Moreau *et al.*, 2000)
GFP- WH1 W54A (1–148)	4.5	(Moreau *et al.*, 2000)
GFP-WH1 (1–148)	4.5	(Moreau *et al.*, 2000)
GFP-N-WASP PolyPro (271–386)	4.5	(Moreau *et al.*, 2000)
GFP-WIP (1–503)	4.14	(Moreau *et al.*, 2000)
GFP-WIP-PolyPro (122–413)	4.17	(Moreau *et al.*, 2000)
GFP-WIPΔWBD (1–413)	4.17	(Moreau *et al.*, 2000)
GFP-WBD (413–503)	3.7	(Moreau *et al.*, 2000)
N-GFP	-	(Rietdorf *et al.*, 2001)
GFP-WBD F454A (413–503)	3.7	this study (see 2.2.1.4.3.3)
GFP-WBD F456A (413–503)	3.7	this study (see 2.2.1.4.3.3)
GFP-WBD F454/456A (413–503)	3.7	this study (see 2.2.1.4.3.3)
GFP-WIP F454/456A (413–503)	3.10	this study (see 2.2.1.4.3.3)
GFP-WIPΔPolyPro (Δ122-413)	4.17	this study (see 2.2.1.4.3.6)
His-WIPΔPolyPro (Δ122-413)	4.24	this study (see 2.2.1.4.3.6)
GFP-N-WASP W54A (1–503)	4.9	this study (see 2.2.1.4.3.5)
GFP-WIP P332A (1–503)	4.25	this study (see 2.2.1.4.3.3)

Table 3: pE/L DNAconstructs

Insert	Used in Figure	Source
GFP-WBD (413–503)	3.9	(Moreau *et al.*, 2000)
GFP-WIP (1–503)	3.9	(Moreau *et al.*, 2000)
GFP-WIPΔWBD (1–413)	4.19	(Moreau *et al.*, 2000)
GFP-WIP-PolyPro (122–413)	4.19	(Moreau *et al.*, 2000)
GFP-N-WASPΔPolyPro (Δ274-385)	4.11	Dr. Violaine Moreau
GFP-WIP A (122–413)	4.22	Dr. Violaine Moreau
GFP-N-WASP (1–503)	4.11	(Frischknecht *et al.*, 1999b)
GFP-WIPΔPolyPro (Δ122-413)	4.19	this study (see 2.2.1.4.6)
His-N-WASP (1–503)	4.6	this study (see 2.2.1.4.3.4)
His-N-WASPΔPolyPro (Δ274-385)	4.6	this Study (see 2.2.1.4.3.4)
GFP-N-WASP (W54A) (1–503)	4.11	this study (see 2.2.1.4.3.5)
His-CRIB (148–273)	-	(Moreau *et al.*, 2000)
His-WIP (1–503)	4.24	this study (see 2.2.1.4.3.6)
His-WIPΔPolyPro (Δ122-413)	4.24	this study (see 2.2.1.4.3.6)
GFP-WBD F454A (413–503)	3.9	this study (see 2.2.1.4.3.3)
GFP-WBD F456A (413–503)	3.9	this study (see 2.2.1.4.3.3)
GFP-WBD F454/456A (413–503)	3.9	this study (see 2.2.1.4.3.3)
GFP-WBD P465A (413–503)	3.12	this study (see 2.2.1.4.3.3)
GFP-WBD K478A (413–503)	3.12	this study (see 2.2.1.4.3.3)
His-WIP P332A (1–503)	4.24	this study (see 2.2.1.4.3.3)

Table 4: miscellaneous vectors

Insert	Used in Figure	Source
Chicken c-Src (1-527)	4.2	(Gonfloni *et al.*, 1997)

2.4 Peptides

All peptides used in this thesis were produced with never ceasing enthusiasm and speed by the peptide synthesis service at Cancer Research UK. The sequences of all peptides were confirmed by mass spectrometry. A cysteine was added to each peptide to allow for covalent coupling to SulfoLink resin (Pierce, Rockford, USA). The SulfoLink resin

is 6% cross-linked agarose, which reacts with free sulfhydryl groups (SH) in a covalent manner. To give the coupled peptides a flexible linkage when bound to the resin two glycines were added following the cysteine. The numbers correspond to the respective amino acids of the protein indicated for each peptide. The additional CGG residues are not shown for clarity in the table below.

Name	Sequence
WIP411-426 (peptide A)	RSGPRPPLPPDRPSAG
WIP422-438 (peptide B)	RPSAGAPPPPPPSTSIR
WIP459-470 (peptide C)	ISDLPPPEPYVQ
WIP492-503 (peptide D)	ERGGPPLPPIPR
WIP451-473 (peptide E)	ESRFYFHPISDLPPPEPYVQTTK
WIP451-461 (peptide F)	ESRFYFHPISD
WIP451-461 E451A	ASRFYFHPISD
WIP 451-461 S452A	EARFYFHPISD
WIP 451-461 R453A	ESAFYFHPISD
WIP 451-461 F454A	ESRAYFHPISD
WIP 451-461 F456A	ESRFYAHPISD
WIP 461-485 (peptide G)	DLPPPEPYVQTTKSYPSKLARNESR
WIP 461-485 (peptide H)	TTKSYPSKLARNESR
WIP 451-461 F454/456A	ESRAYAHPISD
ActA-FPPPP	SFEFPPPPTD
ActA-APPPP	SFEAPPPPTD
CR16 390-400	ESKFTFHSVED
WICH 397-407	ESKYSFHPVED
Nck 329-341	DETPRLPQRNLSL
Nck 329-341 P332A	DETARLPQRNLSL

2.5 Molecular Biology

Standard molecular biology procedures such as agarose gel electrophoresis, Polymerase chain reaction (PCR), restriction digests, ligation reactions were performed as described (Maniatis, 1989)

2.5.1 Transformation of *E.coli*

For preparation of calcium competent cells 1 ml of a 5 ml over night culture of *E. coli* strain DH-5θ was grown in 100 ml LB to an OD_{600} of about 0.4 -0.8. Cells were centrifuged for 5 mins at 3000 g, 4°C and subsequently the pellet was resuspended in 50 ml of ice-cold 0.1 M $CaCl_2$. The suspended cells were left for 30 min to several hours on ice. After a second centrifugation the cell pellet was resuspended in 2 ml 0.1 M $CaCl_2$ and the competent cells 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, incubated for 5 min on ice and plated onto one or two agar plates prewarmed to 37°C containing the correct antibiotic following the rapid transformation protocol by the Pope (Pope and Kent, 1996). Classically ampicillin, chloramphenicol and kanamycin were used at final concentrations of 100, 34 and 30 µg/ml respectively.

2.5.2 Plasmid DNA preparation

A colony from transformed DH5θ cells was picked and grown in a 5ml culture of LB containing the relevant selection antibiotic for the plasmid. From this overnight culture, small scale DNA purifications utilised the Qiagen Mini-prep kit (West Sussex, UK). For large scale DNA purifications the 5ml preculture was added to 100ml LB with antibiotic and grown overnight. Bacteria were pelleted and DNA was prepared from pelleted bacteria using the Qiagen Maxi-prep kit (West Sussex, UK). DNA concentrations were determined using a spectro photometer (Ultraspec 3100 pro, Biochrom Ltd.) measuring the optical density (OD) at 260nm. An OD_{260} of 1 equals a DNA concentration of 50µg/ml. A ratio of OD_{260}/OD_{280}, which measures the DNA versus protein concentration, of 1.8 from maxi prep prepared DNA was considered to be acceptable DNA quality. Even if it was worse I used it since it was too painful to purify the DNA again.

2.5.3 DNA sequencing

Oligonucleotides corresponding to the ends of the respective vector were used to sequence the start and the end of the cloned insert and also to check whether the insert was cloned in frame with the open reading frame. To confirm fidelity of the complete clone primers were designed separated at intervals of 300- 400 bp throughout the

respective gene. PCR reactions using these primers and the template were carried out using the ABI prism BigDye Terminator Cycle sequencing kit (Applied Biosystems). Unincorporated primers and deoxynucleotides were removed from the PCR reaction using the Qiagen nucleotide removal kit (West Sussex, UK). Vacuum dried PCR reactions were subjected automated sequencing using a Perkin Elmer sequencer, ABI prism 377. The fidelity of constructs generated by PCR was confirmed by sequencing and by standard BLAST searches of the nucleotide database at the National Centre for Biotechnology Information (NCBI).

2.5.4 Construction of DNA vectors

2.5.4.1 Subcloning

DNA clones with different N-terminal tags were generated by subcloning of already existing sequenced DNA fragments. All inserts in CB6-GFP, pE/L-GFP and pMW172-His vectors were cloned in an inframe Not1 restriction site downstream of the respective tag and 3' EcoR1 site. Therefore subcloning was always performed via a Not1 and EcoR1 digest to release the insert from the vector. Neither the vector nor any of the clones used in this work have an internal Not1 or EcoR1 restriction site.

2.5.4.2 Polymerase chain reaction (PCR)

New DNA constructs were generated by PCR using primers containing the desired region of the gene sequence. The forward primer contained the Not1 restriction site followed by the gene sequence. The reverse primer contained stop codons and the EcoR1 site in the correct orientation. Three to five base pairs were added to the 5' ends on both primers to allow for efficient digestion of Not1 and EcoR1 in the resulting PCR product. The amplified insert and the vector were digested with Not1 and EcoR1, separated on an 0.8-1.5% agarose gel, purified using the Qiagen gel purification kit and subjected to ligation using T4 DNA ligase (New England Biolabs). Ligations were carried out for minimum in a volume of 10-20μl 30 minutes or overnight at room temperature before tree quarters of the ligation mix was transformed into calcium competent *E.coli* DH5α. Resulting colonies were screened for the correct size of the insert by Not1/EcoR1 digests or by PCR colony screens using vector primers 5' and 3' of the insert. For colony PCR a toothpick of an *E.coli* colony was suspended in the

PCR reaction mix and heated to 95°C for 1 minute to lyse the bacteria followed by a standard PCR protocol.

2.5.4.3 Mutagenic PCR

Point mutations were introduced into the respective clone using the Quik Change Mutagenesis Kit (Stratagene, La Jolla, California, USA) according to the manufacturers recommendations.

2.5.4.3.1 pMW-His-WH1W54A

The WH1 (W54A) DNA insert was obtained digesting Not1/EcoR1 from pE/LGFP-WH1 (W54A) (Moreau *et al.,* 2000) and subcloned into the Not1/EcoR1 sites of pMWHis-Nck (Frischknecht *et al.,* 1999b) (see 2.5.4.1).

2.5.4.3.2 pMW-His-EVH1 (human VASP)

The EVH1 domain was amplified by PCR from a human VASP cDNA template (provided by Dr. Richard Treisman) (Grosse *et al.,* 2003). Using the forward primer 5'CGCCGGATCCGCGGCCGC**ATG**AGCGAGACGGTCATCTGT 3' and the reverse primer 5'CCCGAAT**TC**A**TCA**TCCTTCCAACGCCTCTAGGGC3'. The forward primer incorporated a BamH1(GGATTC) and a Not1(GCGGCCGC) site. The reverse primer contained an EcoR1 restriction site and two stop codons (in bold). The PCR product was digested with Not1/EcoR1 and cloned with Not1 and EcoR1 into pMW-His-Nck vector (see 2.5.4.2). The fidelity of the pMW-His-EVH1 was checked as described in 2.1.1.4.2 and sequencing (see 2.5.3). The BamH1 site was included to be able to clone BamH1 EcoR1 into an untagged pMW vector.

2.5.4.3.3 Generation of pE/L-GFP-WIP and pE/L-GFP-WIP-WBD point mutants

Alanine substitutions of phenylalanine 454, 456, proline 332, 465, and lysine 478 were introduced into pE/L-GFP-WIP and pEL-GFP-WIP-WBD (WBD, WASP binding domain of WIP, residues 413-503) (Moreau *et al.,* 2000) using the Qikchange Site-directed Mutagenesis Kit (see 2.5.4.3). Where required the WIP mutants were subcloned from pE/L-GFP vector into the NotI/EcoRI sites of the CMV driven mammalian GFP expression vector CB6-N-GFP and pE/L-His vectors.

1.1.1.1.4 Generation of pE/L-His-N-WASP, pE/L-His-N-WASPΔPolyPro

N-WASP and N-WASPΔPolyPro inserts were subcloned from the pE/L-GFP vector into the Not1/EcoR1 sites of pE/L-His-Nck (see 2.5.4.1).

2.5.4.3.5 Generation of pE/L-GFP-N-WASP (W54A) and CB6-GFP-N-WASP (W54)

pE/L-GFP-WH1 (W54A) was digested with Not1/BglII as there is a unique BglII restriction site in the WH1 of N-WASP, a 200 bp fragment is released which was subsequently subcloned into pE/L-GFP-N-WASP digested with Not1/BglII. From the resulting vector pE/LGFP-N-WASP (W54A) was subcloned into CB6-GFP-WH1 resulting in CB6-GFP-N-WASP (W54A).

2.5.4.3.6 Generation of pE/L-GFP-WIPΔPolyPro, pE/L-His-WIPΔPolyPro , pE/L-His-WIP and CB6-GFP-WIPΔPolyPro

I deleted the proline rich region of WIP (residues 122-413) via PCR using the Quikchange Site-directed Mutagenesis Kit (2.2.1.4.3). The forward and the reverse primer consisted of base pairs corresponding to the N-terminus and C-terminus of the proline rich region of WIP. The forward primer I used was 5'GCCAACAGGGATAATGATTCTCCCAGGCCTCCCCTTCCTCCT3' and the reverse primer was 5'AGGAGGAAGGGGAGGCCTGGGAGAATCATTATCCCTGTTGGC3'. The insert was subcloned into CB6-N-GFP and pE/L-His-CRIB using Not1/EcoR1 restriction sites that flank WIP. WIP was subcloned from pE/L-GFP-WIP into the Not1/EcoR1 site of pE/L-His-CRIB to generate pE/L-His-WIP.

2.6 Cell culture

Cells were split regularly at 50–80 % confluency up to 40 passages before new stocks were thawed from liquid N_2 lab stocks. Cells were usually not grown to 100% confluency. All cell types used were seeded onto untreated, autoclaved round 11 mm glass coverslips for immunofluorescence imaging. To obtain images of single cells with nice morphology cells with 70-60% confluency at the end of the respective assay were ideal. However, during some experiments, including multiple infections, 100%

confluent cells were required to get enough surviving cells for Western or microscopic analysis.

To generate liquid N_2 stocks of cells a 10 cm diameter tissue culture dish with cells at 70% confluency was trypsinised. Then the cells were pelleted by centrifugation at 1,200 rpm in an Haraeus centrifuge. Subsequently the medium was aspirated, the cells resuspended in 500µl cold FCS and incubated 20min at ice. The appropriate medium containing 20% DMSO was mixed with the cells before they were aliquoted in a cryovial and stored at –80°C for 24 hours before being moved to liquid N_2.

HeLa cells were grown and maintained in minimal essential medium (MEM) (CR-UK research services) supplemented with 10% fetal bovine serum (FBS) (Sigma, Taufkirchen, Germany), 100 µg/ml penicillin, 100 µg/ml streptomycin (Pen/Strep), and 2 mM L-glutamine (CR-UK research services) at 37°C, under 5% CO_2.

SYF +/+ cells were grown in DMEM containing 4.5 g/l D-glucose with 10% FCS (Sigma, Taufkirchen, Germany), 2 mM L-glutamine and Pen/Strep. SYF -/- cells were grown in the same media but instead of 10% FCS heat inactivated 10% newborn calf serum (NCS) (Gibco, Paisely, Scottland) was used.

N-WASP +/+ and N-WASP -/- cells were grown in DMEM containing 4.5 g/l D-Glucose supplemented with 10% FCS, 2 mM L-glutamine, Pen/Strep and 2 mM 2-θθMercaptoethanol. These cells grow extremely fast and therefore were split 1:10 at least or 1:20 over the weekend.

2.6.1 Transfections

I transfected tissue culture cells three different ways depending on the cell type and the assay being performed (see below).

Mouse embryo fibroblasts (MEFs), including N-WASP +/+, N-WASP -/-, SYF +/+ and SYF-/- , generally transfected well with Lipofectamine 2000 (Gibco, California, USA). Transfections were carried out according to manufacturers protocol. Amounts of DNA and transfection reagent were used according to a protocol developed by Dr.

AlfonsoFerran Valderrama. Following the rule that less is sometimes better I used half the amount of CB6 vector DNA (4μg for 3.5cm^2 tissue culture dish) and half the amount of Lipofectamine (typically 8μl per 3.5cm^2 dish). The cells were left in transfection mix without FCS for 4h and then washed once with medium including FCS and allowed to recover over night during which the cells expressed the respective protein. The next day the same medium including FCS was replaced and the cells allowed to recover for 4-8 hours. Subsequently, infections with *Shigella*, EPEC or vaccinia virus were performed as described in section 2.2.2.3.

Since vaccinia virus replicates in the cytoplasm and encodes for its own transcription machinery, the pE/L vector DNA does not have to enter the nucleus and is efficiently transcribed in the cytoplasm of the cell. For infection transfection experiments, cells were infected with vaccinia virus and subsequently transfected with Lipofectin (Gibco, California, USA) (1 μg of pE/L vector DNA per coverslip). For assaying the effect on or localisation of a protein to vaccinia actin tails infected cells were transfected 4 hours post infection (hpi) and subsequently fixed 4hours later (8 hpi) and processed for IF.

Calcium phosphate transfections of CMV based expression vectors (CB6) were used to express proteins in HeLa cells prior to infection with EPEC and *Shigella*. For cells grown in a 3.5 cm diameter culture dish, 0.5 μg of Qiagen midi prep DNA was diluted to a total volume of 50 μl with 0.25 M $CaCl_2$ and incubated at room temperature for about 20 min at room temperature before 50 μl of HBS (280 mM NaCl, 10 mM KCl, 1.5 mM Na_2HPO_4, 12 mM Glucose, 50 mM HEPES, pH 7.05) was added. The resulting mix was incubated for another 10-15 min at room temperature before the mix was added to the medium containing FCS (1.5ml in 3.5 cm diameter culture dish) over the cells. 12-20 hours later cells were washed twice with PBS for a total of 15 min and allowed to recover for various times (minimum two hours – maximum six hours) in MEM. For infection experiments transfected cells were infected 2-24 hours post recovery with the respective pathogen (usually *Shigella* and EPEC).

2.6.2 Infections with vaccinia virus

Wild type vaccinia virus is referred to as WR (Western Reserve strain). Recombinant mutant viruses that carry a mutation in viral genome changing tyrosine 132 of A36R

into phenylalanine is referred to as A36R-Y132F (Scaplehorn *et al.*, 2002). Viral stocks were prepared from 30-35 175cm^2 Falcon flasks (Becton Dickingson, 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 rpm were performed in a Heraeus Megafuge 1.0R (Heraeus, Newtown, CT, USA) unless otherwise stated. Cell pellets were resuspended in 500µl of 10 mM Tris/HCl pH 7.5 and passed through a 22-gauge needle (Becton Dickingson). Trypan blue staining was used to reveal if all cells were broken. Post nuclear supernatant (PNS) was harvested after cell debris was pelleted by centrifugation for 5 min at 3,000 rpm at 4°C. The PNS containing virus particles was aliquoted, stored at –80°C and used for the majority of infections. The virus titer was determined by infecting HeLa cells for 8 hours with various dilutions of virus stock solution and counting the number of infected cells based on immunofluorescence using a viral marker. 100% of infected cells are referred to as multiplicity of infection (moi) 1. All experiments unless otherwise stated were performed by addition of moi 1-2 of virus to ensure complete infection. Vaccinia virus stock solutions were mixed with 10% volume of 2.5 mg/ml trypsin (CR-UK research services), incubated for 30 min at 37°C, and diluted 10 fold in 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 to achieve the desired moi.

To block Src kinases during vaccinia infections cells were treated with PP2 and PP3 as a negative control (see 2.2.2.3). Both inhibitors were used at a final concentration of 100 µM and added at 6hpi as not to interfere with the early stages of virus life cycle.

2.6.3 Infections with bacterial pathogens

Shigella flexneri strain SC301 was a kind but dangerous gift from Dr. Philippe Sansonetti (Institute Pasteur) (Clerc and Sansonetti, 1987). *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. *Shigella* were stored in a 50% glycerol BHI media (CR-UK research services) at –80°C. For infection assays 20 ml BHI-medium

containing 100μg/ul ampicillin were inoculated with *Shigella* and grown overnight at 37°C shaking. 1ml of the overnight culture was used to inoculate 20ml BHI with 100μg/ul ampicillin and cultures were grown to an OD_{600} of 1.8 or 2.2 as surface expression IcsA (a surface protein of *Shigella* essential for actin tail formation see 1.10.4.2) peaked at this density allowing efficient actin tail formation (Goldberg *et al.*, 1994). 200μl of the culture with the correct OD_{600} was spun at 14,000 rpm in a tabletop centrifuge (Eppendorf, Engeldorf, Germany) for 10 seconds, washed twice in PBS before resuspended in 500 μl PBS. Mammalian cells were washed three times in serum and antibiotic free medium before *Shigella* suspended in PBS were added. Of the 500 μl PBS-bacteria suspension 1 μl was used to infect cells on one 11mm coverslip. As soon as apototic blebs were detected, the latest after 2 hours, the medium was replaced with one containing 5-15 μg/ml Gentamycin (Sigma, St.Louis, USA). Gentamycin kills extracellular bacteria but does not cross the plasma membrane of the cell thus leaving intracellular bacteria unharmed. All plastic that came in contact with bacteria was washed in concentrated verkon (CR-UK) before being disposed as S2 garbage. Erlenmeyer flasks were incubated in concentrated verkon over night before being sprayed with ethanol and disposed for reuse.

Enteropathogenic *E.coli* (EPEC) strain E2348/69 was stored as 50% glycerol stock in LB at –80°C (Levine *et al.*, 1978). For infection assays 20ml LB were inoculated and grown over night at 37°C shaking. 200μl of the overnight culture were spun at 14,000 rpm in an Eppendorf centrifuge (Eppendorf, Engeldorf, Germany) for 10 seconds, washed twice in PBS before taken up in 500 μl PBS. Mammalian cells were washed three times in serum and antibiotic free medium before EPEC were added. Of the 500 μl PBS-bacteria suspension 15μl were used to infect cells on 11mm coverslips in 3.5 cm diameter tissue culture dishes. After 1h the cells were washed twice with 2ml PBS to remove excess EPEC. In general the infection was stopped after 2 hours by fixing the cells. However, sometimes the infection was sometimes allowed to proceed for up to five hours because it is easier to see pedestals protruding form the side of the infected cell.

To block Src kinases during EPEC infections cells were treated with PP2 and PP3 (negative control) 1 hour before addition of EPEC at a final concentration of 100 μM

and added. Applying the drugs 1 hour before ensures that Src kinases are inhibited when EPEC establishes contact with the cells. PP2 and PP3 were maintained in the medium until cells were fixed.

Name	Origin	Description	Use
PP2	Calbiochem (Merck Biosciences, Nottingham, UK)	Selective Src family kinase inhibitor (Hanke *et al.*, 1996)	100 μM soluble in DMSO
PP3	Calbiochem (Merck Biosciences, Nottingham, UK)	Negative control for PP2, but inhibits EGF receptor at conc. 2.7 μM (Traxler *et al.*, 1997)	100μM soluble in DMSO

2.7 Immunofluorescence analysis

Cells were fixed with 3% paraformaldehyde (PFA) (Merck, Darmstadt, Germany) in cytoskeletal buffer (CB) (10 mM Mes pH 6.1, 150 mM NaCl, 5mM EGTA, 5 mM MgCl2, 5 mM glucose) for 10 min at room temperature. PFA-fixed cells were permeabilised for 2 min with CB containing 0.1% Triton X-100 (Sigma). Non-specific antibody binding sites were blocked in blocking buffer (TBS (20 mM Tris/HCl pH 7.5, 154 mM NaCl, 2 mM EDTA, 2 mM $MgCl_2$) containing 2% FCS and 1% BSA (Sigma) for 5-30 min (Frischknecht *et al.*, 1999a; Moreau *et al.*, 2000). Subsequently cells were labeled with a combination of primary antibodies diluted in blocking buffer for 30 min. Cells were then washed three times with PBS and incubated with the appropriate secondary antibodies coupled to Alexa-488 or Alexa-568, all obtained from Jackson Immunno Research (Baltimore, USA) for 30 minutes. All incubations were carried out at room temperature in humidified atmosphere. F-Actin was visualized with Alexa-488-phalloidin or Alexa-568-phalloidin (Molecular Probes, Eugene, OR, USA), which labels actin filaments. Cells were washed three times with PBS and once with distilled water and mounted in MOWIOL (Calbiochem, Bad Soden, Germany) mixed with 1/10 of p-phenylendiamine (Sigma) just before use. The use of p-phenylendiamine together with the mounting medium prevents bleaching of the conjugated fluorophore on the secondary antibody. 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 mg/ml 4', 6-diamidine-2'-phenylidole dihydrochloride (DAPI) (Roche Diagnostics,

Mannheim, Germany). Mounted coverslips were dried at 37°C for at least 30 min before examination on the immunofluorescence microscope.

A number of different fixation methods are commonly used for immuno-localisation studies depending on the antibody and epitiope (2.2.3.1). The most widely used fixation method is treatment with formaldehyde, freshly prepared from paraformaldehyd (PFA). I used 3% PFA in CB followed by permeabilisation by 0.1% Triton-X100 for 10 min as the standard fixation. After permeabilisation the cells were washed three times with PBS. However, some epitopes of proteins were not exposed during fixation with PFA and required a different fixation method. Some antibodies give a nicer signal when fixed and extracted at the same time in a mix of 1%PFA with 0.1%Triton X-100 in CB for 10 minutes at room temperature (also called P.T.). While cells were fixed in PFA for 10 min at room temperature, I fixed cells in cold methanol at -20°C for 0.5 to 5 min.

Images were recorded with a Photometrics Cool Snap HQ cooled CCD camera (Universal Imaging Corporation Ltd., Marlow, UK) using the Metamorph software package (Universal Imaging Corporation Ltd., Marlow, UK) attached to an upright Axioplan2 Zeiss microscope (Oberkochen, Germany). Images were processed and annotated with the Adobe software package (Adobe Systems Incorporated, San Jose, CA, USA).

2.7.1 Primary antibodies

W= Western blot, IF= Immunofluorescence

Name	Origin	Description	Use/Fixation
N-WASP (WA)	(Zettl and Way, 2002)	Rat polyclonal specific for WA domain	IF (1:500) PFA, PT
SrcPY418	BioSource, (California, USA)	Rabbit polyclonal, specific for activated Src	IF (1:300)
Src Mab3-27	(Gonfloni *et al.*, 1997)	Mouse monoclonal	IF (1:200)
WIP (15-35)	(Moreau *et al.*, 2000)	Rabbit polyclonal	IF (1:100) PFA, PT
His	Qiagen (, UK)	Mouse monoclonal	W (1:2000)

Name	Origin	Description	Use/Fixation
Nck	Upstate	Rabbit polyclonal	IF (1:100)
	(New York, USA)		W (1:2000)
Grb2	Transduction Lab	Mouse monoclonal	W (1:3000)
	(Lexington, USA)		
GFP (FL)	Santa Cruz	Rabbit polyclonal	W (1:3000)
	(California, USA)		

2.7.2 Secondary antibodies

Name	Origin	Use
Mouse-HRP	Jackson Immunno Research	W
	(Baltimore, USA)	(1:3000)
Rabbit-HRP	Jackson Immunno Research	W
	(Baltimore, USA)	(1:3000)
Rat-HRP	Jackson Immunno Research	W
	(Baltimore, USA)	(1:5000)
Alexa-568-anti rat	Jackson Immunno Research	IF (1:500)
	(Baltimore, USA)	
Alexa-488 anti rabbit	Jackson Immunno Research	IF (1:300)
	(Baltimore, USA)	
Alexa-488 anti mouse	Jackson Immunno Research	IF (1:300)
	(Baltimore, USA)	
Phalloidin-Alexa-568	Molecular Probes	IF (1:800)
	(Leiden, Netherlands)	
Coumarin-350	Molecular Probes	IF (1:50)
	(Leiden, Netherlands)	

2.8 Quantification of pathogen induced actin polymerisation

Quantifications of actin tails or pedestals were performed as described (Frischknecht *et al.*, 1999b; Moreau *et al.*, 2000). Briefly, for each experiment the presence of a single actin tail or pedestal was scored in transfected versus non-transfected cells. The presence of a single actin tail or pedestal within a cell was scored as positive as it is hard to quantify a reduction in actin tails or pedestal as their numbers naturally vary

greatly between cells. Since not all untransfected infected cells showed actin tails or pedestals the data was normalised to the number of infected cells that were not transfected but showed actin tails or pedestals. All experiments showing standard deviations from the mean were at least performed three times independently from each other. Generally when the effect of dominant negative construct was quantified medium to high expression of the respective protein were scored. Localisation studies on the other hand were done by observing the localisation of low level expressing proteins.

2.9 Preparation of HeLa cell extracts

Infected or uninfected HeLa cells were washed with cold PBS containing 1 mM Na_3VO_3 (PBS-VO), scraped in the same buffer, and centrifuged for 10 min at 2,000 rpm at 4°C. Na_3VO_3 was added to block phosphatases to preserve phosphorylation state of the proteins in the cell extract. For experiments with "over-stressed" cells based on their morphology 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 (sodiumdodecylsulphate) page sample buffer (50% glycerol, 3% SDS, 10% 0.5M 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. These samples represent "whole cell lysates", which were used for Western analysis. Alternatively cell pellets were resuspended in 1 volume 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 mg/ml leupeptin, 20 mg/ml chymostatin, 20 mg/ml pepstatin A and 20 mg/ml antipain) (Sigma) and extracted for 1.5 hours to overnight on a rotating wheel at 4°C. The resulting extracted cells were centrifuged for 15 min at 14,000 rpm on Eppendorf centrifuge at 4°C and protein concentration of the supernatant ("cell extract") determined using the BioRad Protein Assay (BioRad, München, Germany). A dilution series of 1 mg/ml BSA was used to generate a standard calibration curve. These cell extracts were subsequently used for pull down experiments.

2.10 Precipitation of histidine tagged proteins out of cell extracts

Cell extracts from infected or uninfected cells were diluted in 1x extraction buffer (12.5 mM Tris/HCl pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, 50 mM NaCl, 0.5% Triton X-100, 0.25% Nonidet P-40) including a protease inhibitor cocktail (0.4 mM phenylmethylsulfonyl fluoride, 20 mg/ml leupeptin, 20 mg/ml chymostatin, 20 mg/ml pepstatin A and 20 mg/ml antipain) (Sigma) to a protein concentration of 3-5 mg/ml, and incubated either for 1-2 hours or overnight at 4°C with Nickel agarose (Qiagen) or with various WIP peptides (see 2.4) coupled to SulfoLink resin. Generally 50µl of a 50% resin slurry in PBS was used for 200µl extract. After centrifugation at 500 rpm on Eppendorf centrifuge for 30 sec at 4°C, the supernatant was collected and the beads washed three times with 1x extraction buffer. Both supernatants and beads were diluted to an equal volume with SDS-PAGE sample buffer and boiled for 5-10min before being subjected to SDS-PAGE or western blot analysis where required.

2.11 SDS-PAGE and Western blotting

Cell extracts were subjected to SDS-PAGE on either 10, 12.5 or 15% gels depending on the size of protein (Laemmli, 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 polymerised with 40 ml N,N,N',N'-tetramethylene-diamine (TEMED) (Sigma) and 0.25 ml 10% ammonium persulfate (APS). Single gels were run at 6-12 W power in running buffer (10x stock: 10 g/l SDS, 30 g/l Trizma Base (Sigma), 144 g/l Glycine (Merck)). Where required gels were subjected to SDS PAGE gels were subjected to semi-dry blotting onto nitrocellulose membranes for 1 h at 150 mA. 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 and demarcate the size of the protein standards. Western blots were first incubated in blotto (PBS containing 0.1% Tween-20 (PBS-T) and 5% non-fat dry milk) for 10min at room temperature and then incubated 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 5-10min with blotto, incubated for 45-60 min at room temperature with the appropriate secondary antibody coupled to horseradish peroxidase (HRP) (Jackson Immunno Research, Baltimore, USA). Membranes were then washed at least 3-5 times for 10min with blotto and then at least

5 times with PBS-T to ensure removal of milk. Western blots were developed using the ECL system according to the manufacturer's instructions (Amersham International, Braunschweig, Germany). Blots were reprobed where necessary after stripping in strip buffer (2% SDS and 0.06 M Tris/Hcl, pH 6.8 supplemented with 0.78 ml θ-mercaptoethanol) for 45min at 50°C and subsequently extensively washed in PBS-T prior to the addition of primary antibody in blotto.

2.12 Expression of proteins in *E.coli*

All His tagged pMW172 clones (see 2.1.1.1, table1) were transformed into *E.coli* strain BL21 (DE3) and protein produced via leaky expression by growth overnight in LB with ampicillin at 30 °C. The bacteria were pelleted by centrifugation at 5,000 rpm in a Haerus centrifuge and the supernatant was discarded. Subsequently the bacterial pellets were frozen and stored at –20°C. The soluble fraction was prepared as described previously (Way *et al.,* 1992). When His tagged proteins were purified Imidazole pH8.0 was added to a final concentration of 25 mM to reduce background binding of positively charged amino acids to the nickel resin. His-Nck was purified as previously described (Moreau *et al.,* 2000) and eluted from the nickel resin by 2 elutions with 1ml PBS containing 250mM Imidazole pH8 and one 1ml elution with PBS containing 500mM Imidazole pH8.0. Subsequently the fractions were analysed by SDS-PAGE, pooled as required and dialysed against 4L PBS over night at 4°C. From 0.5L of a bacterial culture grown as described above I routinely got 13mg pure His-Nck.

2.13 Peptides and pull down assays

Based on the Ellmans reaction, which detects reactive sulfhydryl groups, saturating amounts of all peptides were coupled via the N-terminal cysteine residue to SulfoLink resin according to the manufacture's instructions (Pierce, Rockford, USA). Equivalent volumes of BL21 (DE3) soluble fraction containing His-WH1, His-WH1-W54A, His-VASP-EVH1 or His-Nck were incubated with a constant volume of peptide saturated resin in the presence of 500 mM NaCl, 0.5 % Triton X-100, 25% Sucrose, 1.0 mM EDTA, 50 mM Tris pH 8.0 at room temperature for 30 minutes. The resin was washed 3 times with 1.0 M NaCl, 1.0 % Triton X-100, 25% Sucrose, 1.0 mM EDTA and 50 mM Tris pH 8.0. For His-Nck interaction with the WIP peptide pull downs and the His-WH1 His EVH1 competition experiment the buffer conditions for binding were the same except 150mM NaCl and 0.1%

Triton X-100 were used. The resin was washed 3 times in the binding buffer. Equivalent samples of washed resin were then subjected to SDS-PAGE. All data presented are from binding assays performed at the same time with equivalent amounts of soluble fraction. The gels were incubated in a staining solution (0.5% Comassie Brilliant Blue dissolved in 50% MeOH, 10% actetic acid) for 30 minutes followed by destaining with high destain solution (50% MeOH, 10% Acetic acid) for 5min and low destain solution (5% MeOH, 10% Acetic acid) for 30min to visualise proteins.

2.14 Peptide scan to identify Nck binding site in WIP

Spot synthesis covering the complete sequence of WIP was performed according to Frank 1996 (Frank and Overwin, 1996) by Dr. Juergen Wehland (Department of Cell Biology, Braunschweig, Germany). Peptides covering the entire amino acid sequence of WIP were synthesised as 15 amino acids long peptides that overlapped by 12 residues. A total of 164 peptides (15 mers) were spotted onto a cellulose filter sheet, which I received dry.

Far western was established as follows. The filter is moistened first with 100% EtOH followed by 3 times washes with PBS-T for 5min. Subsequently non-specific binding sites on the filter were blocked by 2h incubation in blotto (PBS-T containing 5% milk powder). As a blocking solution everything that works for Western blots is suitable for these membranes. Purified His-Nck (see 2.1.9) was diluted to a concentration of 200-300µg/ml in blotto. The WIP peptide scan was incubated in total volume of 2-3ml in a sealed plastic bag for 1h at room temperature or at 4°C over night on a spinning wheel. The filter was then washed 3 times for 10min with blotto followed by incubation with the primary antibody raised against the His-tag for 1h at room temperature. After 3 times 10min washing with blotto the membrane was incubated with secondary antibody. To avoid high background it is important to wash the membrane stringently for 10min with first PBS-T, second with PBS-T containing 0.5M NaCl followed by a PBS-T/0.5% Triton X-100 wash. The final 10min wash is again in PBS-T before the membrane was developed using the ECL-kit (see 2.1.7).

The bound protein and the antibodies can be removed by stripping the membrane. All stripping steps are performed 3 times and last 10min. First the membrane is washed in

H_2O, followed by incubation in buffer A (8M Urea, 1% SDS, 0.5% θ-Mercaptoethanol, which has to be freshly added every time) in a sonicating water bath XB2 (Grant Instruments, Cambridge, UK). Subsequently the peptide scan membrane is washed with buffer B (50% EtOH, 40% H_2O, 10% acetic acid) and finally treated with 100% EtOH then dried at room temperature and stored at –20°C.

I checked the stripping efficiency and found that primary and secondary antibodies were removed efficiently but I found it impossible to remove recombinant His-Nck from the filter containing the WIP peptide.

Chapter 3

3 Characterisation of a N-WASP/WIP complex

3.1 Introduction

Wiskott-Aldrich Syndrome protein (WASP) is the founding member of the Wiskott-Aldrich syndrome (WAS) protein family (see 1.10). Although we know that mutations in the WAS gene are causing the disease we still lack knowledge of the molecular basis of its pathogenesis. Recent work reported a point mutation in WASP (Arg138Pro) causing thrombocytopenia, one of the symptoms of WAS. This mutation severely reduced WASP expression levels and significantly disrupts the interaction with WIP (Luthi *et al.,* 2003). It is an attractive hypothesis that these two observations are related and that an interaction with WIP is required in order to protect WASP from being degraded although this remains to be established. It is however striking that more 85% of all known missense mutations resulting in WAS occur within the N-terminal WH1 domain of WASP (Schindelhauer *et al.,* 1996). Although it has been suggested that this domain binds PIP_2 (Miki *et al.,* 1996), to date the only well characterised property of the WH1 domain is to bind to WIP-like proteins (see 1.10.1). This together with the finding that 95% of WASP is complexed to WIP in lymphocytes (Sasahara *et al.,* 2002) supports the idea that WASP/WIP interactions are important for normal WASP function. It is however unknown whether a complex of WASP and WIP plays a role in the etiology of WAS.

N-WASP/WIP complex in filopodia formation

Filopodia are long thin cellular processes enriched in long parallel actin filaments, which are organised in tight bundles (Small *et al.,* 2002). They frequently occur at the leading edge of motile cells and are essential for cell motility (Svitkina *et al.,* 2003). The Rho GTPase Cdc42, N-WASP and more recently WIP have been shown to induce filopodia formation upon overexpression (Hall, 1998) (Martinez-Quiles *et al.,* 2001; Miki *et al.,* 1998a). As it is appears there is more than one signalling pathway to induce filopodia, it is important to determine whether Cdc42, N-WASP and WIP are part of the

same pathway (Snapper *et al.,* 2001). Consistent with this notion it was shown that N-WASP and WIP interact *in vivo* and that Cdc42, WIP and N-WASP can form a trimolecular complex *in vitro* (Martinez-Quiles *et al.,* 2001). N-WASP and Cdc42 stimulated filopodia formation could be inhibited by microinjection of WIP antibodies and WIP induced filopodia are inhibited by N-WASP antibodies (Martinez-Quiles *et al.,* 2001). Thus WIP, N-WASP and Cdc42 appear to be involved in the same pathway to induce filopodia. However, from these studies it is not clear how the N-WASP/WIP complex forms or is regulated by Cdc42.

N-WASP/WIP complexes in the actin based motility of pathogens

Pathogens like *Shigella flexneri* and vaccinia virus are widely used model systems to study signalling events leading to actin polymerisation. These two pathogens recruit a complex of N-WASP and WIP as well as the Arp2/3 complex in order to polymerise actin on their surface (Frischknecht and Way, 2001; Goldberg, 2001). However, they developed different strategies to recruit the N-WASP/WIP complex to their surfaces (Figure 1.17).

The first evidence that N-WASP function directly depends on WIP came from experiments with vaccinia virus exploiting the ability of the virus to induce actin tails (Moreau *et al.,* 2000). N-WASP recruitment to vaccinia virus strictly depends on its N-terminal WH1 domain and overexpression of the WH1 domain of N-WASP inhibited vaccinia virus actin tail formation (Moreau *et al.,* 2000). Previous results from yeast two hybrid studies and biochemical interaction analysis have revealed that the WH1 domain of N-WASP binds to the C-terminal WASP binding domain of WIP (WIP-WBD) (Moreau *et al.,* 2000; Ramesh *et al.,* 1997)(Figure 1.16). This suggested that the overexpression of the WH1 domain of N-WASP blocks recruitment of endogenous N-WASP to virus particles thereby inhibiting Arp2/3 complex recruitment. WIP localises to virus particles, which is consistent with the notion that it is involved in vaccinia virus actin tail formation (Moreau *et al.,* 2000). Furthermore, overexpression of the WIP-WBD inhibited actin tail formation of vaccinia virus by preventing N-WASP recruitment to vaccinia virus, thereby blocking Arp2/3 complex dependent actin polymerisation beneath the CEV particles (Moreau *et al.,* 2000). Experiments using N-WASP deficient cell lines showed that in the absence of N-WASP vaccinia actin tail formation as well as the recruitment of WIP to vaccinia virus particles are abolished

(Snapper *et al.*, 2001). This suggested that WIP and N-WASP are recruited as a complex to vaccinia virus. Taken together these experiments suggest that an N-WASP/WIP complex plays a pivotal role in the actin-based motility of vaccinia virus. In addition these experiments showed that the N-WASP/WIP complex is maintained via the interaction of the N-terminal WH1 domain of N-WASP with the WIP-WBD (Moreau *et al.*, 2000). Therefore it was suggested that vaccinia virus uses WIP as an adaptor protein in order to recruit N-WASP to its surface.

Shigella like vaccinia virus recruits endogenous N-WASP and WIP to its surface (Moreau *et al.*, 2000). Furthermore it has been shown that in the absence of N-WASP WIP is not recruited to the surface of *Shigella* suggesting that N-WASP and WIP are recruited as a complex (Snapper *et al.*, 2001). Since there is a direct interaction between N-WASP and the *Shigella* surface protein IcsA, it is likely that WIP is downstream of N-WASP in the *Shigella* actin signalling cascade (Egile *et al.*, 1999)(Figure 1.11). Consistent with this speculation the WBD domain of WIP (WIP-WBD) localised to the surface of *Shigella* (Moreau *et al.*, 2000). However, in contrast to vaccinia overexpression of the WIP-WBD has not effect on *Shigella* induced actin tails. Thus in the case of *Shigella*, WIP might act downstream of N-WASP (Figure 1.11).

In N-WASP deficient cell lines WIP is not recruited to vaccinia virus (Snapper *et al.*, 2001) suggesting that N-WASP and WIP are recruited as a complex. Further evidence that an N-WASP/WIP complex is involved in vaccinia actin tail formation is based on the overexpression of the WIP-WBD inhibiting recruitment of N-WASP (Moreau *et al.*, 2000). Although the WIP-WBD domain is very small it is still possible that it binds to other proteins besides N-WASP. Furthermore at the time it was not clear whether the interaction of N-WASP and WIP is direct. Thus we decided to determine the molecular nature of the N-WASP/WIP complex.

3.2 Results

3.2.1 Possible insights in WH1 binding specificity

WH1 domains are closely related to EVH1 domains, which are conserved in the Ena/vasodilator-stimulated phosphoprotein (VASP) family (Volkman *et al.,* 2002). VASP proteins are molecules known to be involved in many signalling processes regulating the actin cytoskeleton (Krause *et al.,* 2003). They play a role in the regulation of cell movement and shape as well as the motility of the intracellular pathogens such as *Shigella flexneri* and *Listeria monocytogenes* (Bear *et al.,* 2001; Smith *et al.,* 1996). The EVH1 domain of ENA/Evl/Mena have been crystallised in combination with a proline rich ligand peptide derived from the *Listeria* surface protein ActA, which contains the consensus EVH1 binding motif FPPPP (Fedorov *et al.,* 1999; Niebuhr *et al.,* 1997; Prehoda *et al.,* 1999). The strong sequence conservation between the WH1 and the EVH1 domains, including the residues involved in co-ordinating EVH1 binding to FPPPP ligands, suggests that the WH1 domain may also bind a proline rich ligand (Callebaut *et al.,* 1998; Fedorov *et al.,* 1999; Prehoda *et al.,* 1999; Renfranz and Beckerle, 2002). Consistent with this notion the WASP binding domain (WBD) in WIP consists of 26% proline residues and four proline sequence motifs, one of which (DLPPPEP), is closely related to the VASP EVH1 binding motif (Ramesh *et al.,* 1997) (Figure 3.1A). Furthermore, mutation of tryptophan 54 in the N-WASP WH1 domain to alanine abolishes its ability to interact with WIP (Moreau *et al.,* 2000). The equivalent residue in the EVH1 domains of VASP family members Evl and Mena is essential in co-ordinating the interaction with proline rich sequences containing an FPPPP motif (Fedorov *et al.,* 1999; Prehoda *et al.,* 1999). This strongly suggests that the WH1 domain would also bind to a short proline rich motif in the WIP-WBD.

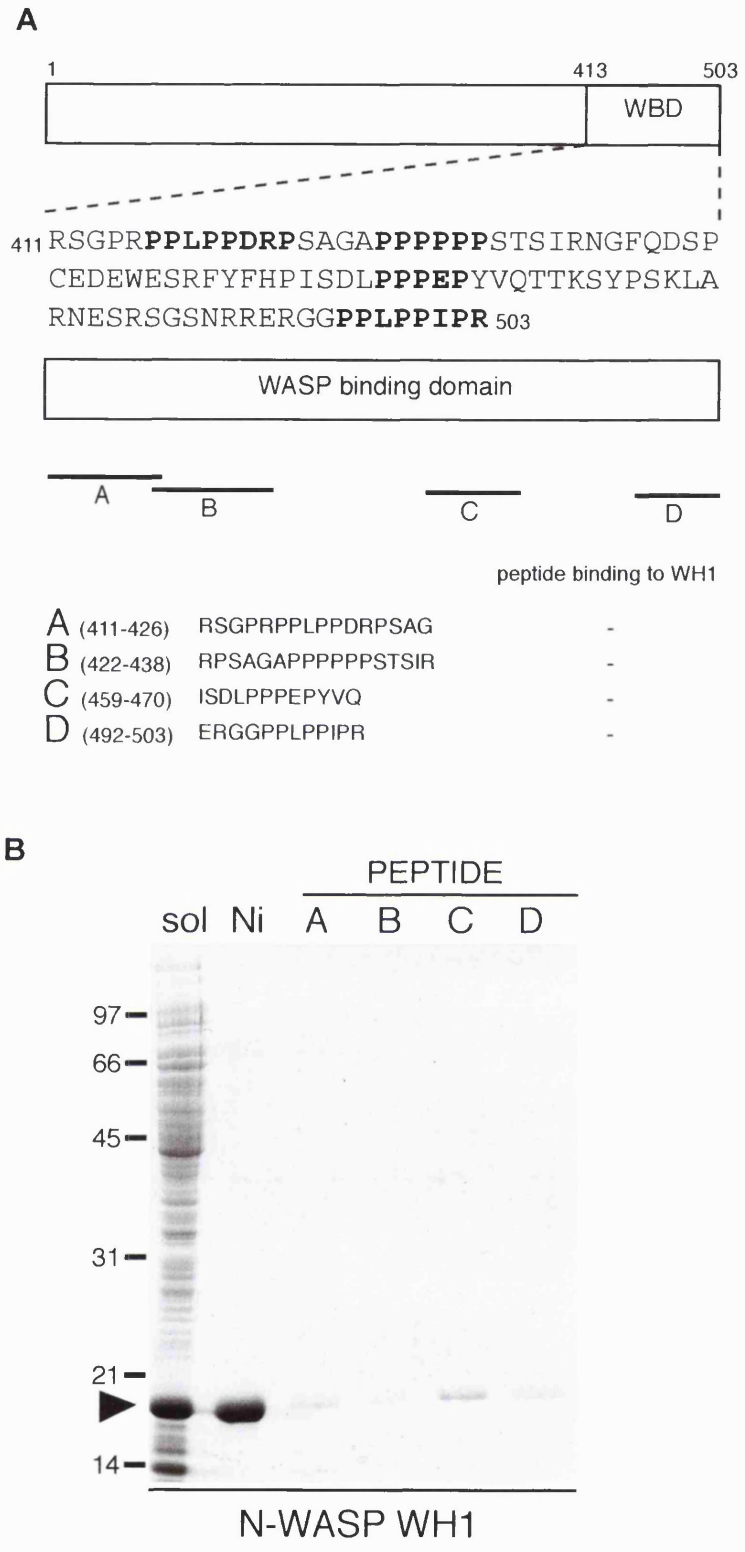


Figure 3.1. Mapping of the WH1 binding site in WIP *in vitro*.
(A) Schematic representation of WIP showing the relative position of the WASP binding domain (WBD), its amino acids and the peptides (A-D) used for *in vitro* binding studies. The amino acid sequence of these peptides and their N-WASP WH1 binding activity is indicated. **(B)** Coomassie stained gel showing that the His-tagged WH1 domain of N-WASP (indicated by an arrowhead) is retained from a soluble *E. coli* extract (sol) by nickel resin (Ni) but not by peptides A-D.

3.2.2 Identification of the WH1 binding motif in WIP

To examine whether the WH1 domain does indeed interact with proline rich ligands we sought to characterise the N-WASP binding motif in the WASP binding domain of WIP (residues 413-503) (Figure 3.1A). Peptides corresponding to the four proline-rich sequences in the WIP-WBD were tested for their capacity to retain the N-WASP WH1 domain from an *E.coli* soluble fraction (Figure 3.1B). Surprisingly, all four peptides failed to show significant binding (Figure 3.1B), suggesting either that the interaction site is elsewhere or that efficient WH1 binding requires a larger sequence motif than is required for the EVH1 domain.

Studies investigating the mammalian WIP homologues CR16 and WIRE, have demonstrated that exon 7 of CR16 and the equivalent region in WIRE, which are 50% and 47% identical to residues 448-481 of human WIP respectively, interact with N-WASP (Ho *et al.,* 2001; Kato *et al.,* 2002) (Figure 3.2). We therefore performed identical pull down assays with a larger peptide corresponding to residues 451-473 of WIP (peptide E, Figure 3.3A). This larger peptide was found to be extremely efficient at binding the N-WASP WH1 domain (Figure 3.3B). As expected from our previous observations (Moreau *et al.,* 2000), the WH1-W54A mutant protein failed to bind residues 451-473 of WIP (Figure 3.3C). The strong binding of WH1 to residues 451-473 of WIP (ESRFYFHPISDLPPPEPYVQTTK) and the lack of significant binding to the shorter peptide (ISDLPPPEPYVQ) prompted us to investigate whether the WH1 binding site was in fact in the N-terminal half rather than the proline rich C-terminal half of peptide E (Figure 3.3B). We therefore performed pull down assays using a peptide corresponding to residues 451-461 of WIP (ESRFYFHPISD). We found that residues 451-461 of WIP (peptide F) are sufficient to efficiently bind the WH1 domain of N-WASP and do not shown significant binding to the WH1 W54A mutant that is deficient in its ability to target to vaccinia virus or bind WIP (Figure 3.3C) (Moreau *et al.,* 2000). Surprisingly given our original hypothesis the WH1 binding motif of WIP is not proline rich nor has it any common features to classical EVH1 ligands. This suggested that despite the predicted conserved structural fold between EVH1 and WH1 domains their respective ligand specificity is different.

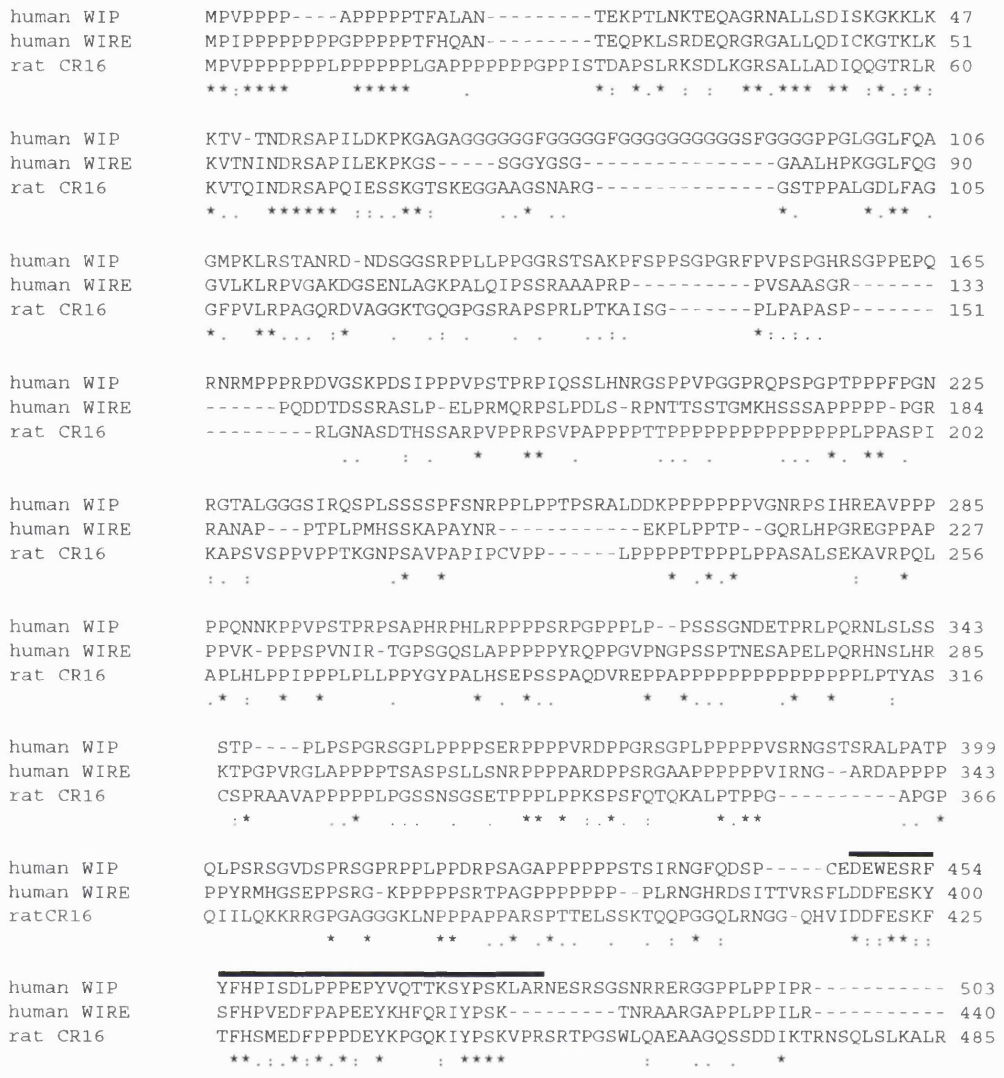


Figure 3.2. Sequence comparison of CR16, WIP, and WIRE.
Alignment of human WIP, human WIRE and rat CR16. Identical residues among the three proteins are marked with a star, and similar residues are indicated with a double dot. Conserved substitutions fall in three distinct groups are: 1) small plus hydrophobic (AVPMIL, incl.aromatic FWY), acidic (DE), basic (RHK) and hydroxyl, amine and basic Q (STYHCNGQ). A single dot indicates semiconserved changes meaning that the amino acids in the alignment can be grouped in the same class of amino acids. The black line highlights the alternatively spliced exon 7 (CR16 only), which is crucial for N-WASP binding which contains peptide C. Note that in the region of CR16 exon7 the sequence identity is particularly high.

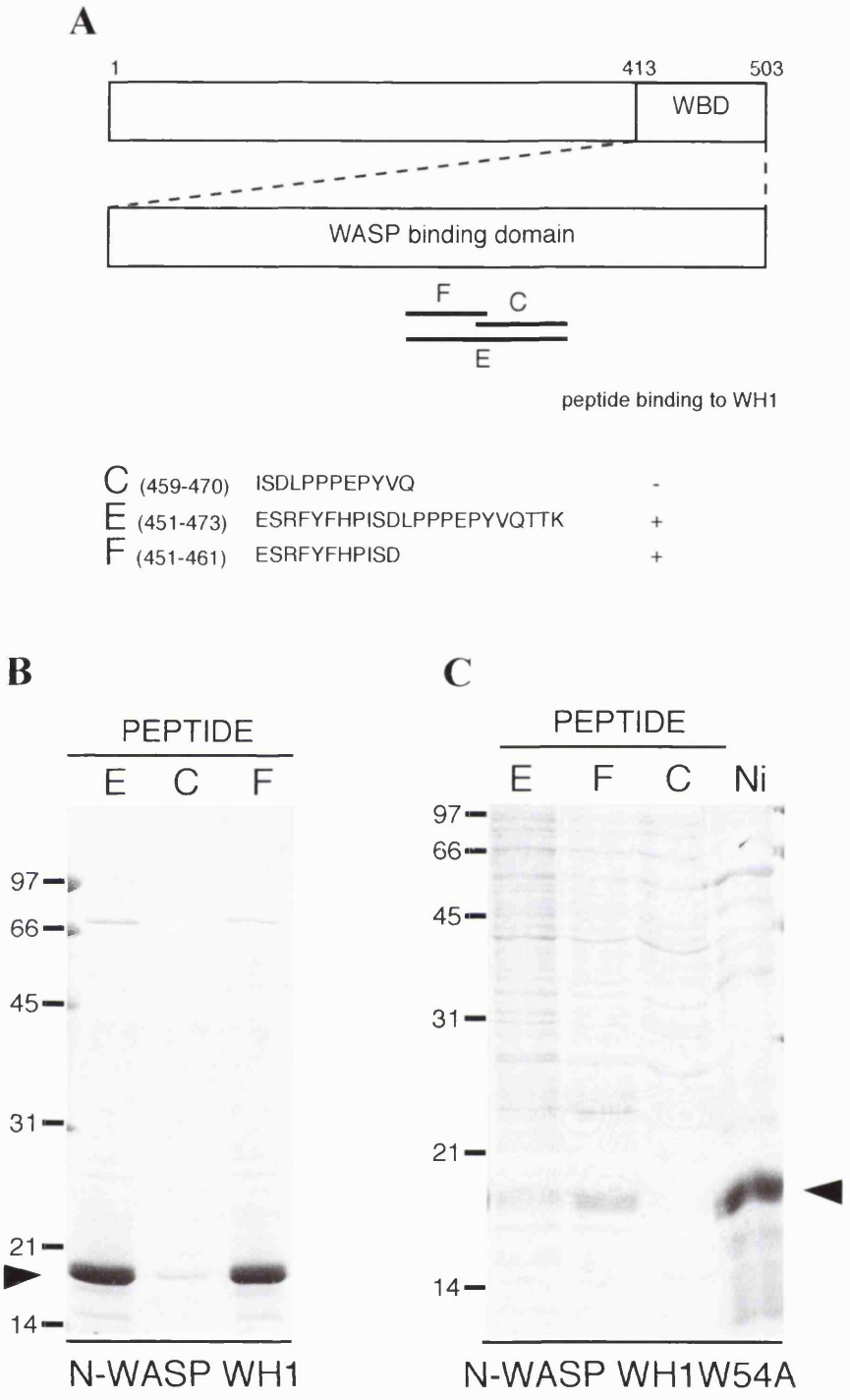


Figure 3.3. Identification of the WH1 binding site in WIP.
(A) Schematic representation of WIP showing the relative position of the WASP binding domain of WIP (WBD) and the peptides (C, E, F) used for *in vitro* binding studies. The amino acid sequence of these peptides and their N-WASP WH1 binding activity is indicated. **(B)** Coomassie stained gel showing that residues 451-461 of WIP (peptide F) are sufficient to interact with the WH1 domain of N-WASP (indicated by an arrowhead). **(C)** Coomassie stained gel showing that neither peptide E nor F can interact with the WH1 W54A domain of N-WASP (indicated by an arrowhead).

3.2.3 WH1 and EVH1 domains bind to distinct sequence motifs

VASP as well as N-WASP and WIP are recruited to vaccinia virus (Frischknecht *et al.*, 1999a). Although it is known that N-WASP and WIP play a role in the actin based motility of vaccinia virus nothing is known about the function and mechanism of recruitment of VASP in this process (Krause *et al.*, 2003). The EVH1 domain of VASP is required for targeting Ena/VASP proteins to *Listeria* surface, as well as to focal adhesions (Carl *et al.*, 1999; Gertler *et al.*, 1996; Niebuhr *et al.*, 1997). Identification of the EVH1 binding motif (DL/FPPPP) was facilitated by characterisation of the interaction between VASP and the proline-rich repeats of ActA, the bacterial surface protein responsible initiating the actin based motility of *Listeria* (Ball *et al.*, 2000; Niebuhr *et al.*, 1997; Renfranz and Beckerle, 2002). VASP binding studies have also been performed on known SH3 and WW/WP proline rich ligands (Niebuhr *et al.*, 1997). These data however, do not rule out the possibility that the EVH1 domain of Ena/VASP family members might also bind residues 451-461 of WIP, albeit with reduced affinity. To test whether residues 451-461 of WIP are specific for the WH1 domain, I examined the binding properties of the EVH1 domain from VASP to peptide F, which strongly binds to the WH1 domain of N-WASP. Although the VASP EVH1 domain was able to bind a control FPPPP peptide found in ActA, it was unable to bind the peptide corresponding to residues 451-461 of WIP (Figure 3.4B). Thus although the WH1 and EVH1 domains have a similar structural fold and are thus often grouped together (Ball *et al.*, 2000; Niebuhr *et al.*, 1997; Renfranz and Beckerle, 2002; Volkman *et al.*, 2002), they do in fact recognise distinct binding motifs.

A

		peptide binding to WH1	peptide binding to EVH1
F (451-461)	ESRFYFHPISD	+	-
ActA FPPPP	SFEFPPPPTD	-	+
ActA APPPP	SFEAPPPPTD	-	-

B

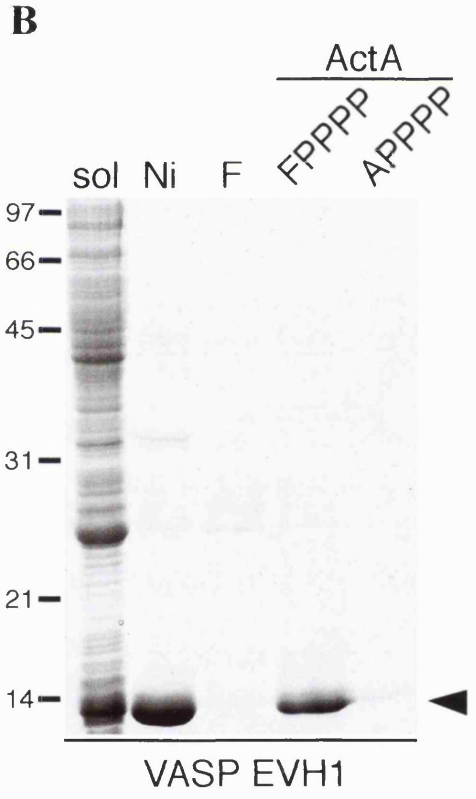


Figure 3.4. The WH1 binding motif in peptide F does not bind to the structurally related EVH1 domain.
(A) The amino acid sequence of peptides that were tested for their ability to retain the WH1 and EVH1 domain from soluble *E.coli* extract are shown. Their N-WASP WH1 and VASP EVH1 binding activity is indicated. **(B)** Coomassie stained gel showing that His- tagged EVH1 domain of VASP (indicated by an arrowhead), from a soluble *E. coli* extract (sol), binds to the nickel resin (Ni) and the positive control ActA peptide FPPPP but not the negative control ActA peptide APPPP or residues 451-461 of WIP (peptide F).

3.2.4 The WH1 binding motif in WIP is conserved in WIP homologues.

WIP is the most studied member of a family of proteins consisting of WIP, WIRE and CR16, which all interact with N-WASP. Overall WIP shares 41% sequence identity to WIRE and is 25% identical to CR16 (Figure 3.2). Consistent with the high degree of similarity WIP, WIRE and CR16 bind to both monomeric and filamentous actin as well as localise to filopodia (Ho *et al.,* 2001; Kato *et al.,* 2002). WIP and WIRE can induce filopodia suggesting they function in the same pathway than N-WASP (Kato *et al.,* 2002; Martinez-Quiles *et al.,* 2001). The alignment of the N-WASP WH1 binding motif that I have identified in WIP, with the corresponding region of CR16 and WIRE reveals that it is a highly conserved amino acid sequence (Figure 3.5A). Pull down assays with the equivalent sequence motifs of CR16 and WIRE confirmed that they are also capable of binding the WH1 domain of N-WASP (Figure 3.5B). It was noticeable however, that the CR16 peptide consistently retained less WH1 than the other two peptides suggesting it may have a lower affinity for N-WASP than either WIP or WIRE derived peptides. Database searches with the N-WASP binding motif in WIP showed that the sequence was only present in WIP homologues. The WH1 binding motif is also conserved in the more divergent verprolin (End5), the WIP homologue in yeast that interacts with Las17 (Bee1), the yeast homologue of WASP (Evangelista *et al.,* 2000; Madania *et al.,* 1999; Naqvi *et al.,* 1998). WIP family proteins share a particular high degree of sequence identity in their respective WBD (Figure 3.2) suggesting that the interaction with N-WASP is a conserved important property of this protein family.

A

human	WIP (451-461)	E	S	R	F	Y	F	H	P	I	S	D
human	CR16 (390-400)	E	S	K	F	T	F	H	S	V	E	D
human	WIRE (397-407)	E	S	K	Y	S	F	H	P	V	E	D
S. cerivisiae	VRP (777-787)	D	S	R	F	K	W	T	N	V	S	Q
S. pombe	VRP (270-280)	H	G	R	F	H	F	K	D	D	S	Y
consensus		-	S	+	F	/	F	/	/	/	/	/

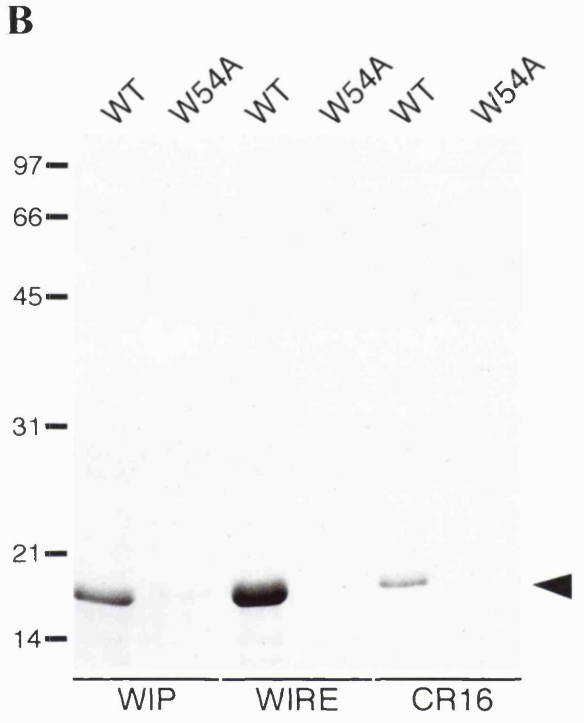


Figure 3.5. The N-WASP binding motif in WIP is conserved in WIP, WIRE and CR16.

(A) Alignment of the WIP homologues CR16 and WIRE, which bind N-WASP reveals the WH1 binding motif in WIP is highly conserved. The motif is also readily observed in verprolin, the yeast homologue of WIP. The consensus shows positions where four out of the five aligned proteins have identical residues or a conserved change. +/- indciates a conserved charged amino acid. **(B)** Coomassie stained gel showing that the motif found in WIP, WIRE and CR16 (indicated in A) binds the N-WASP WH1 domain but not the mutant WH1-W54A (indicated by an arrowhead).

3.2.5 Phenylalanine 454 and 456 of WIP are involved in WH1 binding *in vitro*.

Looking at the sequence comparison of the WH1 binding motif (peptide F) in WIP homologues it is noticeable that the N-terminal part shows a greater conservation to the C-terminal half (Figure 3.5A). Given that WIP, WIRE and CR16 all bind to N-WASP would suggest that the WH1 binding motif resides within the N-terminal half of peptide F. To identify which residues are required for the interaction of WIP with the WH1 domain of N-WASP, we therefore analysed the effects of alanine substitution on the most conserved residues in the motif (Figure 3.6A). We found that only changes of the two central phenylalanine residues to alanine had any noticeable affect on WH1 binding (Figure 3.6B). Substitution of phenylalanine 454 resulted in reduced binding, while changing phenylalanine 456 to alanine almost completely abolished the interaction with the WH1 domain. When both phenylalanine 454 and 456 were substituted for alanine all binding was eliminated (Figure 3.6B). The *in vitro* binding assays demonstrated that although the N-WASP WH1 binding motif contains a number of conserved residues, the principal residues involved in binding are the central phenylalanines.

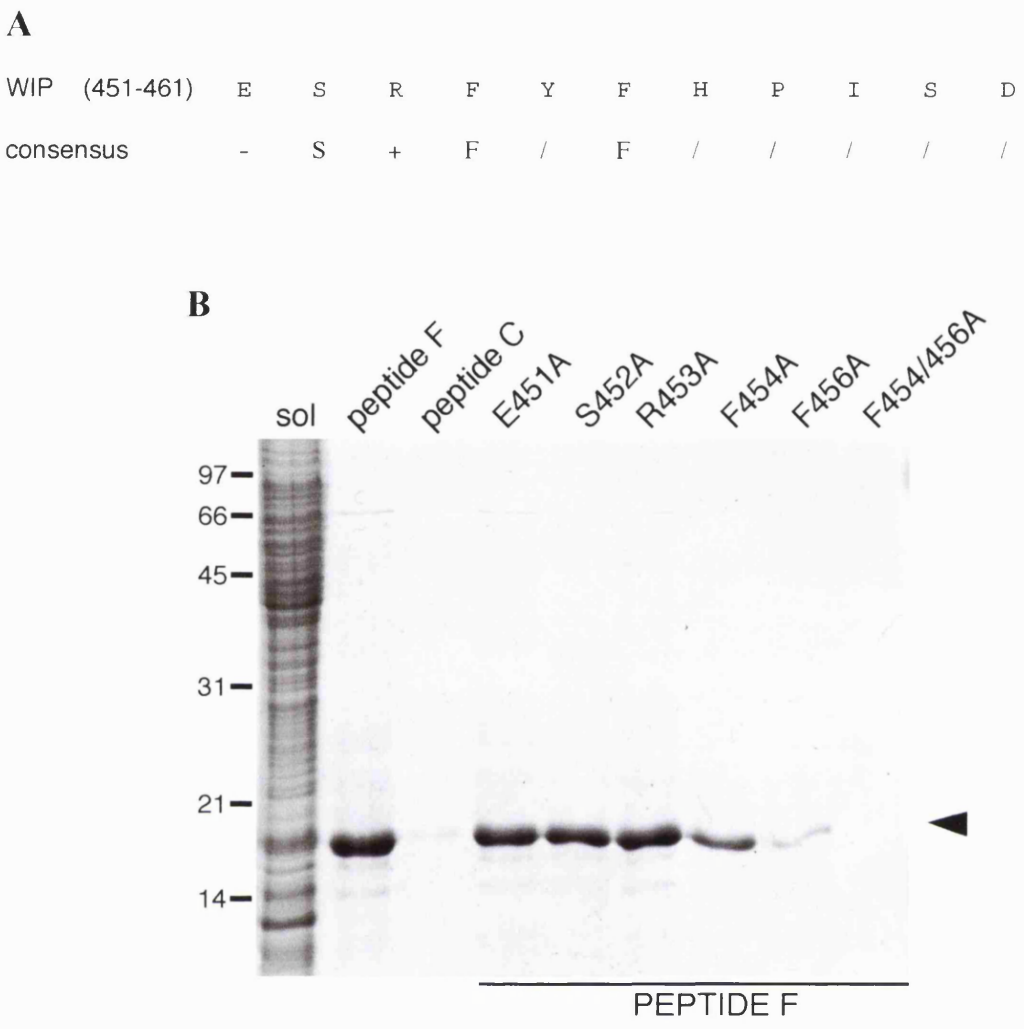


Figure 3.6. Phenylalanines 454 and 456 of WIP are required to bind the WH1 domain *in vitro*.

(A) The amino acid sequence of the minimal WIP peptide that binds to N-WASP is shown together with the consensus binding sequence derived from sequence alignments with WIP family members (see Figure 3.5A). **(B)** Coomassie stained gel showing the effects of alanine substitutions on the ability of residues 451-461 of WIP (peptide F) to retain the WH1 domain (indicated by an arrowhead) from identical amounts of soluble *E. coli* extract (sol). Tested point mutants in peptide F are indicated above the gel. Substitution of phenylalanine 454 and 456 to alanine leads to reduced binding while mutation of both residues completely abolishes the interaction.

3.2.6 Analysis of the effect of mutation of phenylalanine 454 and 456 in the WASP binding domain of WIP (WIP-WBD) *in vivo*.

To confirm whether our *in vitro* biochemical observations reflect the *in vivo* requirements for WIP binding to N-WASP we took advantage of the *Shigella* and vaccinia pathogen systems. Recruitment of WIP to *Shigella* is dependent on its ability to interact with N-WASP via its WBD (Moreau *et al.,* 2000). However, the overexpression of the WBD does not interfere with *Shigella* induced actin polymerisation. Therefore it is possible to use recruitment of WIP-WBD to *Shigella* as a read out for its ability to interact with N-WASP to confirm the *in vitro* results. Consistent with previous observations of Moreau *et al.,* 2000 the WIP-WBD when expressed in infected cells is recruited to *Shigella* nucleating actin tails (Figure 3.7). We therefore examined the ability of GFP-tagged WIP-WBD, containing the phenylalanine 454 and 456 to alanine substitutions, to be recruited to *Shigella.* We found that GFP-WBDF454A was largely cytoplasmic but on rare occasions was capable of being recruited *Shigella* nucleating actin tails (Figure 3.7). In contrast mutation of phenylalanine 456 to alanine resulted in a complete lack of recruitment, as did substitution of both phenylalanine residues (Figure 3.7). Thus mutation, which affects the binding capacity of WIP peptides to the WH1 domain in vitro, might lower the affinity of mutated WIP-WBD to N-WASP *in vivo.* It is likely that WIP-WBD harbouring mutations in the conserved phenylalanines were not able to compete with endogenous WIP for N-WASP binding resulting in a loss of recruitment to *Shigella.*

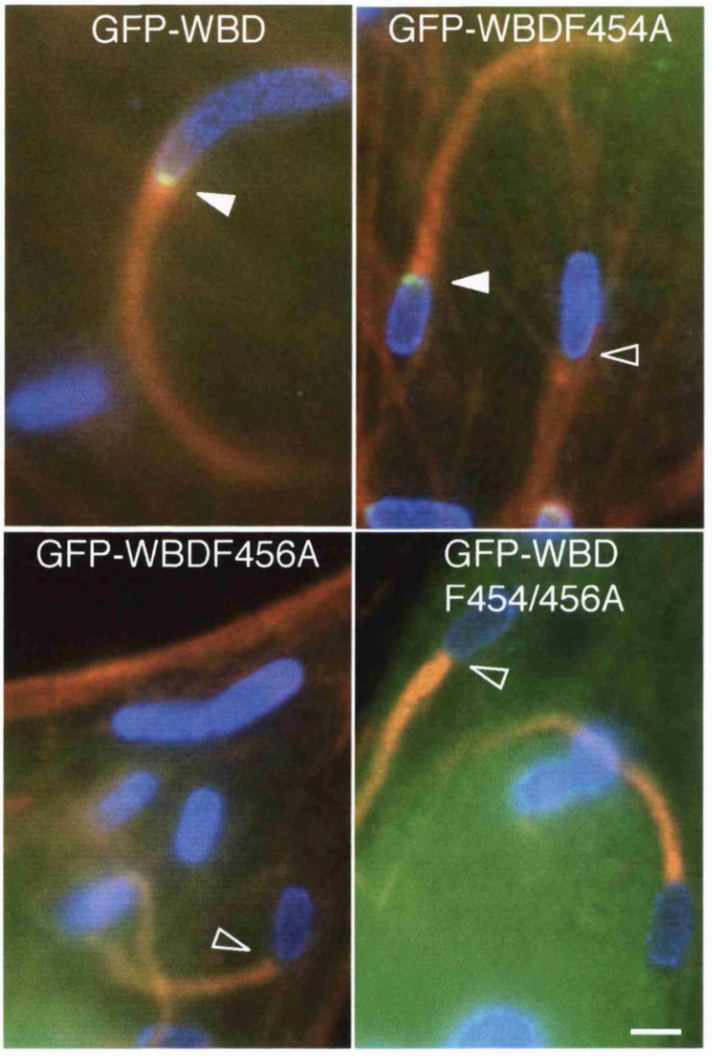


Figure 3.7. Mutation of phenylalanine 454 and 456 in the WASP binding domain of WIP (WIP-WBD) disrupt the localisation of WIP-WBD to *Shigella*. Immunofluorescene images showing the localisation of GFP tagged of WIP-WBD mutants (green), as indicated above the panels, to *Shigella* (blue) nucleating actin tails (red). GFP-WBDF454A in contrast to GFP-WBD is weakly recruited to *Shigella* (open arrow head). In rare cases however, as shown here, GFP-WBDF454A is localised to the bacterium (filled arrow head). In contrast, GFP-WBDF456A and GFP-WBDF454/456A are never recruited to the bacterium (open arrow heads). Scale bar is 2 μm. According to the current model for *Shigella* actin based motiltiy the overexpression of GFP-WBD does not inhibit *Shigella* actin tail formation suggesting that WIP is downstream of N-WASP (see Figure 1.11).

While recruitment provides a qualitative assay, it gives no quantitative measure of the effect of these mutations on their affinity for N-WASP *in vivo*. Like *Shigella,* vaccinia recruits N-WASP and WIP, but in contrast to *Shigella* the actin-based motility of vaccinia is disrupted by the overexpression of WIP-WBD, as it appears to inhibit recruitment of N-WASP to virus particles (Moreau *et al.,* 2000) (Figure 3.8). Therefore, by examining the ability of the different phenylalanine to alanine mutations in WIP-WBD to inhibit vaccinia induced actin tail formation, it is possible to obtain more quantitative data that can be compared with our *in vitro* studies. GFP-WIP-WBD inhibited vaccinia induced actin tail formation by 71.8 ± 4.8% (Figure 3.9). In contrast, GFP alone did not block N-WASP recruitment and gave an 8.5 ± 1.7% reduction in actin tail formation when compared to untransfected controls on the same coverslip (data not shown and Figure 3.8). Expression of GFP-WIP-WBDF454A resulted in a significant reduction in the level of inhibition to 33.6 ± 16.2%. This was reduced still further in the case of GFP-WIP-WBDF456A to 17.8 ± 5.2% and to 13.5 ± 7.6% when both mutations were combined (Figure 3.9). Consistent with its lack of inhibition of actin tails in contrast to GFP-WIP-WBD GFP-WIP-WBDF454/456A did not block recruitment of N-WASP to vaccinia (Figure 3.8). Taken together our *in vivo* data using the *Shigella* and vaccinia pathogen systems confirm that the identification of the N-WASP WH1 binding motif in WIP *in vivo*. However, the evidence that WIP plays a functional role in the actin-based motility of vaccinia virus is only based on overexpression of WIP-WBD, which could affect other proteins than N-WASP. Thus it was reassuring that the dominant negative effect of the WIP-WBD on vaccinia actin tails was abolished in WIP-WBD mutants lacking the ability to bind to N-WASP.

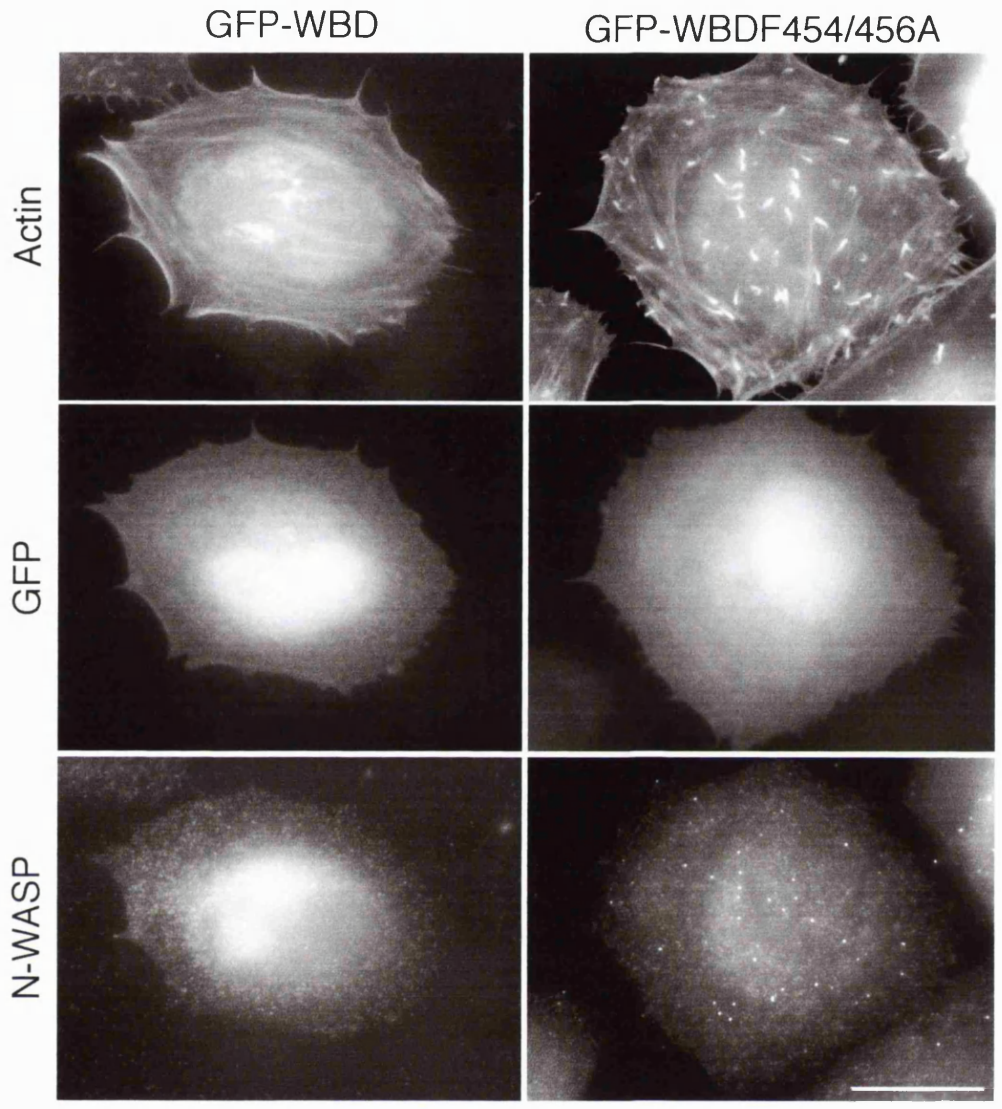


Figure 3.8. N-WASP recruitment to vaccinia virus particles is not affected by overexpression of the WIP-WBDF454/456A.
Immunofluorescence images showing that in contrast to GFP-WBD, expression of GFP-WBDF454/456A does not block the recruitment of endogenous N-WASP to vaccinia and actin tail formation. Scale bar is 20 μm

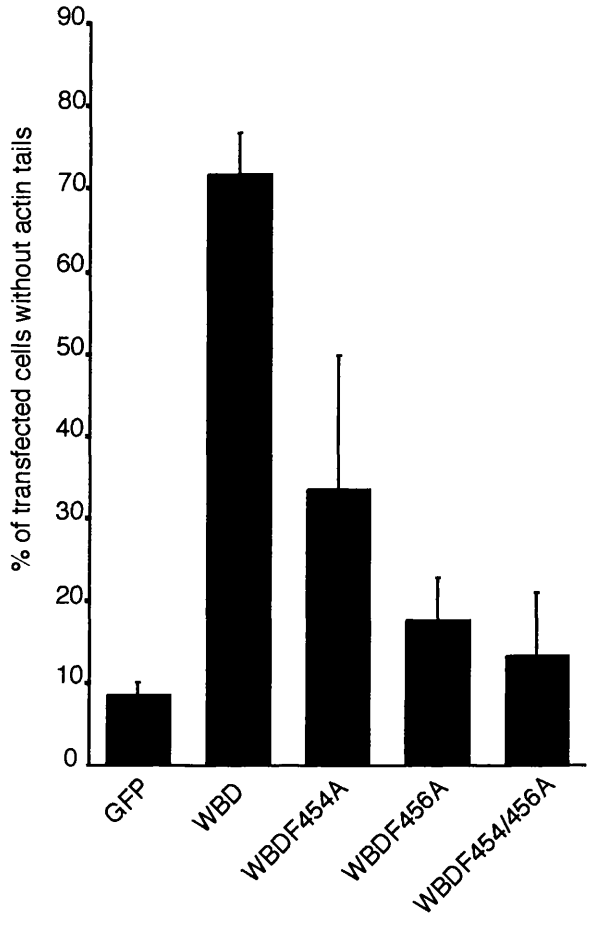


Figure 3.9. Overexpression of WIP-WBD F454/456A does not block vaccinia actin tail formation.
Quantification of vaccinia induced actin tail efficiency in cells overexpressing the indicated GFP construct. The data represent the average from three independent experiments and error bars represent standard deviations from the mean.

3.2.7 Interaction of the WIP-WBD with N-WASP is required for recruitment of WIP to vaccinia and *Shigella*.

Observations by Snapper *et al.,* 2001 have demonstrated that in the absence of N-WASP, vaccinia is unable to recruit WIP, consistent with the suggestion that both proteins are recruited as a complex (Moreau *et al.,* 2000; Snapper *et al.,* 2001). While this study formally shows N-WASP is essential for actin-based motility of vaccinia it does not rule out the possibility, that additional WH1 independent interactions might also contribute to WIP recruitment and stabilisation of the complex on the virus. To explore this possibility we examined the localisation of full length WIP containing the phenylalanine 454 and 456 alanine substitutions in *Shigella* and vaccinia infected cells. GFP-WIP-F454A was still recruited to *Shigella* and vaccinia nucleating actin tails albeit at reduced levels when compared to GFP-WIP (Figure 3.10). This is consistent with its apparent weaker binding of the corresponding peptide to the N-WASP WH1 domain *in vitro* (Figure 3.4). GFP-WIPF456A or double phenylalanine mutants were never recruited to either *Shigella* or vaccinia (Figure 3.10). It was noticeable that all GFP-WIP mutants had a more pronounced association along the length of the actin tail than wild type WIP. This was especially apparent in the case of *Shigella* induced actin tails (Figure 3.10). This localisation is consistent with the observation that the protein is associated with actin stress fibres *in vivo* and can bind actin filaments *in vitro* (Martinez-Quiles *et al.,* 2001; Vetterkind *et al.,* 2002).

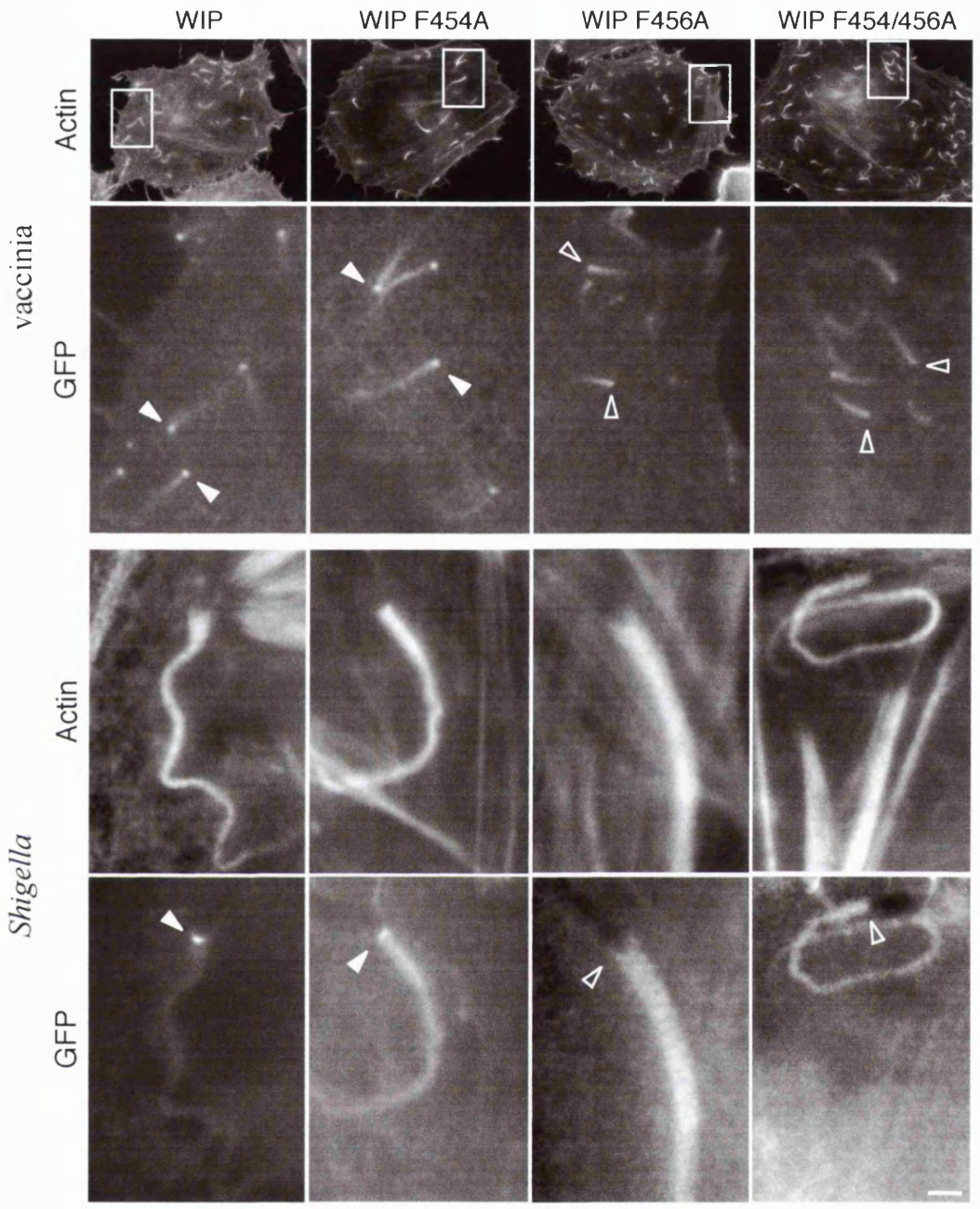


Figure 3.10. Interaction with N-WASP is required for recruitment of WIP to vaccinia and *Shigella*.
Immunofluorescene images of cells infected with vaccinia or *Shigella* expressing the GFP-WIP mutants F454A, F456A and F454/456A. GFP-WIP, and to a lesser degree GFP-WIP-F454A are recruited to vaccinia or *Shigella* nucleating actin tails (filled arrow heads). In contrast, GFP-WIP-F456A and GFP-WIP-F454/456A are not recruited to the pathogen surface (open arrow heads) but are observed down the actin tail. Scale bar is 2 μm.

3.2.8 WIP interacts with N-WASP via an extended surface

Taken together the observations are consistent with the hypothesis that I identified a high affinity-binding site for the WH1 domain of N-WASP in WIP family members. Disruption of this binding site in WIP ablates the ability of WIP to target to vaccinia virus and *Shigella flexneri.* Although this clearly indicates that this minimal peptide motif is important it does not rule out that there are more binding interfaces between N-WASP and WIP. Consistent with this hypothesis Volkman *et al.,* 2002 defined residues 461-485 (DLPPPEPYVQTTKSYPSKLARNESR) as the minimal binding site for the WH1 domain of N-WASP (Volkman *et al.,* 2002). The authors report the structure of the WH1 domain complexed to the minimal WIP peptide sequence. Overall the structure is similar to the other EVH1 domains from Mena, Homer and VASP (Barzik *et al.,* 2001; Beneken *et al.,* 2000; Fedorov *et al.,* 1999; Prehoda *et al.,* 1999). However, unlike classical 6-10 amino acid long EVH1 ligands, the minimal WH1 ligand wraps around an extended surface of the WH1 domain. In contrast to my studies, their experiments were achieved by a Far Western approach using a series of N-terminal deletions of biotinylated WIP peptides derived from the WIP-WBD. However, due to the large initial deletions the authors never tested the binding motif I identified. Thus I wanted to test whether I could repeat their results with my *in vitro* binding assay. I saw that a peptide corresponding to WIP residues 461-485 (peptide G) binds very efficiently to the N-WASP WH1 domain, confirming the results obtained by Volkman *et al.,* 2002. I already knew from previous experiments that a peptide containing the putative EVH1 binding motif DLPPPEP (peptide C) was not sufficient to bind to the N-WASP WH1 domain (Figure 3.11B). However, since I observed a very strong interaction of peptide G with the WH1 domain, I decided to test if the C-terminal region of peptide G is sufficient to retain the WH1 domain from soluble *E.coli* extracts. Consistent with the structure, which postulates that the WH1 ligand wraps around an extended surface, I could not detect any binding with a peptide comprising amino acids 471-485 (Figure 3.11B, peptide H). Thus in addition to WIP residues 451-461 (peptide F) residues 461-485 (peptide G) constitutes an additional larger binding surface for the WH1 domain of N-WASP. However, these experiments indicate that the interaction between the WH1 domain of N-WASP and peptide G is weaker than with peptide F. Therefore many weak interactions over an extended surface might be needed to stabilise the binding of peptide G with the WH1 domain of N-WASP.

The structure of the WH1 domain of N-WASP revealed that the DLPPPEP motif in WIP contacts the WH1 domain (Volkman *et al.,* 2002). However, this motif is not sufficient to establish a stable contact to the WH1 domain (Figure 3.11B). Volkman *et al.,* 2002 speculated that a C-terminal basic stretch of amino acid in peptide G (Figure 3.11A) is important to make a stable contact with the WH1 domain of N-WASP. Based on the structure, I decided to test whether the proline rich (DLPPPEP) and/or the basic motif are relevant for an N-WASP/WIP interaction *in vivo* using vaccinia virus induced actin tails as a quantitative read out. I found that the mutating the conserved P465 and K478 to alanine reduced the ability of the mutated WBD domain to block vaccinia actin tails to 32.2 ± 17.9% and 52.7 ± 13.4% respectively when compared to the wild type WBD of WIP (Figure 3.12B). These observations are consistent with the structural predictions that these two motifs in WIP make contacts to the WH1 domain of N-WASP. However, the inhibitory effect on vaccinia actin tails was not as strong as with mutants in the short WH1 binding motif (Figure 3.12, 3.9). These observations are consistent with binding studies showing that a large continuous stretch of amino acids of WIP (peptide G) is required to establish a stable contact between peptide G and the WH1 domain. Therefore single point mutants in the WIP-WBD residing in the region of peptide G are predicted to have a weaker effect on the interaction with N-WASP explaining the weaker inhibition on vaccinia actin tails.

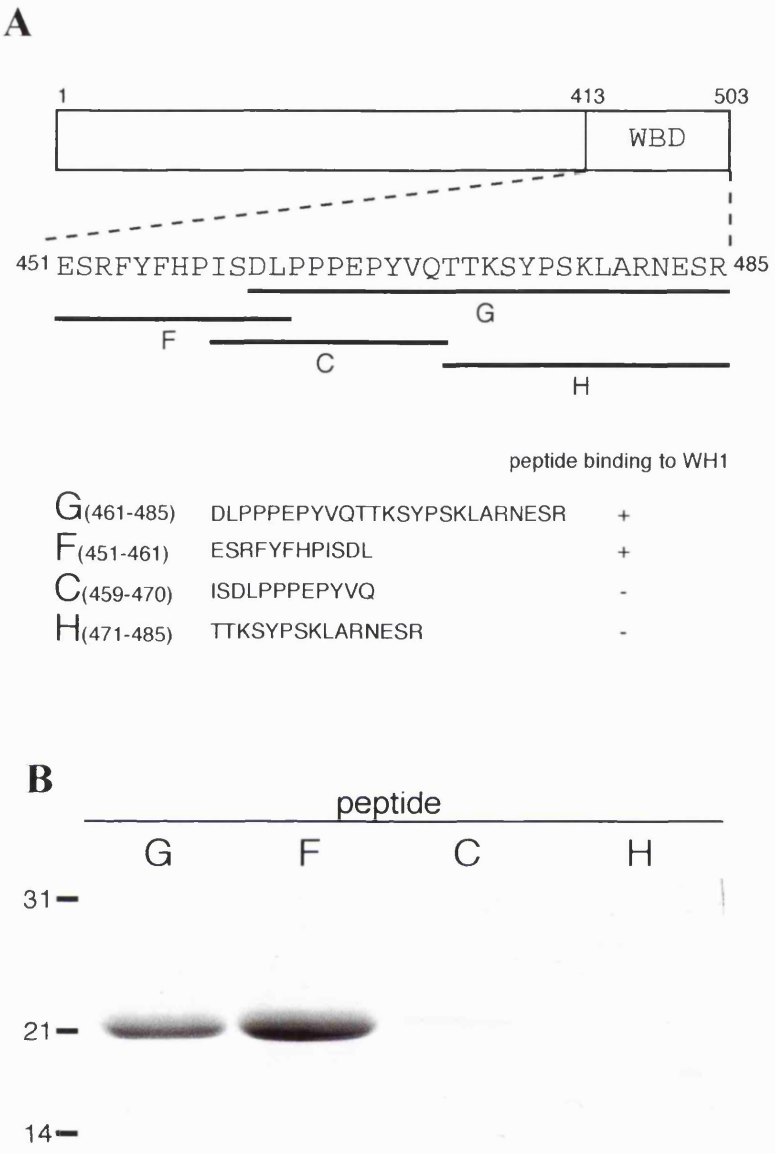


Figure 3.11. Further characterisation of the WH1 binding site in WIP.
(A) Schematic representation of WIP showing the relative position of the WASP binding domain of WIP (WBD), its amino acids and the peptides (G,F,C and H) used for *in vitro* binding studies. The amino acid sequence of these peptides and their N-WASP WH1 binding activity is indicated. (**B)** Coomassie stained gel showing that the His-tagged WH1 domain of N-WASP is retained from a soluble *E. coli* extract (sol) by peptides G and F but not by peptides C and H.

A

		proline rich motif	basic region
human WIP	461-485	DLPPFEPYV--QTTKSYPSKLARNESR	
human WIRE	407-431	DFPAPEEYK--HFQRIYPSKTNRAARG	
rat CR16	432-456	DFPPPDEYK--PGQKIYPSKVPRSRTP	
S. cerivisiae VRP	701-730	PPPPPSPSTMDTGTSNSPSKNLKQRLF	

B

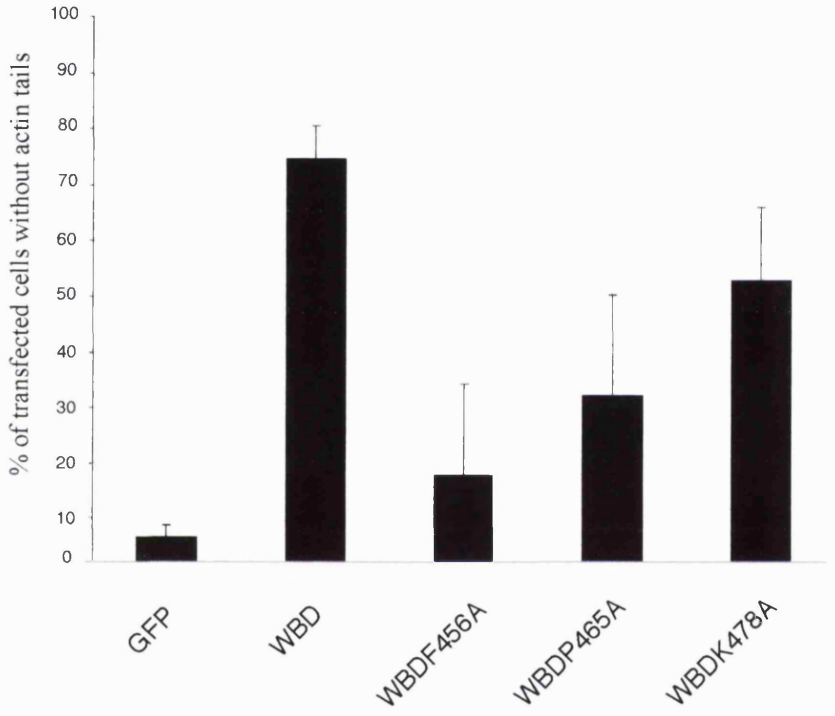


Figure 3.12. The proline rich motif and the basic region in the WH1 domain binding peptide 461-485 are important to interact with N-WASP *in vivo*.
(A) Alignment of WIP homologues in the region comprising the WASP binding peptide 461-485 reveals two highly conserved regions, a proline rich and a C-terminal region containing two conserved basic amino acids. Conserved regions are shown in grey boxes and white letters indicate the amino acids that were changed to alanines in the WIP-WBD. **(B)** Quantification of vaccinia induced actin tail efficiency in cells overexpressing the indicated GFP construct. The data represent the average from four independent experiments and error bars represent standard deviations of the mean.

3.3 Discussion

The data presented and discussed show that although EVH1 domains of VASP family proteins and the WH1 domain of N-WASP have the same structural fold (Volkman *et al.,* 2002), their mechanism of binding to their respective ligands is completely different. EVH1 domains bind to short proline rich sequences whereas the WH1 domain of N-WASP binds to a single long motif comprising at least three conserved sequence motifs within residues 451-485 of WIP (Figure 3.5, 3.12). I have biochemically identified a sequence motif in WIP, which when disrupted *in vivo* ablates the ability of WIP to be recruited to *Shigella* and vaccinia virus presumably because it can not interact with N-WASP *in vivo*. Furthermore I have confirmed structural data *in vivo* suggesting that a conserved proline rich stretch of amino acids in WIP encompassing P465 and a basic region around K478 are important for the interaction of WIP with N-WASP *in vivo*.

What determines the specificity of the WIP N-WASP interaction?

One important issue remains as to how WH1 and EVH1 domains achieve their respective specificity since the minimal binding motif in WIP identified by Volkmann *et al.,* 2002 (peptide G, 461-485) cross-reacts with the EVH1 domain of Mena (Volkman *et al.,* 2002). In contrast, the small hydrophobic sequence motif in WIP (peptide F, 451-461) does not bind the EVH1 domain of VASP (Figure 3.4A). Therefore I examined whether amino acids C-terminal to residue 461 were important for the cross reactivity with the EVH1 domain. I tested whether a peptide containing the putative EVH1 binding motif DLPPPEP (peptide E, 451-473) can bind to the EVH1 domain. I found that peptide E retains the His-EVH1 domain of VASP from soluble *E.coli* extract, which confirms results from Volkmann *et al.,* 2002 (Figure 3.13, lane1). The supernatant from this assay still contained soluble His-EVH1 domain indicating that the EVH1 domain saturated the binding capacity of peptide E. To test whether the binding determinants of the WH1 and the EVH1 domain on peptide E overlap, I performed a competition experiment. I loaded peptide E with the EVH1 domain and tested the ability of the resulting saturated peptide E/His-EVH1 complex to retain the WH1 domain of N-WASP out of soluble *E.coli* extract. The WH1 domain can compete off the EVH1 domain although not to completion (compare lane1 and lane 4 in Figure

3.13). This indicates that WH1 domain has a higher affinity to peptide E than the EVH1 domain and that both domains share some overlapping binding surfaces. I therefore like to suggest that the hydrophobic sequence motif containing F454 and F456 is an important specificity determinant for the N-WASP WIP interaction. Consistent with this suggestion the conserved hydrophobic sequence motif in WIP 451-461 is exclusively found in WIP family members.

N-WASP and WIP are almost always found in a complex in resting cells (95% of WASP is complexed to WIP in lymphocytes) (Martinez-Quiles *et al.,* 2001; Sasahara *et al.,* 2002). This is achieved by high affinity binding of N-WASP to WIP by covering an otherwise exposed hydrophobic surface of the WH1 domain. I have shown and discussed data illustrating that WIP contains at least three different motifs important for N-WASP binding, which consist of a small hydrophobic epitope and a longer linear amino acid sequence containing a proline rich motif and a stretch of basic amino acids. Thus the N-WASP-WIP interaction represents a hybrid class of protein-protein interactions. Most protein-protein interactions fall in two major classes: (1) interactions that involve docking of two large folded protein surfaces, and (2) interactions that involve binding of a short, largely linear peptide epitope on a protein surface (Stanfield and Wilson, 1995). Using a combination of different modi of docking the N-WASP/WIP complex is maintained via a specific high-affinity protein-protein interaction.

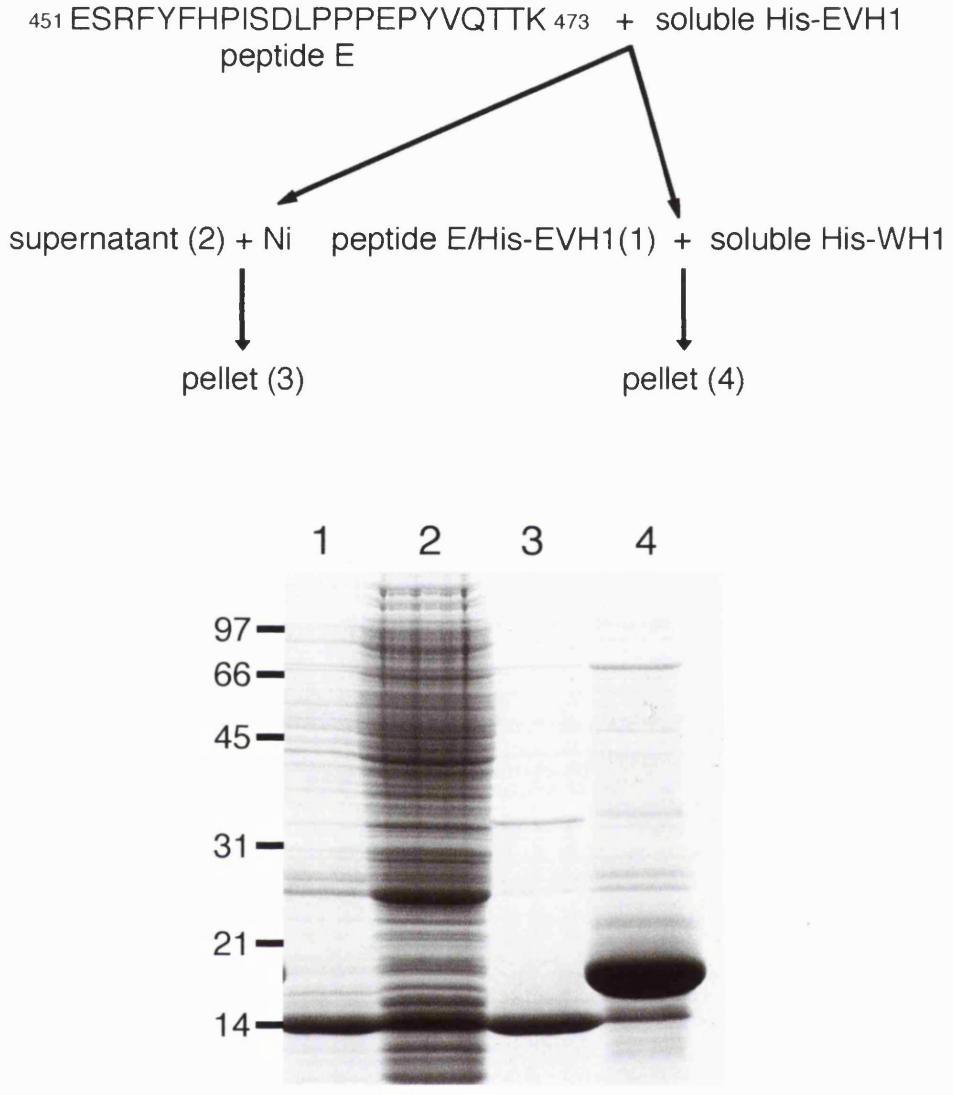


Figure 3.13 . The WH1 and EVH1 domains compete for similar binding sites on peptide E.

Scheme showing the order of the competition assay between the EVH1 of VASP domain and the WH1 domain of N-WASP. Peptide E containing a putative EVH1 binding motif DLPPPEP retained bacterial expressed His-EVH1 domain from soluble *E.coli* extracts (1). The binding sites of the EVH1 domain on peptide E were saturated since soluble His-EVH1 (2) could still be bound to nickel resin (Ni) shown in (3). An *E.coli* extract containing soluble His-WH1 was passed over peptide E loaded with His-EVH1 domain. The pellet in lane 4 shows that the His-WH1 domain competes off some of the His-EVH1 domain bound to peptide E.

Chapter 4

4 A comparison between actin based motility of vaccinia virus and EPEC

4.1 Introduction

Vaccinia virus infects the host cell and replicates close to the nucleus in so called viral factories. After replication vaccinia moves to plasma membrane surfing on microtubules using the plus end directed motor kinesin (Rietdorf *et al.,* 2001). After fusing with the plasma membrane the virus induces actin ploymerisation in form of so called actin tails (Cudmore *et al.,* 1995). EPEC never enters the host cell but attaches to the plasma membrane of the host cell in order to induce actin polymerisation from the outside like vaccinia virus (Rosenshine *et al.,* 1996).

It has been shown that an integral viral membrane protein A36R is essential for vaccinia actin actin tail formation (Wolffe *et al.,* 1998). A36R induced actin polymerisation requires phosphorylation of tyrosines 112 (Frischknecht *et al.,* 1999b). A second phosphorylation of tyrosine 132 plays a secondary role, making actin tail formation more efficient (Scaplehorn *et al.,* 2002). This situation is reminiscent to EPEC induced pedestal formation, which depends on the phosphorylation of bacterial protein Tir at tyrosine 474 (Kenny, 1999). A sequence comparison in this region of A36R around tyrosine 112 and Tir surrounding tyrosine 474 reveals a high degree of identity (Figure 4.1). The sequence around Tyr 112 is similar to the optimal Src kinase substrate sequence (DEEIYEEFF)(Songyang and Cantley, 1995), suggesting that Src family kinases play an important role in vaccinia induced actin tail formation. This appears to be the case since actin tails are blocked by the Src-family kinase inhibitor PP1 (Frischknecht *et al.,* 1999b). They are unaffected by treatment with PP3, a chemical compound that is very similar to PP2 but does not have an effect on Src-family kinases (Traxler *et al.,* 1997). Furthermore it was shown that the overexpression of a dominant negative c-Src severely reduces the formation of actin tails (Frischknecht *et al.,* 1999b). However, it is not known whether Src kinases are involved in EPEC pedestal formation.

Vaccinia and EPEC induce actin polymerisation depending on tyrosine phosphorylation, recruitment of adaptor proteins such as Nck and WASP/N-WASP, which activate the Arp2/3 complex at the surface of these pathogens. However it is

currently not clear whether EPEC and vaccinia recruit these factors in the same way. Generally this chapter seeks to compare vaccinia virus and EPEC actin signalling cascades.

4.2 Results

4.2.1 Do Src kinases play a role in EPEC induced pedestal formation?

The high degree of sequence identity around Tyr112 of A36R and Tyr474 of EPEC Tir suggested that Src kinases might also be responsible for the phosphorylation of Tir (Figure 4.1). Furthermore, the sequence around Tyr474 is very similar to the Src consensus phosphorylation site (DEEIYEEFF) (Songyang *et al.*, 1993). Therefore I examined whether Src-family kinases are required for EPEC induced actin polymerisation.

It is not an unreasonable assumption that the kinase phosphorylating Tir should colocalise with its substrate at the tip of EPEC induced pedestals. I examined the localisation of Src to see whether it is a potential candidate for Tir phosphorylation. I used overexpression of a chicken c-Src clone in EPEC infected HeLa cells to examine Src localisation. By immunofluorescence analysis I detected a strong signal at the bacteria-cell interface where pedestals were formed (Figure 4.2A). As a positive control I overexpressed chicken c-Src in vaccinia infected HeLa cells and confirmed its localisation at the tip of vaccinia induced actin tails (Figure 4.2A). Out of a range of commercially available antibodies only an antibody raised against a phosphopeptide corresponding to the activated phosphorylated form of Src gave a positive signal at EPEC induced pedestals (Figure 4.2B). Using this antibody endogenous Src was also detected at the tip of vaccinia actin tails (Figure 4.2B). These observations suggest a possible role of Src in pedestal formation.

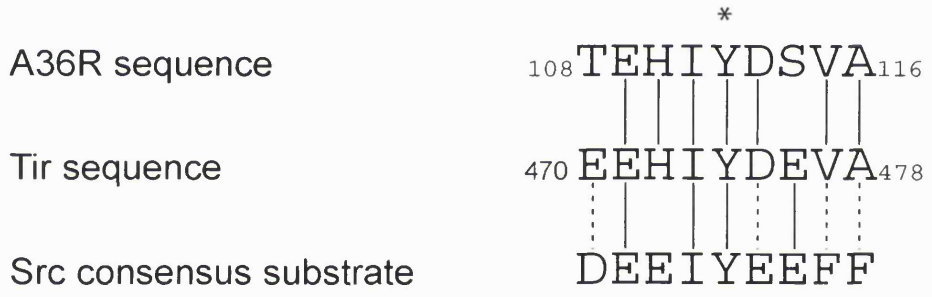


Figure 4.1. A36R and Tir display high identity to the optimal Src consensus phosphorylation site in the indicated region.
Solid lines indicate identical residues while dotted bars indicate similarity based on the groupings (A, V, F, M, I, L), (D, E, K, R), (S, T, Y, H, C, N, Q, W) and (G). The star indicates the tyrosine phosphorylation site.

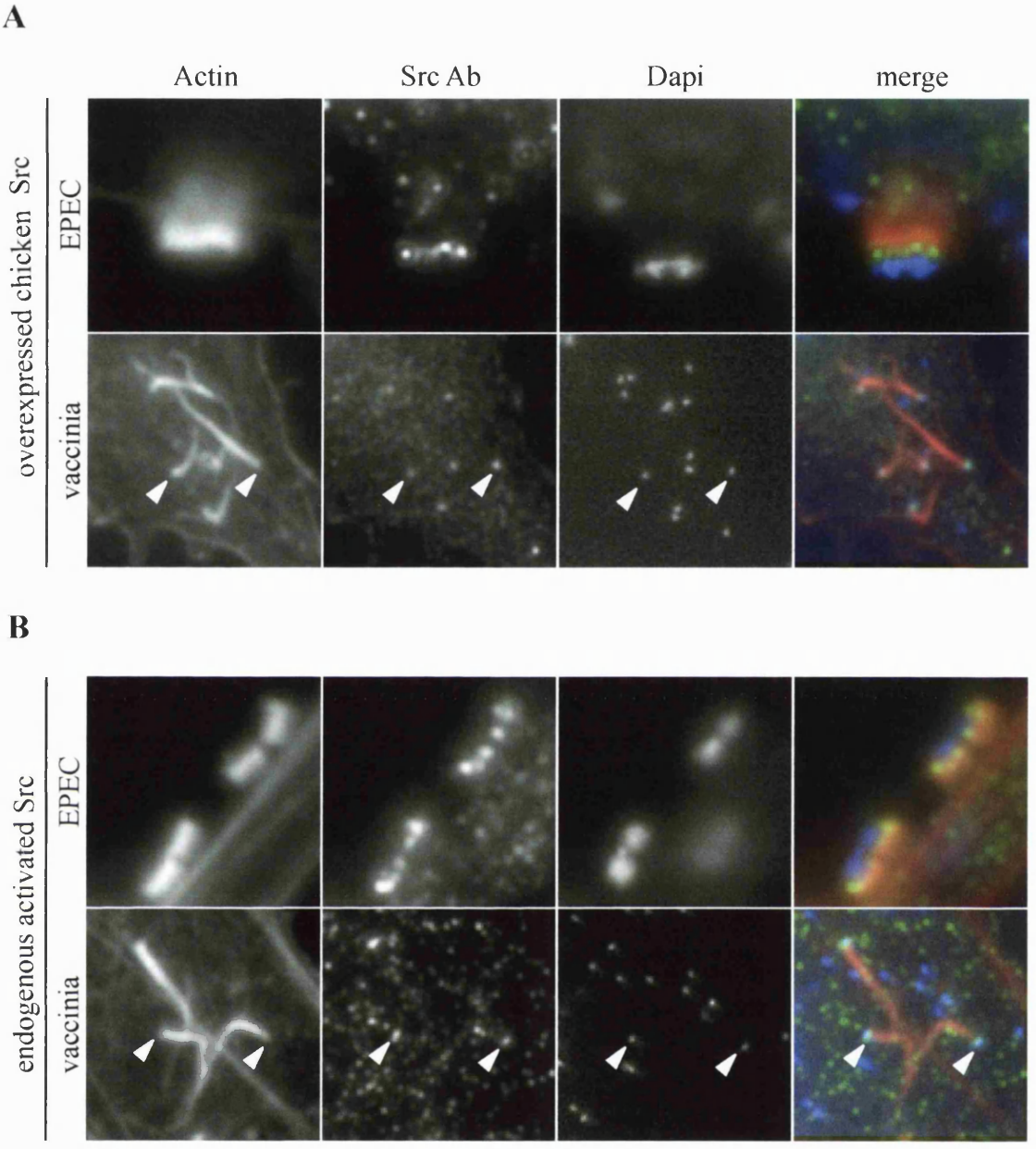


Figure 4.2. Src is recruited to the tip of EPEC actin pedestals and vaccinia actin tails.

Immunofluorescene images showing that overexpressed chicken Src **(A)** and endogenous activated Src **(B)** were recruited to vaccinia virus and EPEC. Chicken Src was detected with a monoclonal antibody, specifically recognising chicken Src (A, green). Endogenous Src was detected with an antibody raised against a pospho-peptide in which Tyr 416 is phosphorylated, which is phosphorylated upon Src activation (B, green). Bacteria and viruses were visualised by DAPI staining (blue), filamentous actin was stained with phalloidin (red). Arrow heads point to vaccinia virus particles, Src localisation and the tip of vaccinia actin tails.

The antibody used to detect endogenous Src was raised against the active form of the tyrosine kinase. This suggests that EPEC pedestal formation, like vaccinia actin tail formation, is dependent on Src kinase activity. To examine if this is the case I treated EPEC and vaccinia infected cells with the general Src kinase inhibitor PP2. In contrast to vaccinia virus, EPEC pedestal formation was completely unaffected by PP2 treatment, indicating that Src kinase activity does not play a role in EPEC induced actin polymerisation (Figure 4.3). Thus although the endogenous activated Src is recruited to EPEC pedestals its kinase activity is not required for EPEC induced actin polymerisation.

Src has also been suggested to function as a scaffolding protein, which could also be important for EPEC induced pedestal formation (Kaplan *et al.,* 1995; Schlaepfer *et al.,* 1997). Therefore I wanted to investigate whether genetic deficiency of the ubiquitously expressed Src family kinases, Src, Fyn and Yes (SYF) affects EPEC induced pedestal formation. Mouse embryo fibroblasts (MEFs) lacking Src, Fyn and Yes (SYF-/-) and the SYF +/+ parental cell line were infected with EPEC and tested for their capacity to induce pedestals. EPEC induced actin pedestals in both cell lines (Figure 4.4). Furthermore I could not detect activated Src in the SYF -/-, although it was present on pedestals formed in SYF positive MEFs (Figure 4.4). This clearly demonstrated that the ubiquitously expressed Src, Fyn and Yes are not essential for EPEC to induce actin polymerisation. Furthermore this result showed that the signal detected by the antibody raised against the activated form of Src is specific. Together these results showed that similar to vaccinia, EPEC recruits endogenous activated Src. However in contrast to vaccinia actin tail formation, EPEC pedestals seem to be formed independently of Src-family kinases.

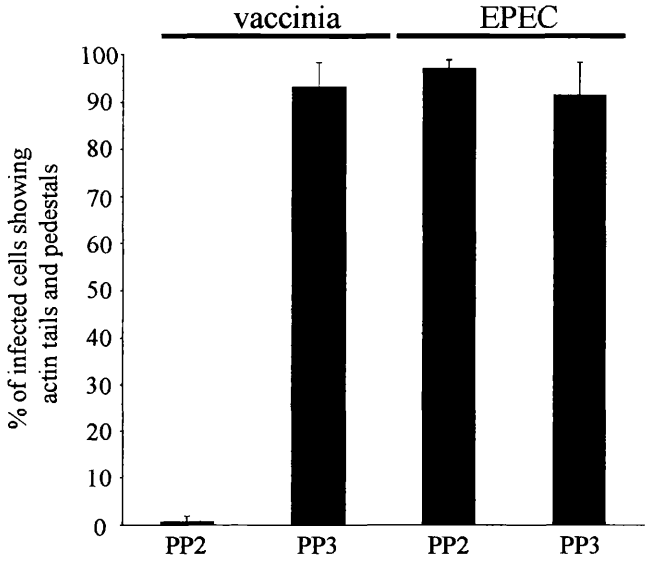


Figure 4.3. EPEC pedestal formation is not dependent on Src kinase activity. Quantification of the effect of PP2 treatment on vaccinia actin tail and EPEC induced pedestal formation in infected HeLa cells. In contrast to vaccinia actin tail formation EPEC pedestal formation was not sensitive to treatment with the Src kinase inhibitor PP2. PP3 is a very similar chemical compound to PP2 but does not affect Src kinases. PP3 treatment neither affected vaccinia actin tails nor EPEC pedestals. Error bars represent the standard deviation calculated from three independent experiments.

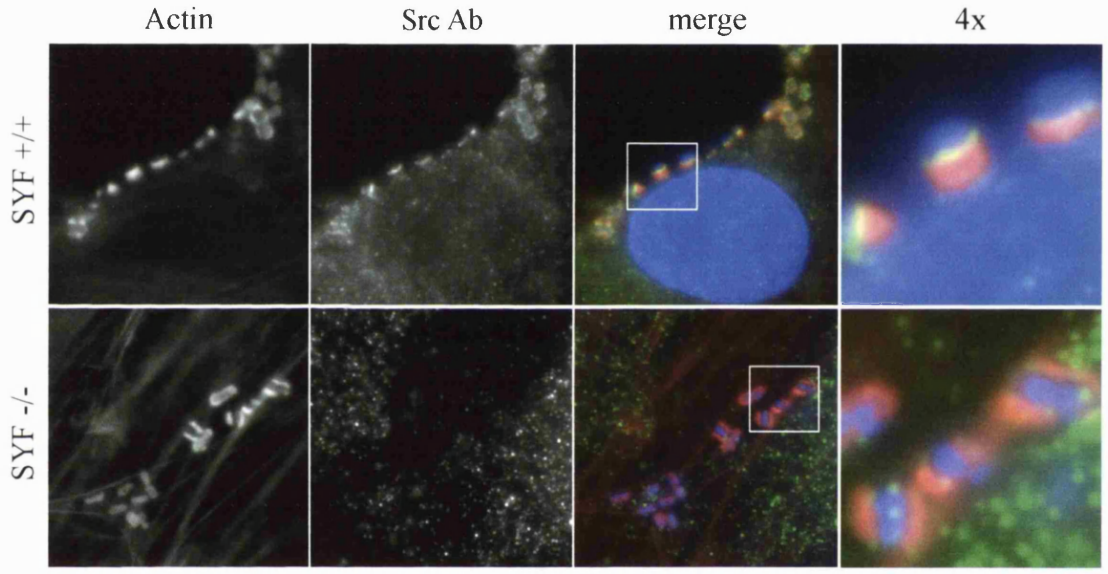


Figure 4.4. Src, Fyn and Yes are not required for EPEC actin pedestal formation. Mouse embryo fibroblasts (MEFs) containing the ubiquitously expressed Src kinases Src, Fyn and Yes (SFY+/+) and MEFs lacking Src, Fyn and Yes (SYF -/-) were infected with EPEC (blue). Both cell lines were able to form pedestals (red) but MEF SYF -/- were not positive for Src (green).

4.2.2 How does EPEC recruit N-WASP?

Initial evidence suggested that WASP is recruited through its CRIB domain to EPEC actin pedestals (Kalman *et al.*, 1999). The CRIB region of N-WASP binds strongly to the Rho family GTPases Cdc42 (Miki *et al.*, 1998a). Given the potential of Cdc42 to activate N-WASP, Rho GTPases represented attractive candidates that mediate WASP/N-WASP recruitment to EPEC pedestals. Since pedestals are resistant to the Rho GTPase inhibitor Toxin B (ToxB) it was suggested that WASP is indirectly recruited via a ToxB resistant Rho-family GTPase (Kalman *et al.*, 1999). This idea is however controversial given that a mutant of N-WASP that lacks the CRIB domain is still recruited to EPEC induced pedestals in N-WASP deficient cells (Lommel *et al.*, 2001). Lommel *et al.*, 2001 showed that an N-terminal N-WASP construct, including the WH1 and the CRIB domain, is recruited to EPEC pedestals in N-WASP deficient mouse embryo fibroblasts. Deletion of this domain lead to a loss of recruitment of N-WASPθCRIB to EPEC. Taken together these data indicate that the WH1 domain of N-WASP might be important for N-WASP recruitment to EPEC. Since the authors were not able to show that the WH1 domain on its own is sufficient to target to EPEC pedestals the possibility remains that the CRIB domain plays a helper role in recruitment of N-WASP to EPEC. Therefore I wanted to investigate the targeting mechanism of N-WASP using localisation studies and overexpression of GFP expression constructs in EPEC infected HeLa cells as well as N-WASP deficient fibroblasts.

Is N-WASP recruited via its CRIB?

I confirmed results of Lommel *et al.*, 2001 showing that the CRIB domain is not recruited to EPEC pedestals by itself (Figure 4.5 A) (Lommel *et al.*, 2001). Consistent with these observation it was demonstrated that deletions in the CRIB domain encompassing the CRIB motif and the basic region did not affect the localisation of full length N-WASP to EPEC pedestals and its ability to rescue EPEC pedestal formation in N-WASP-/- cells (Lommel *et al.*, 2001). This shows that the CRIB domain is not sufficient to recruit N-WASP to EPEC pedestals suggesting that its recruitment does not require an interaction with Cdc42 or PIP_2, which binds to the basic region of N-WASP. Furthermore the overexpression of a GFP-CRIB construct does not efficiently block

pedestal formation (Figure 4.7). These results are consistent with experiments showing that EPEC pedestal formation is insensitive to the ToxB. In addition pedestal formation is not blocked by the overexpression of dominant negative forms of Rac or Cdc42 (Ben-Ami *et al.,* 1998). Together these observations strengthen the view that EPEC induced pedestal formation is independent of Rho family GTPases.

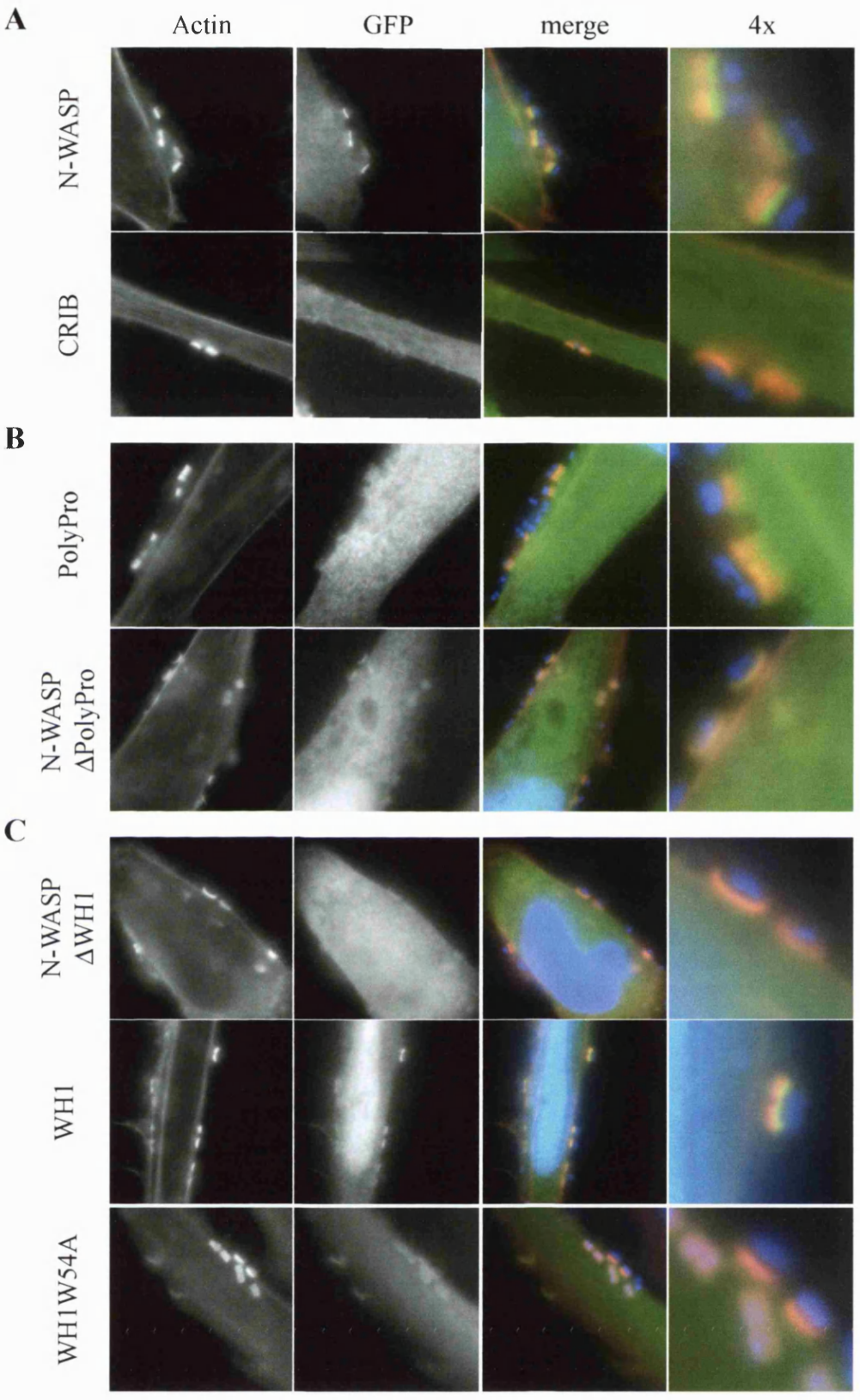


Figure 4.5. The WH1 domain is required for recruitment of N-WASP to EPEC in HeLa cells.

Immunofluorescence analysis of EPEC infected cells expressing the indicated GFP tagged N-WASP construct (green), showing that neither the (**A**) CRIB nor (**B**) the PolyPro of N-WASP are recruited to EPEC. N-WASPΔPolyPro was weakly recruited to EPEC pedestals (**B**). N-WASP constructs containing the WH1 domain (**A**) and (**C**) are recruited to EPEC actin pedestals (red). N-WASP constructs lacking or carrying a disrupted (W54A) WH1 domain are not recruited to EPEC pedestals (**C**). For a diagram of the used GFP-N-WASP constructs please refer to Figure 4.13.

Is N-WASP recruitment to EPEC or vaccinia dependent on its proline rich region?
The SH2/SH3 adaptor proteins such as Nck and Grb2 are found associated with EPEC pedestal and have been reported to bind N-WASP via its proline rich region (Carlier *et al.,* 2000; Goosney *et al.,* 2001; Gruenheid *et al.,* 2001; Rohatgi *et al.,* 2001). Thus Nck and Grb2 are potential candidates to recruit N-WASP to EPEC. A mechanism where SH3 containing adaptor proteins recruit N-WASP directly, would suggest that the proline rich region of N-WASP is important for its recruitment to EPEC actin pedestals.

I found that N-WASP is complexed with Nck and Grb2 *in vivo* depending on the presence of its proline rich region (Figure 4.6). However, a GFP tagged construct comprising the proline rich region of N-WASP (GFP-PolyPro$_{NW}$) by itself was not efficiently recruited to the bacterium in HeLa cells (Figure 4.5 B). In addition overexpression of GFP-PolyPro$_{NW}$ did not efficiently block pedestal formation (Figure 4.7). N-WASP lacking the proline rich region (N-WASPΔPolyPro) was weakly recruited to EPEC pedestals (Figure 4.5 B). The lack of convincing recruitment can possibly be explained by the fact that wild type N-WASP is more efficiently recruited than N-WASPΔPolyPro in HeLa cells. Therefore I wanted to test the recruitment and the ability to rescue EPEC actin pedestals of N-WASPΔPolyPro in cells lacking N-WASP.

Using cell lines derived from N-WASP deficient mice (see 2.1), it has been shown that N-WASP is essential for the actin based motility of EPEC and vaccinia virus and that this defect can be rescued by ectopic N-WASP expression (Lommel *et al.,* 2001; Snapper *et al.,* 2001). I confirmed these results showing that wild type GFP-N-WASP localised to and rescued EPEC pedestals and vaccinia virus actin tails very efficiently (Figure 4.9 to 4.12). GFP- N-WASPΔPolyPro was recruited to EPEC but not to vaccinia virus in fibroblasts lacking N-WASP (Figure 4.9, 4.11). Consistent with this GFP-N-WASPΔPolyPro did rescue EPEC pedestal formation but not vaccinia actin tail formation (Figure 4.9, 4.11). Thus N-WASP recruitment to vaccinia virus is not only dependent on its WH1 domain but also on its proline rich domain (Moreau *et al.,* 2000) (Figure 4.11, 4.12). In contrast to vaccinia, N-WASP lacking the proline rich domain is able to rescue EPEC pedestal formation in N-WASP deficient cells, which is consistent

with results of Lommel *et al.,* 2001. However, the actin pedestal rescue efficiency was lower than with wild type N-WASP (Figure 4.10), suggesting that proteins binding the proline rich region (e.g. Nck or Grb2) might be important for efficient actin pedestal formation.

Taken together the proline rich domain of N-WASP is neither necessary nor sufficient for N-WASP recruitment to EPEC in HeLa as well as N-WASP-/- cells. In contrast to EPEC N-WASP recruitment to vaccinia virus requires its proline rich region, which is essential for vaccinia actin tail formation. The results on N-WASP localisation to EPEC and vaccinia in HeLa cells and N-WASP -/- cells are summarised in Figure 4.13.

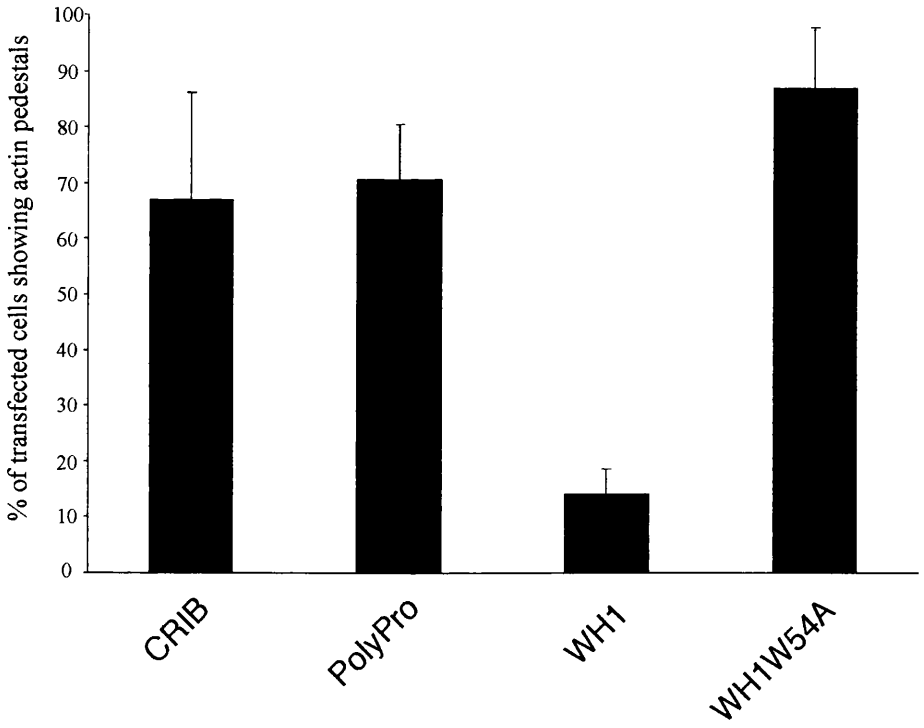


Figure 4.6. Effects of overexpression of GFP tagged N-WASP domains on EPEC pedestal formation.
Quantification of EPEC pedestal formation in cells overexpressing the indicated GFP–N-WASP constructs. Values are means +/- standard deviation from three independent experiments in which 50 infected cells were examined.

Is the WH1 domain important for recruitment of N-WASP to EPEC?

N-terminally truncated N-WASP constructs, which could still activate the Arp2/3 complex *in vitro*, were not recruited to actin pedestals in N-WASP deficient mouse fibroblasts (Lommel *et al.,* 2001; Prehoda *et al.,* 2000). This suggests that the N-terminal WH1 domain of N-WASP might be important for its recruitment to the attachment sites of EPEC.

As with vaccinia, the WH1 domain alone is efficiently recruited to pedestals in EPEC infected HeLa cells (Moreau *et al.,* 2000) (Figure 4.5 C). A GFP-N-WASP construct lacking the WH1 domain (GFPΔWH1) did not localise to EPEC in HeLa cells (Figure 4.5 C). Supporting these observations N-WASP lacking its N-terminus, including the WH1 and the CRIB domain, did not rescue pedestal formation in N-WASP -/- cells (Lommel *et al.,* 2001). N-WASP just lacking its WH1 domain could not localise to EPEC nor rescue actin pedestals in N-WASP deficient fibroblasts (Figure 4.9, 4.10). Similarly GFP-N-WASPΔWH1 did not localise to vaccinia virus particles and hence the virus was unable to form actin tails in N-WASP-/- cells transfected with GFP-N-WASPΔWH1 (Figure 4.11, 4.12). Thus similar to vaccinia virus the WH1 domain is important for recruitment of N-WASP to EPEC induced pedestals.

The WH1 domain has been shown to be involved in protein-protein interactions and phospholipid binding (Miki *et al.,* 1996; Moreau *et al.,* 2000). However, more recent studies revealed that the N-WASP WH1 domain does not bind to PIP_2, as previously reported by Miki *et al.,* 1996 (Volkman *et al.,* 2002). It was shown that the WH1 domain of N-WASP binds to sequences only found in WIP family members (see Chapter 3). This interaction is abrogated when tryptophan 54 (W54) in the WH1 domain of N-WASP is mutated to alanine (Moreau *et al.,* 2000; Zettl and Way, 2002). No efficient recruitment to EPEC pedestals was observed when a GFP-WH1 construct containing the W54A mutation (WH1W54A) was expressed in HeLa cells (Figure 4.5 C). Furthermore the GFP-WH1W54A lost its dominant negative effect on EPEC actin pedestal formation as compared to the wild type WH1 domain (Figure 4.7). These experiments suggest that Trp54 of N-WASP plays an important role in targeting N-WASP to EPEC and vaccinia virus. However, the point mutation in W54A in the

separated domain might behave differently in the context of the full-length molecule. Alternatively N-WASP could be targeted by multiple compensatory interactions to EPEC pedestals or vaccinia actin tails. Therefore I wanted to examine whether the WH1 domain is the only region needed for N-WASP recruitment to pedestals. I found that introducing the W54A into full length N-WASP abrogated its localisation to EPEC and to vaccinia virus in HeLa cells (Figure 4.8). It is possible that N-WASP carrying the point mutation W54A can not compete with endogenous N-WASP and hence does not localise to EPEC or vaccinia virus in HeLa cells. Thus I examined whether GFP-N-WASP (W54A) can be recruited to EPEC and vaccinia virus in cells lacking N-WASP. In contrast to HeLa cells, GFP-N-WASP (W54A) was recruited to EPEC pedestals and vaccinia actin tails in cells lacking N-WASP (Figure 4.9, 4.11). The rescue efficiencies of GFP-N-WASP (W54A) as compared to wild N-WASP were however lower for EPEC pedestals and vaccinia actin tails (Figure 4.10, 4.12).

Taken together these results show that the WH1 domain is absolutely required for vaccinia and EPEC induced actin polymerisation. A point mutation W54A in theWH1 domain of N-WASP in the context of the full length molecule abrogated targeting of GFP-N-WASP (W54A) to EPEC and vaccinia in HeLa cells but not in N-WASP-/- cells. However, the efficiencies of EPEC pedestals and vaccinia actin tails were significantly reduced when rescued with GFP-N-WASP (W54A). The deletion of the WH1 or the point mutation W54A interferes with the interaction of N-WASP with WIP (Moreau *et al.,* 2000; Zettl and Way, 2002). Therefore these observations suggested that an N-WASP WIP interaction is important but not essential for vaccinia and EPEC to induce actin polymerisation.

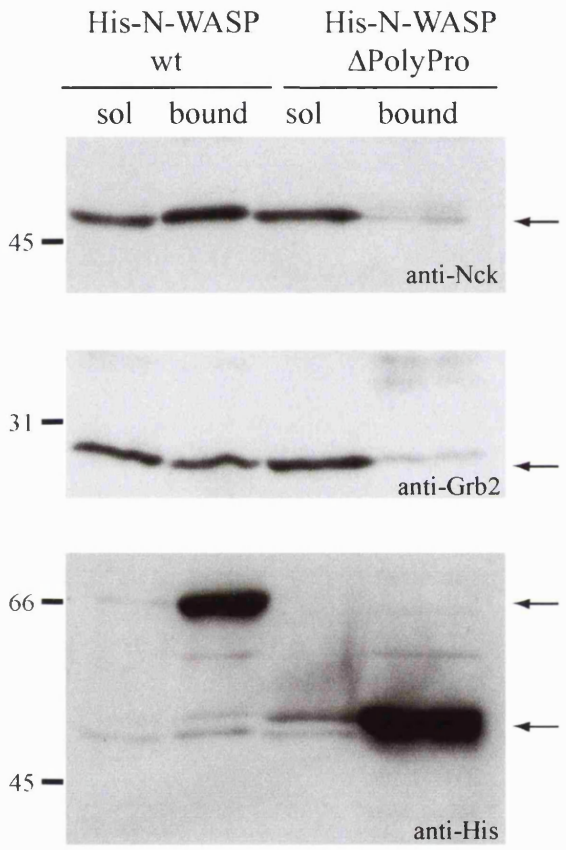


Figure 4.7. Grb2 and Nck bind to the proline rich region of N-WASP.
His-N-WASP and His-N-WASPΔPolyPro were expressed in vaccinia infected HeLa cells under the control of a viral promotor (pE/L). Cell extracts were passed over a nickel resin and the soluble (sol) and bound fractions were analysed for the presence of Nck, Grb2 and His tagged N-WASP constructs. The same western blot was reprobed three times using the indicated antibodies. The positions of the Nck, Grb2, His-N-WASP and His-N-WASPΔPolyPro are indicated by arrows.

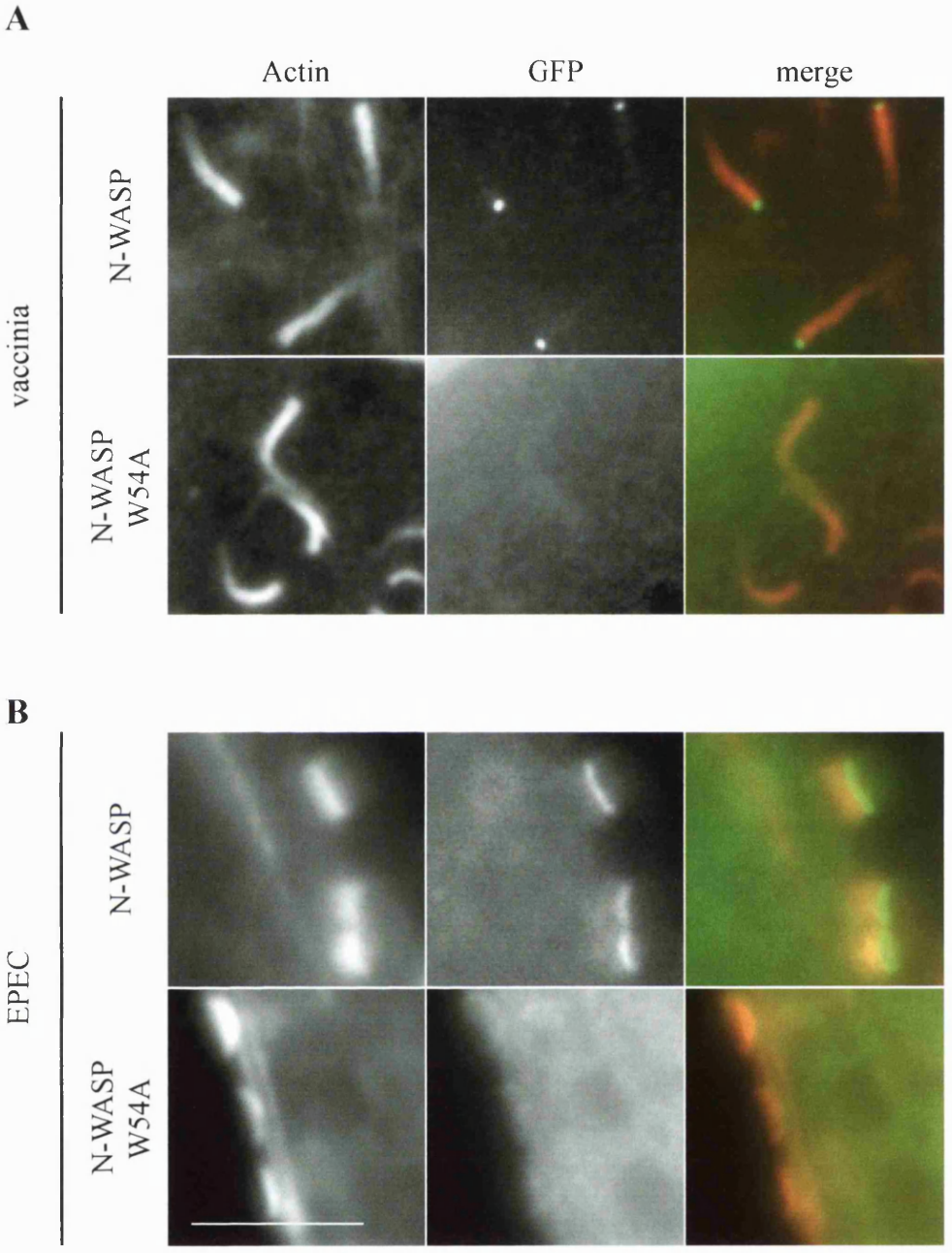


Figure 4.8. Changing Trp54 to Ala in the WH1 domain in full length N-WASP disrupt its localisation to vaccinia and EPEC in HeLa cells.
Immunofluorescene images showing localisation of the indicated GFP-N-WASP constructs in vaccinia **(A)** and EPEC **(B)** infected HeLa cells. Filamentous actin was visualised by phalloidin staining (red) and GFP localisation are shown in green in the merged image. Scale bar represents 10 μm.

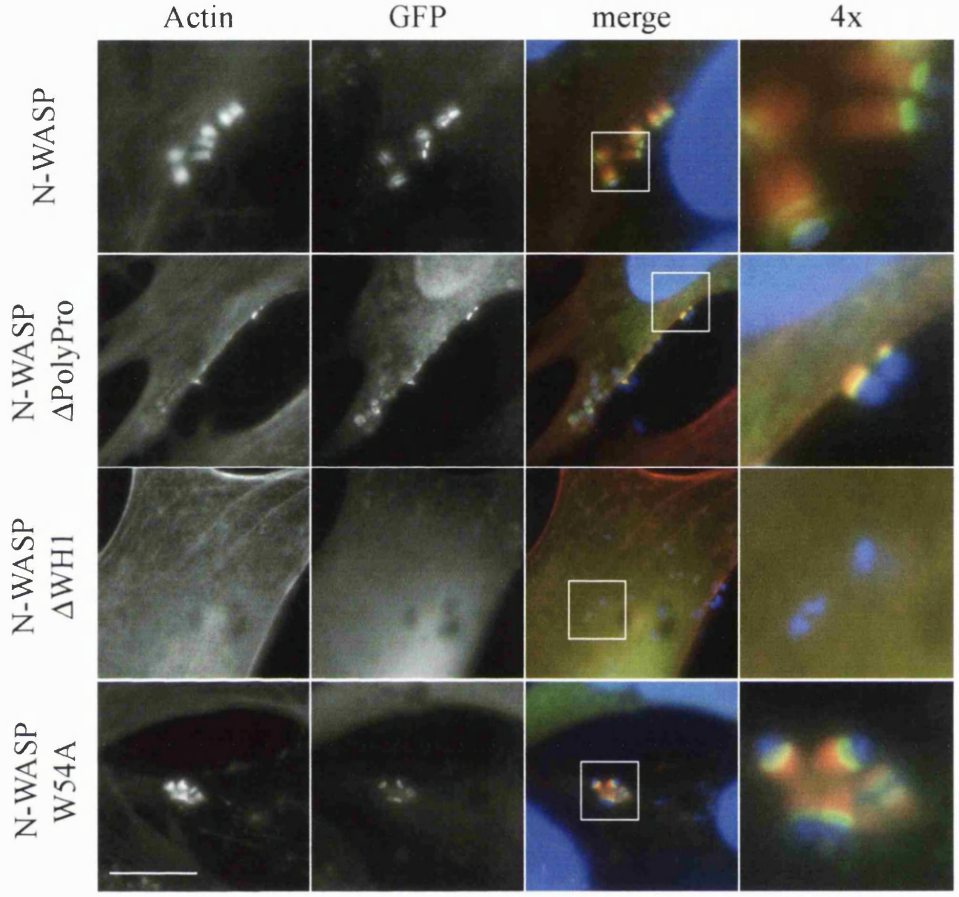


Figure 4.9. The WH1 domain is required for EPEC pedestal formation. Immunofluorescence analysis of EPEC infected N-WASP -/- mouse embryo fibroblasts expressing the indicated GFP–N-WASP constructs (green). The WH1 domain is essential for N-WASP to rescue EPEC pedestals (red). N-WASP constructs lacking the proline rich region or carrying a single point mutation in the full length molecule still localise and rescue pedestals. Bacteria were visualised by DAPI staining shown in blue in the merged images. Magnifications of the white boxes are shown on the right. Scale bar represents 10μm.

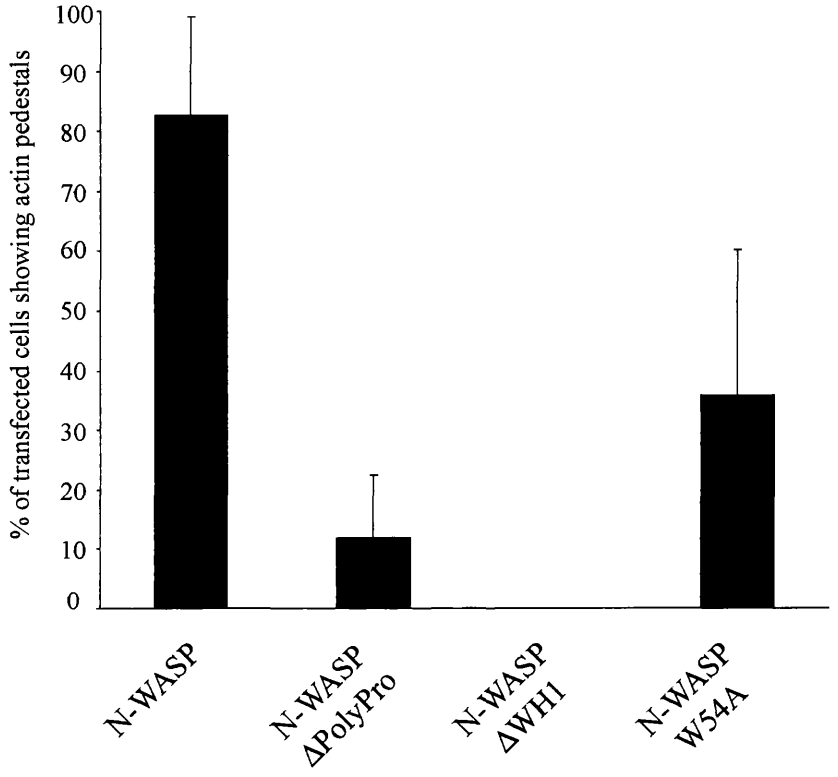


Figure 4.10. The proline rich region as well as the WH1 domain of N-WASP are important for efficient rescue of EPEC actin pedestal in N-WASP-/- cells.
Wild type N-WASP efficiently rescued EPEC pedestal formation. Deletion of the proline rich region of N-WASP and disruption of the WH1 domain (W54A) severely reduced rescue efficiencies compared to wild type N-WASP. Deletion of the WH1 domain completely ablates the ability of N-WASP to rescue EPEC induced pedestals. Values are mean +/- standard deviation from three independent experiments in which 50 cells were examined.

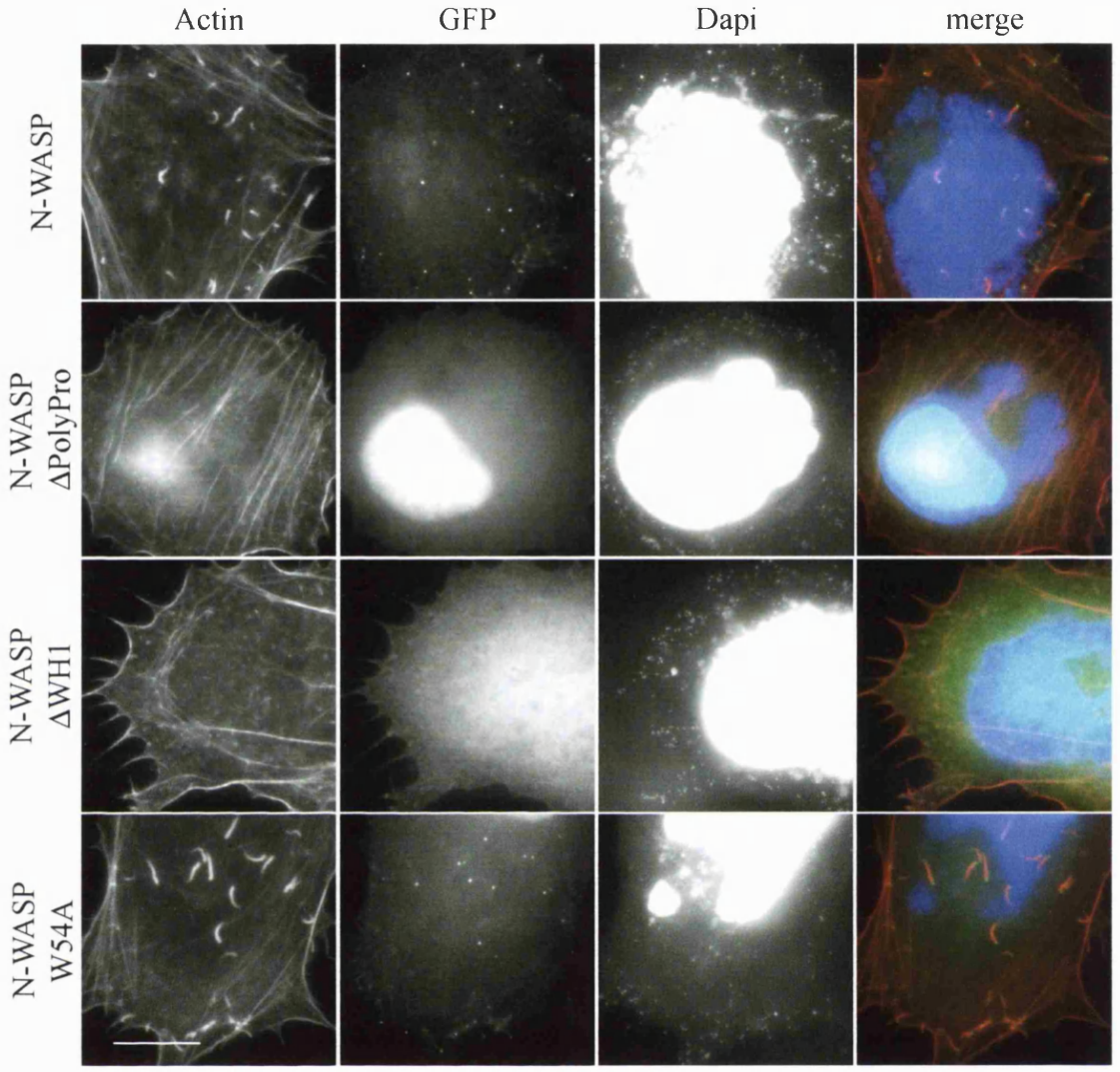


Figure 4.11. The WH1 domain and the proline rich domain are essential to mediate N-WASP recruitment to vaccinia virus in N-WASP -/- cells. Immunofluorescence analysis of vaccinia infected cells lacking N-WASP expressing the indicated GFP–N-WASP constructs (green). The WH1 domain and the proline rich region are essential for N-WASP to rescue vaccinia actin tails (red). Vaccinia virus particles and the nucleus are visualised by DAPI staining (blue). Scale bar represents 10 µm. Note that the GFP tag can causes proteins to localise into the nucleus, which could be the reason for the strong nuclear signal observed when GFP-N-WASPΔPolyPro is expressed in N-WASP-/- cell lines.

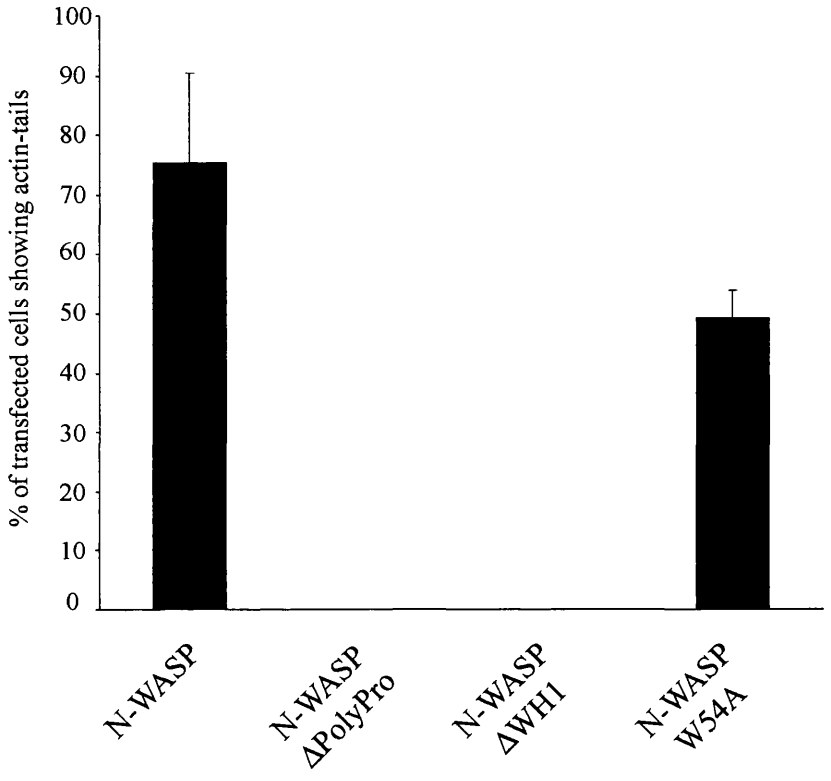
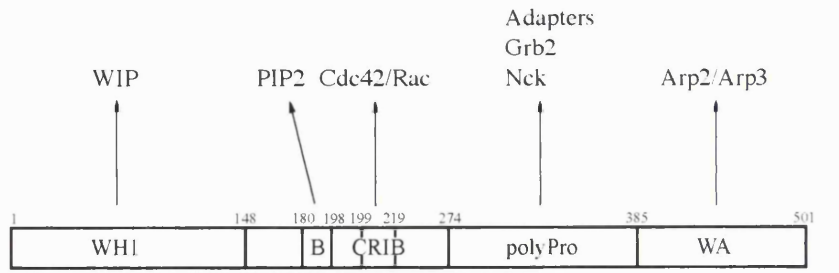


Figure 4.12. Quantification of vaccinia actin tail rescue effiencies in N-WASP-/- cells.

Wild type N-WASP efficiently rescued vaccinia actin tails, whereas deletion of the proline rich region or the WH1 domain of N-WASP does not. Mutation of tryptophan 54 to alanine in N-WASP reduced actin tail rescue efficiency. Values are means +/- standard deviation from three independent experiments in which 50 cells were examined.



	Targeting to EPEC		Targeting to vaccinia	
	HeLa	N-WASP -/-	HeLa	N-WASP -/-
CRIB	–	n.d	–	n.d
polyPro	–	n.d	–	n.d
N-WASP ΔpolyPro	+	+	n.d	–
ΔWH1	–	-	–	–
WH1	+	n.d	+	n.d
WH1W54A	–	n.d	–	n.d
N-WASP W54A	–	+	–	+

Figure 4.13. Recruitment requirements of N-WASP to vaccinia actin tails and EPEC pedestals.
Schematic representation of N-WASP, showing the positions of the WASP homology domain 1 (WH1), the basic region (B), Cdc42/Rac-interactive binding motif (CRIB), proline-rich SH3-adaptor-binding region (polyPro) and the Arp2/3 complex binding domain (WA). The respective interaction partners are indicated above the schematic of N-WASP. The positions of the domains are indicated as are the GFP-tagged constructs used in this study. The abilities of these constructs to be recruited to EPEC on actin pedestals and to vaccinia actin tails are indicated for HeLa cells and N-W -/- fibroblasts (right). N.d stands for not determined.

4.2.3 Is WIP required for EPEC pedestal formation?

WIP interacts with the WH1 domain of N-WASP and also binds Nck (Anton *et al.*, 1998; Ramesh *et al.*, 1997). Since N-WASP is recruited to EPEC via its WH1 domain, WIP is an ideal candidate responsible for recruitment of N-WASP to EPEC.

In order to get more direct evidence whether WIP is involved in EPEC pedestal formation, I examined its localisation in EPEC infected HeLa cells. GFP-WIP and endogenous WIP were efficiently recruited to the tip of EPEC pedestals (Figure 4.14). Overexpression of the WBD of WIP blocks the formation of vaccinia actin tails (Moreau *et al.*, 2000). This is thought to sequester endogenous N-WASP thereby blocking its recruitment to virus particles (Moreau *et al.*, 2000). To examine whether WIP is also required for EPEC pedestal formation I overexpressed GFP-WBD in EPEC infected HeLa cells. Overexpression of GFP-WBD efficiently blocked pedestals and N-WASP recruitment to EPEC (Figure 4.15, 4.16). Furthermore overexpression of GFP-WBDF454/456A does not block vaccinia actin tail formation and does not alter the localisation of endogenous N-WASP WASP (Zettl and Way, 2002). These results suggest that GFP-WBDF454/456A is not able to interact with N-WASP *in vivo*. Therefore I wanted to investigate whether the dominant negative effect on EPEC pedestal formation of GFP-WBD was mediated via N-WASP. Similarly to vaccinia I saw that overexpression of GFP-WBDF454/456A does neither affect pedestal formation nor N-WASP recruitment (Figure 4.15, 4.16). These observations indicate that similar to vaccinia WIP is important to recruit N-WASP to EPEC induced actin pedestals.

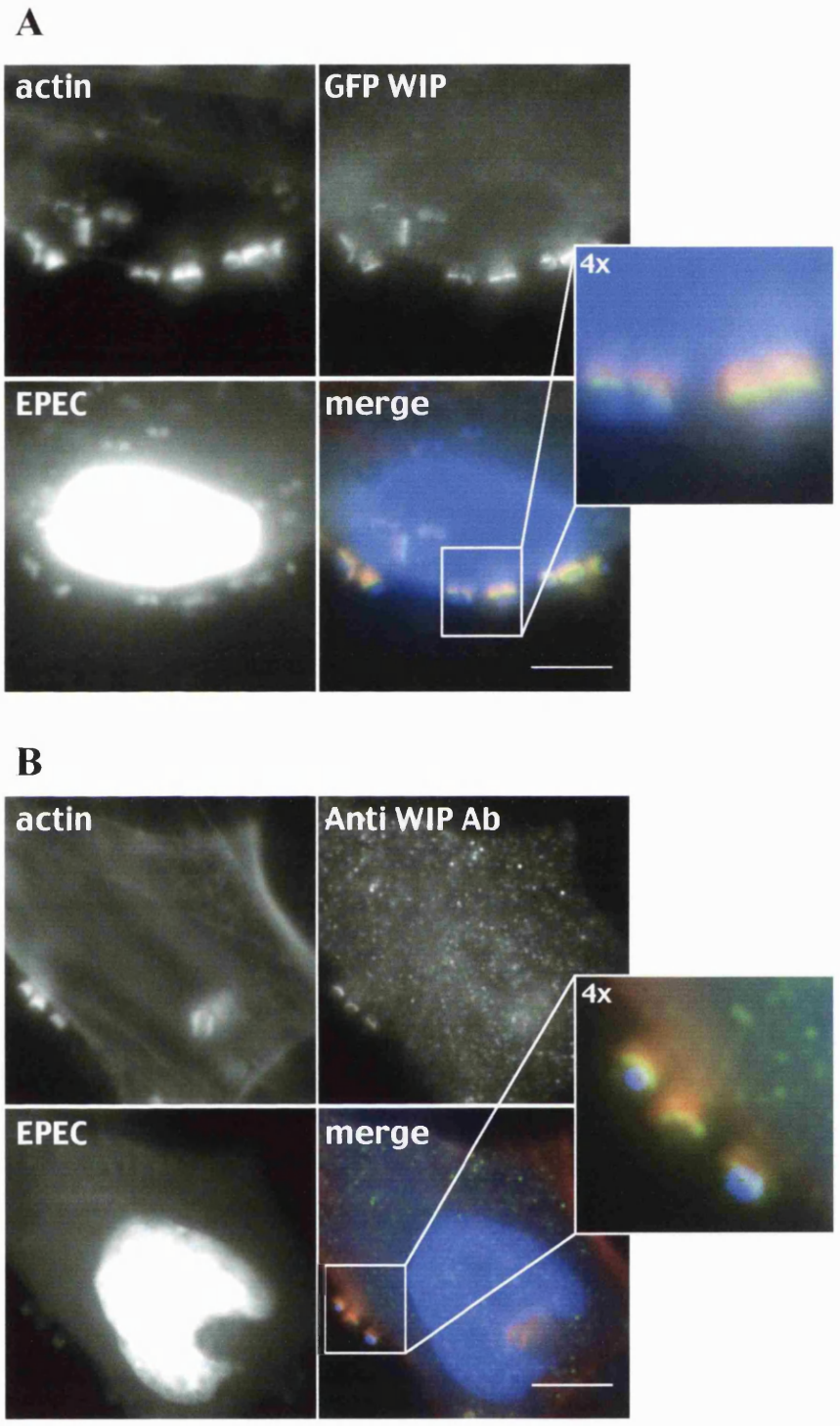


Figure 4.14. GFP-WIP and endogenous WIP are recruited to the tip of EPEC actin pedestals.
Immunofluorescence analysis showing that GFP-WIP **(A)** (green) and endogenous WIP (green). **(B)** are recruited to EPEC (blue) induced actin pedestals (red). Note that the GFP signal (green) is separated from the actin pedestal (red) indicating recruitment of WIP to the tip of actin pedestals. Scale represents 10 µm.

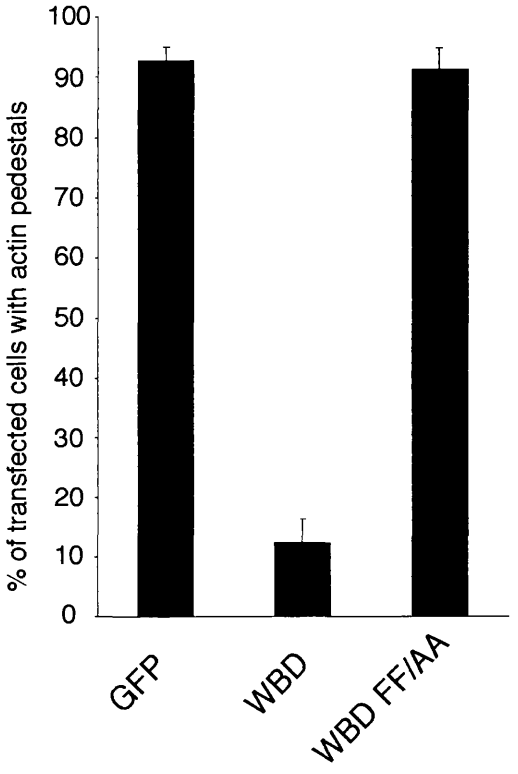


Figure 4.15. WIP is involved in the actin pedestal formation of EPEC. Quantification of EPEC pedestal formation shows that overexpression of the WASP binding domain of WIP (WIP-WBD) but not WIP (WBDF454/456A) or GFP, inhibits formation of EPEC pedestals. Values are means +/- standard deviaton from three independent experiments in which 50 infected cells were examined.

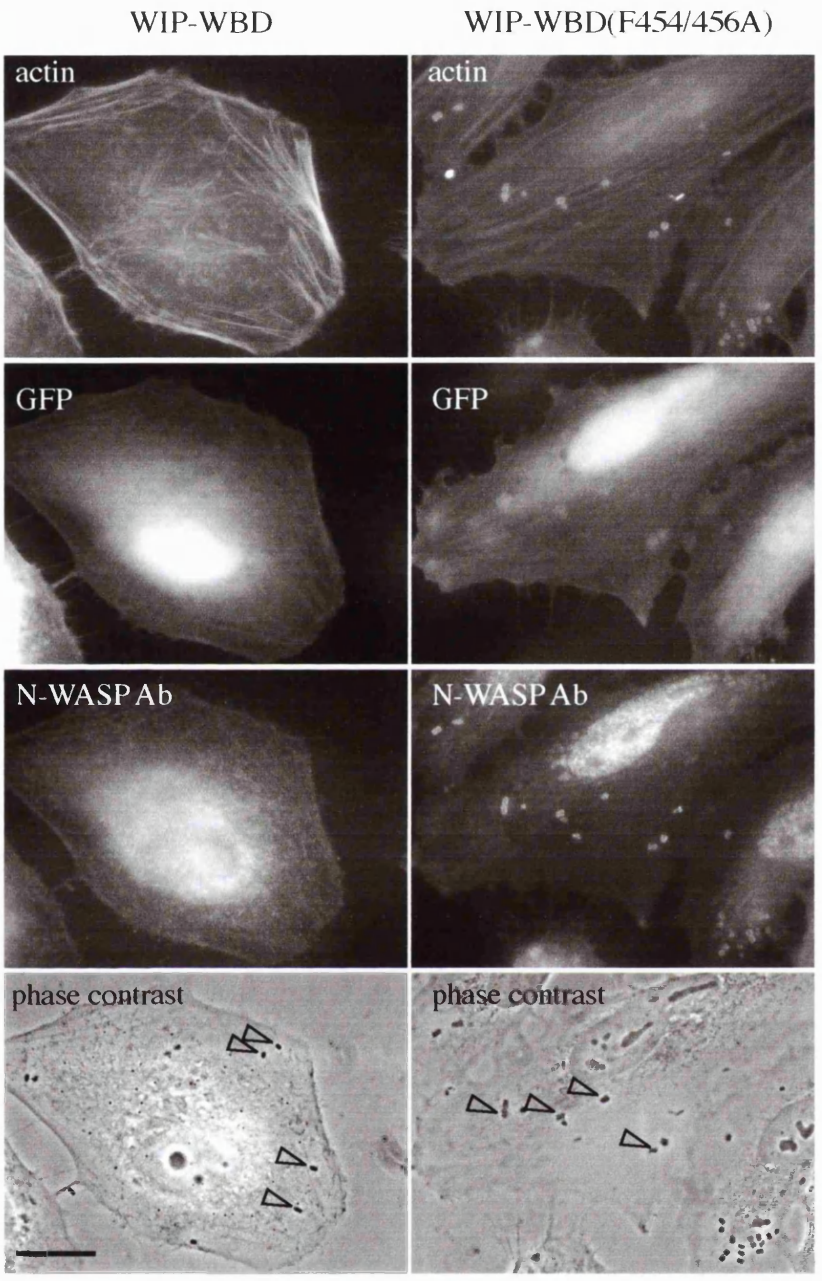


Figure 4.16. Effects of overexpressions of WBD and WBD (F454/456A) on N-WASP recruitment to EPEC induced actin pedestals.
Immunofluorescence analysis of EPEC infected HeLa cells overexpressing the indicated GFP construct showing pedestals and recruitment of N-WASP are blocked by WASP binding domain of WIP (WIP-WBD). EPEC induced pedestals and N-WASP recruitment are not affected by the overexpression of WIP-WBD (F454/456A). Black open arrow heads point to EPEC. Scale bar represents 10 μm.

4.2.4 How is WIP recruited to EPEC?

In the absence of N-WASP, WIP is not recruited to vaccinia virus particles (Snapper *et al.,* 2001). The recruitment of N-WASP to vaccinia virus particles depends on the WH1 domain, which binds to WIP. Conversely the recruitment of WIP to vaccinia virus particles depends on its WASP binding domain (WBD), suggesting that WIP and N-WASP are recruited as a complex to vaccinia virus (Moreau *et al.,* 2000). Therefore I wanted to investigate how EPEC recruits WIP.

A construct lacking the WIP-WBD (GFPWIPΔWBD) was recruited along the actin pedestals as well as along vaccinia actin tails (Figure 4.17, 4.18). Furthermore a construct with mutations in the N-WASP binding site in WIP (GFPWIPF454/456A) localised along the EPEC pedestals as well (Figure 4.17). This demonstrated that the WIP-WBD is not necessary for the recruitment of WIP along actin pedestals but plays a role in anchoring WIP to the tip of EPEC pedestals. Thus WIP can be recruited independently of N-WASP along EPEC actin pedestals and vaccinia actin tails.

Thus I wanted to further investigate which domain of WIP is required for its localisation along EPEC pedestals. The central part of WIP is proline rich and was reported to bind to Nck (Anton *et al.,* 1998), which is essential for EPEC pedestal formation (Gruenheid *et al.,* 2001). I therefore investigated whether WIP is recruited via its proline rich domain to EPEC. The proline rich domain of WIP (GFP-PolyPro$_{WIP}$) on its own was recruited to the bacterium co-localising with the actin pedestal rather than being recruited to the tip (Figure 4.17). This recruitment is not due to trapping effects caused by the dense actin since GFP-PolyPro$_{WIP}$ was not recruited to vaccinia virus actin tails (Figure 4.18). Supporting the idea that WIP is recruited via Nck to EPEC pedestals high expression levels of GFP-PolyPro$_{WIP}$ blocked EPEC pedestal formation so that only 38.7 ± 9% of transfected infected cells formed pedestals (Figure 4.19). Although Nck also binds to the proline rich region of N-WASP (Figure 4.6) 70.7 ± 9.8% of EPEC infected cells overexpressing GFP-PolyPro$_{N\text{-}WASP}$ induced pedestals (Figure 4.19). Consistent with idea that GFP-PolyPro$_{WIP}$ rather than the proline rich domain of N-WASP blocks pedestal formation GFP-PolyPro$_{N\text{-}WASP}$ is not recruited to EPEC induced pedestals in HeLa cells (Figure 4.5 B). Deletion of the proline rich domain of WIP (GFP-WIPΔPolyPro) lead to a loss of WIP recruitment to EPEC actin pedestals and

vaccinia virus actin tails (Figure 4.17, 4.18). GFP-WIPΔPolyPro interacted with the WH1 domain of N-WASP demonstrating that the overall fold of the protein was not disrupted by the deletion of the proline rich region (Figure 4.20). This showed that the proline rich region of WIP plays an essential role in WIP recruitment to EPEC and vaccinia virus. Furthermore it suggested that the N-terminus of WIP (residues 1-127), which binds to G- and F-actin (Martinez-Quiles *et al.,* 2001), is not important for the localisation of WIP along actin pedestals and vaccinia actin tails. Taken together these data showed that WIP recruitment to EPEC actin pedestals and vaccinia actin tails was dependent on is interaction with N-WASP as well as on its proline rich region. Since N-WASP and WIP are unlikely to interact with their respective proline rich regions (Zettl and Way, 2002), it is likely that in addition to N-WASP there is a third protein involved in the recruitment of WIP to vaccinia and EPEC, which could be Nck.

Vaccinia virus does not recruit WIP and Nck in the absence of N-WASP (Snapper *et al.,* 2001), suggesting that N-WASP, WIP and Nck recruited as a trimolecular complex. In order to test whether WIP and Nck need N-WASP to be recruited to EPEC actin pedestals I investigated the localisation of endogenous WIP and Nck in N-WASP deficient cells (N-WASP-/-). In N-WASP-/- cells I could not detect WIP or Nck on EPEC. However endogenous WIP and Nck were found on the tip of actin pedestals formed upon EPEC infection of the parental N-WASP +/+ mouse embryo fibroblasts (Figure 4.21). Together these data suggested that WIP and Nck were recruited to the tip of EPEC pedestals in a complex with N-WASP.

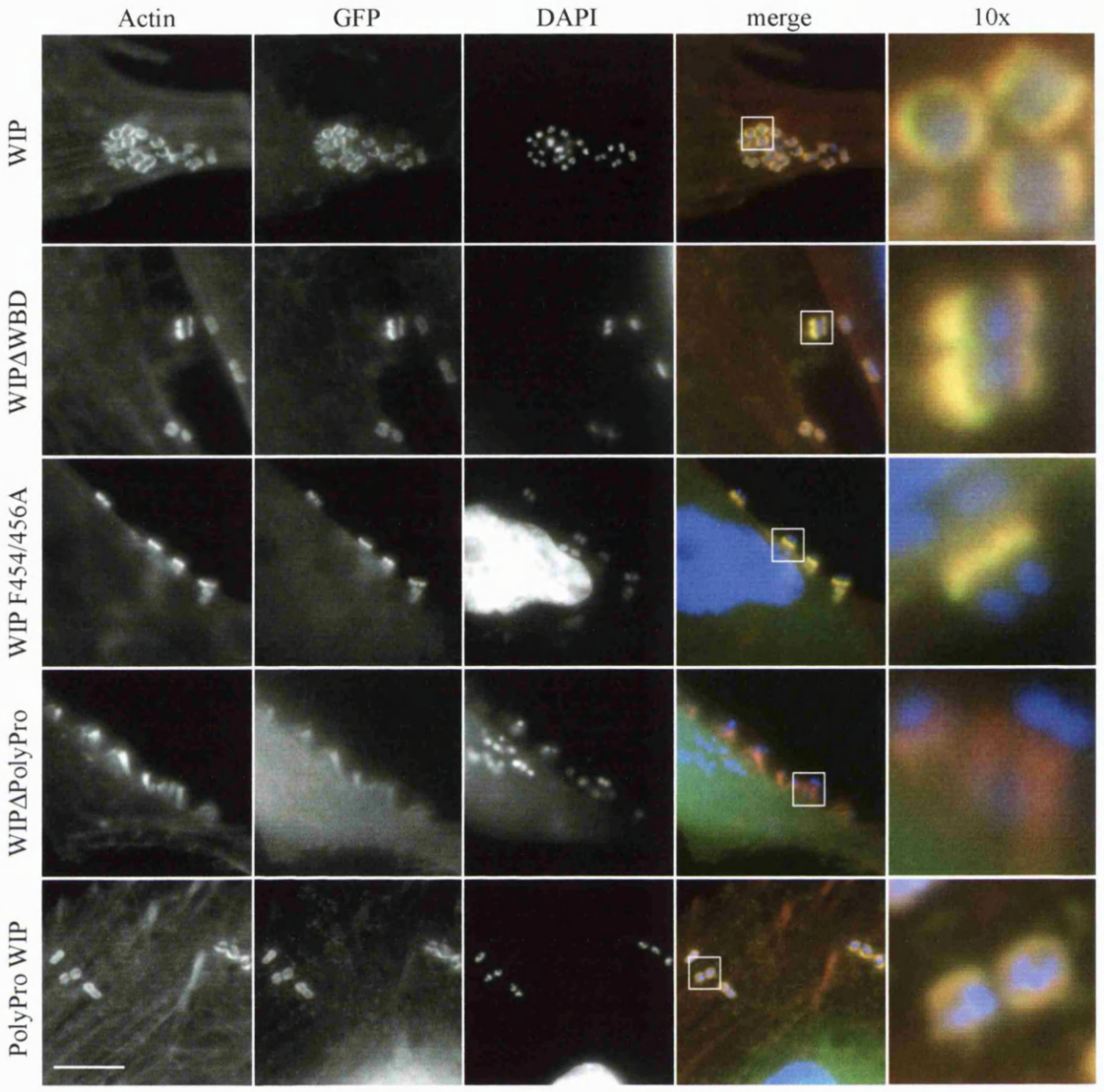


Figure 4.17. The proline rich region and the WASP binding domain of WIP (WIP-WBD) mediate recruitment of WIP to the tip of EPEC pedestals.
Immunofluorescence analysis showing that GFP-WIPΔWBD and GFP-WIPF454/456A are recruited along EPEC induced actin pedestals. Similarly, the proline rich region of WIP alone colocalises with actin pedestals. (compare merge images of GFP-WIP and PolyPro WIP). No recruitment to EPEC pedestals was observed with GFP-WIPΔPolyPro. Filamentous actin was visualised with phalloidin (red), EPEC DNA was stained by DAPI (blue) and the GFP signal of the respective WIP construct is shown in green in the merged image. To show the difference between localisation to the tip of the pedestal (GFP-WIP) and along the pedestal the boxed area of the merged image is shown 10x magnified. Scale bar represents 10 μm.

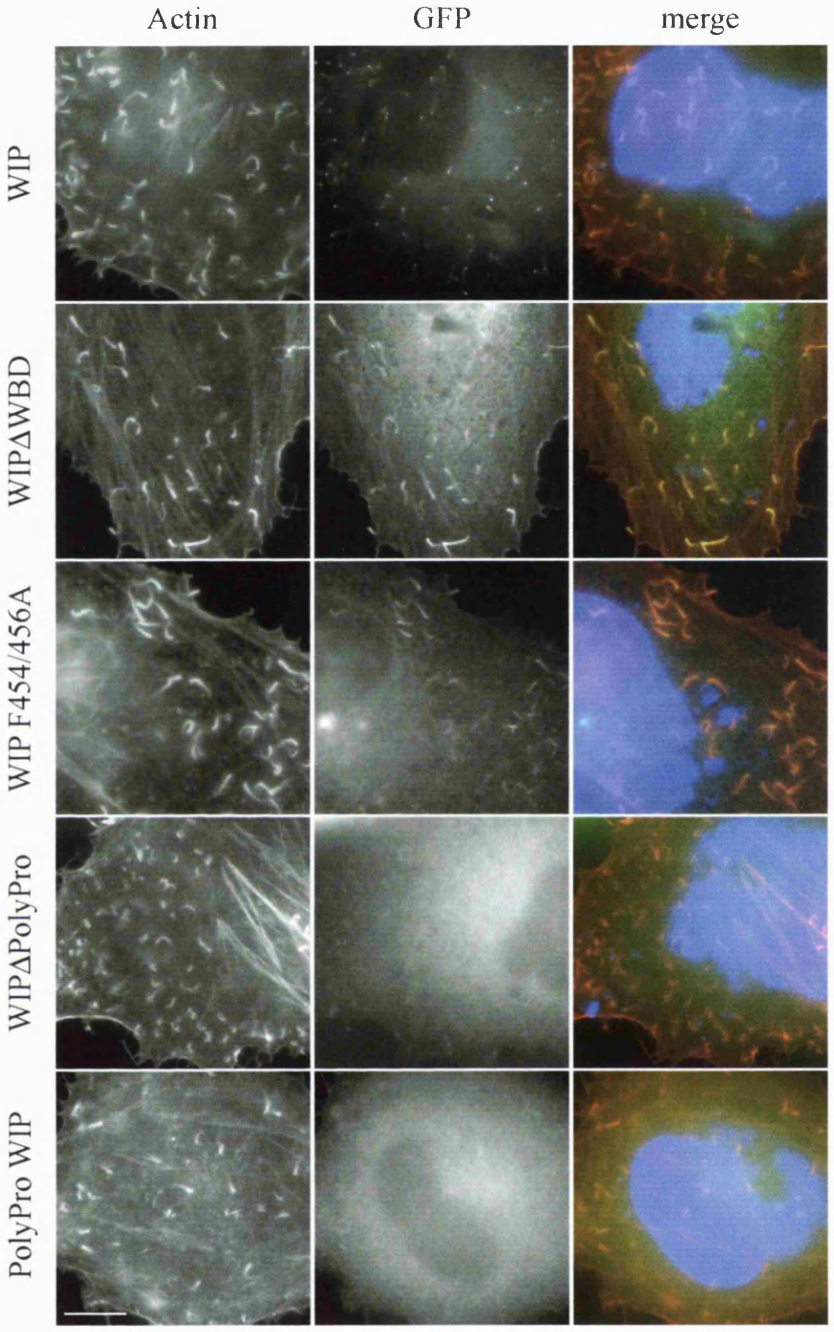


Figure 4.18. The proline rich region and the WASP binding domain (WBD) of WIP mediate recruitment of WIP to the tip of vaccinia actin tails.
Immunofluorescence analysis showing that GFP-WIPΔWBD and GFP-WIPF454/456A are recruited along vaccinia actin tails. No recruitment to vaccinia actin tails was observed with GFP-WIPΔPolyPro and with GFP-PolyProWIP alone. Filamentous actin was visualised with phalloidin (red), vaccinia virus DNA was stained by DAPI (blue) and the GFP signal of the respective WIP construct is shown in green in the merged image. Note that the yellow colour indicates colocalisation of actin and the GFP labelled WIP construct. Scale bar represent 10 μm.

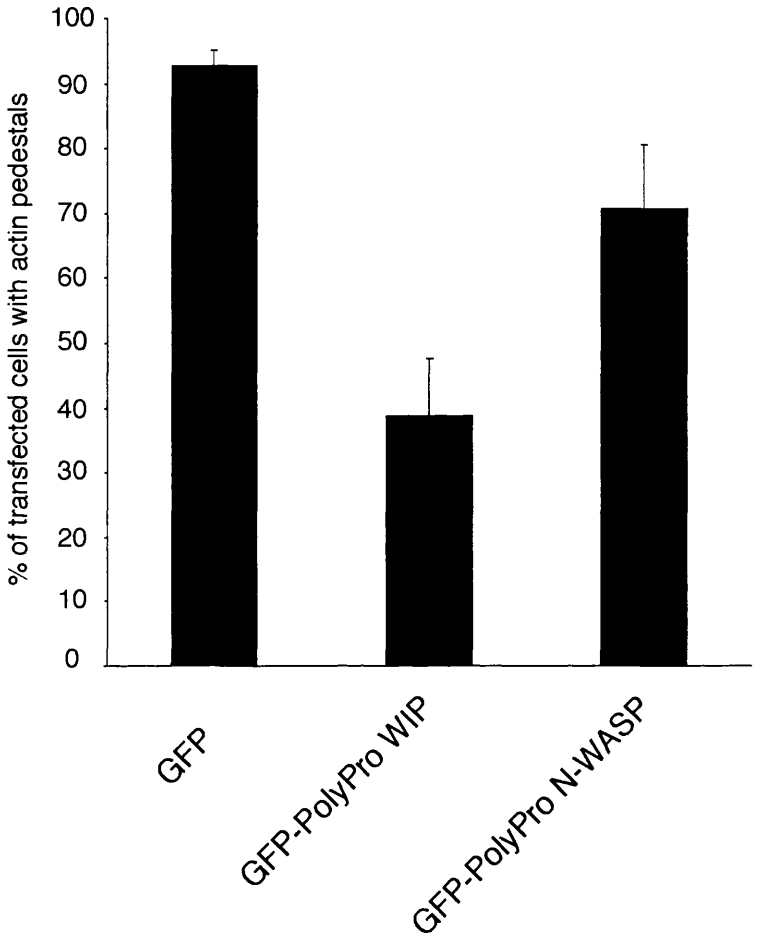


Figure 4.19. The proline rich region of WIP inhibits actin pedestal formation of EPEC.

Quantification of EPEC pedestal formation shows that the overexpression of the proline rich region of WIP is significantly more effective in blocking EPEC induced pedestals than the proline rich region of N-WASP. The data represent the mean from three independent experiments and error bars represent standard deviations from the mean.

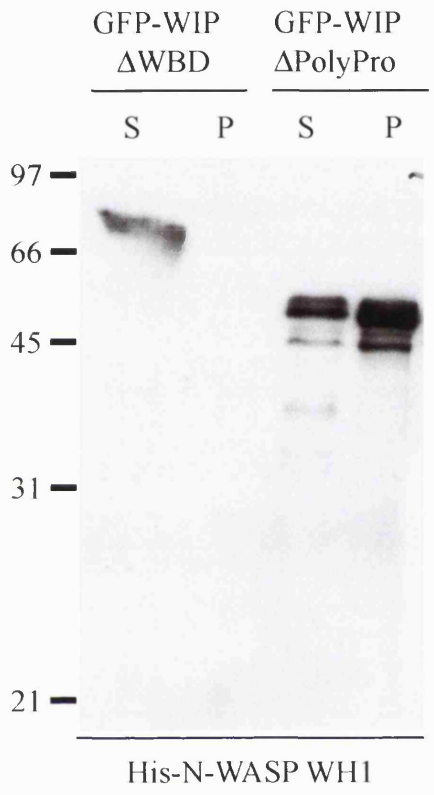


Figure 4.20. Deletion of the proline rich region of WIP does not affect the interaction with N-WASP.
Indicated GFP tagged versions of WIP were expressed in HeLa cells and tested for their ability to bind to the bacterially expressed His-WH1 domain of N-WASP. WIP lacking its proline rich domain interacted with the WH1 domain, whereas a construct lacking the WASP binding domain (WBD) cannot bind to the His-WH1 domain of N-WASP.

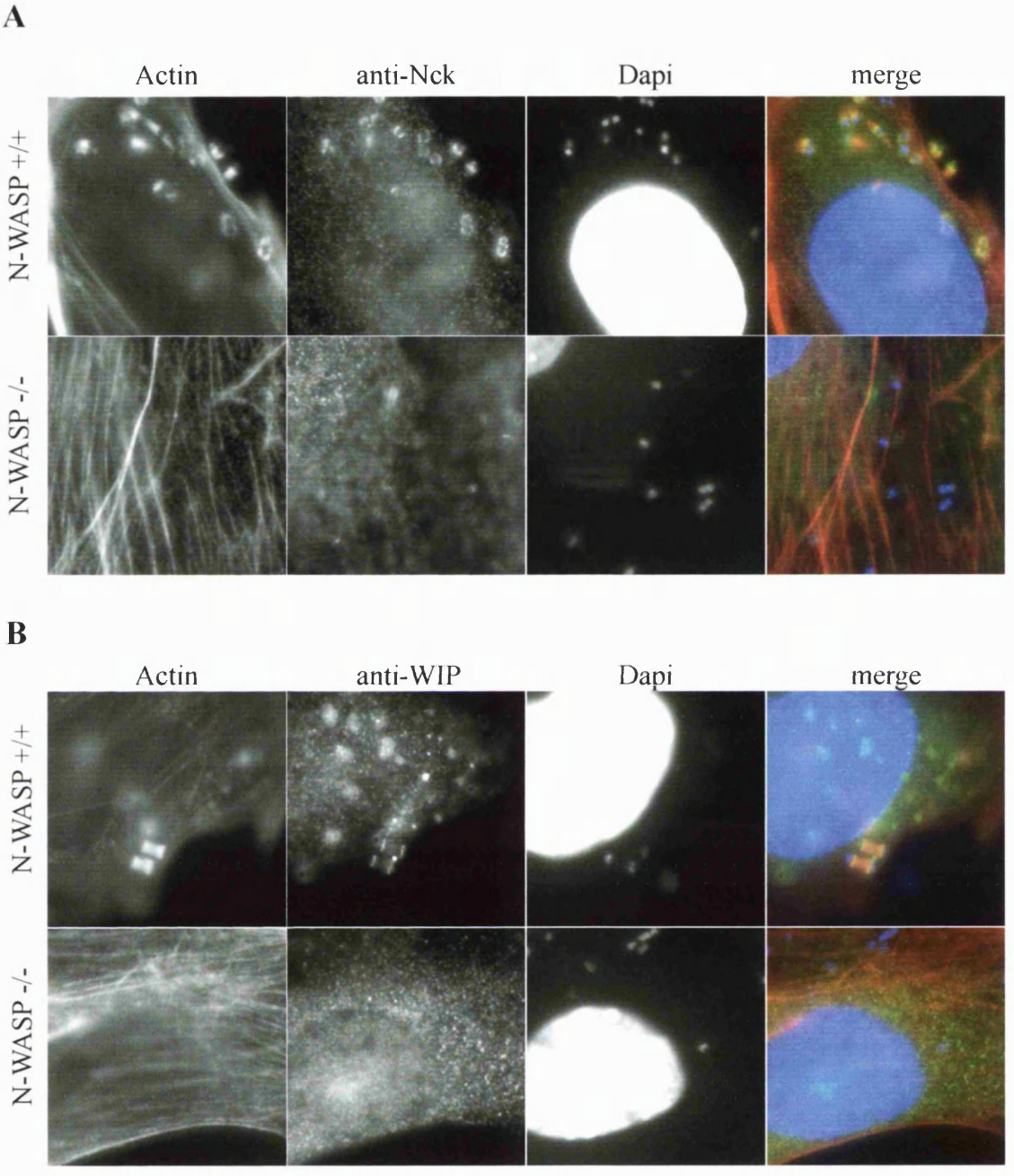


Figure 4.21. Nck and WIP are not recruited to EPEC in N-WASP -/- cells. Mouse embryonic fibroblasts (MEF) with (N-WASP+/+) and without N-WASP (N-WASP-/-) were infected with EPEC. **(A)** Endogenous Nck (green in merge) was detected at the tip of EPEC actin pedestals in N-WASP+/+ but was not associated with bacteria when N-WASP is absent. **(B)** Similarly WIP (green in merge) was only associated with the tips of EPEC actin pedestals in EPEC infected MEFs with N-WASP but not in MEFs without N-WASP. Bacteria were visualised by DAPI staining (blue) and filamentous actin was stained with phalloidin (red).

4.2.5 Is WIP recruitment to vaccinia or EPEC dependent on Nck?

Nck and WIP play a crucial role for EPEC pedestal and vaccinia actin tail formation (Frischknecht *et al.,* 1999b; Gruenheid *et al.,* 2001; Moreau *et al.,* 2000) (Figure 4.15, 4.16). Nck is complexed to WIP *in vivo* and yeast two hybrid studies demonstrate that the interaction site of Nck in WIP mapped to the proline rich region (Anton *et al.,* 1998). Since WIP lacking its proline rich region did not localise to EPEC and vaccinia (Figure 4.17, 4.19) I wanted to test whether the interaction of Nck and WIP is required for the recruitment of WIP to vaccinia and EPEC. Since Nck seemed to be a promising candidate protein I decided to map the binding site of Nck in WIP and investigate how mutations in that binding site would affect the localisation of WIP to EPEC and vaccinia.

Since the Nck/WIP interaction studies done by Anton *et al.,* 1998 were carried out using a partial WIP clone comprising WIP residues 321-503, I decided to do a more complete analysis. I expressed GFP tagged WIP constructs in HeLa cells and tested their capacity to interact with His-Nck produced in *E.coli.* Nck bound to GFP-WIP and to GFP-PolyPro$_{WIP}$ but not to constructs corresponding to the N- and C-terminus of WIP (Figure 4.22). These results are consistent with the hypothesis that Nck binds the proline rich domain of WIP and that this interaction might be important for WIP recruitment to EPEC and vaccinia virus. However, it is not clear if Nck is the only protein binding to the proline rich region of WIP or whether this interaction is direct. Therefore I wanted to determine the Nck binding site in WIP.

It has been shown that Nck interacts with WIP via its second SH3 domain (Anton *et al.,* 1998). Early studies indicated that SH3 binding motifs to date in all known examples are proline rich and characterised by a conserved PxxP motif, where P is proline and x is any amino acid (Ren *et al.,* 1993). From structural studies on SH3 domains bound to their respective ligands it is clear that the binding surface of SH3 domain is hydrophobic and consists of three shallow pockets defined by conserved aromatic residues (reviewed in (Kay *et al.,* 2000)). Two of three ligand pockets are occupied by two hydrophobic prolines, whereas the third one in most cases interacts with a basic residue distal to the core PxxP motif. For Nck domains the SH3 binding motif has been carefully mapped showing a strong preference for a serine residue downstream of the

core binding motif (PxxPxRxxS) (Zhao *et al.*, 2000). An alanine mutagenesis of PxxP motifs was not feasible since the proline rich domain of WIP (122-413) contains more than 20 putative Nck binding sites. Since SH3 domains interact with rather small epitopes of 8-12 amino acids (Ren *et al.*, 1993) I decided to perform an immobilised peptide array assay. A set of immobilised overlapping synthetic 15mer peptides covering the entire sequence of WIP was used to identify the WIP binding site for Nck. I incubated the membrane with recombinant His-Nck protein, purified from *E.coli*, and after several stringent washes detected an interaction by western blot analysis using an antibody raised against the His tag. In total I found five positive signals three of which did not fall in the proline rich domain of WIP. It was reassuring to find that the by far strongest signal corresponded to a previously predicted Nck binding motif in WIP (TPRLPQRNLSLSSST) (Figure 4.23A) (Zhao *et al.*, 2000). I confirmed the results from the immobilised peptide array assay with a second method using a peptide derived from WIP containing the identified Nck binding motif (DETPRLPQRNLSL). The peptide was chosen in such a way that the putative Nck binding motif lied in the centre of the peptide. This peptide coupled to a resin retained recombinant expressed His-Nck from a soluble *E.coli* extract very efficiently (Figure 4.23B). I decided to change P332 in the PxxP motif in order to confirm that WIP contains a classical Nck SH3 binding site. The mutant peptide (DETARLPQRNLSL) did not interact with soluble His-Nck (Figure 4.23B). This result showed that Nck interacts directly with a WIP derived peptide sequence via classical SH3 binding motif, which is consistent with the previously predicted Nck SH3 binding motif PxxPxRxxS (Zhao *et al.*, 2000).

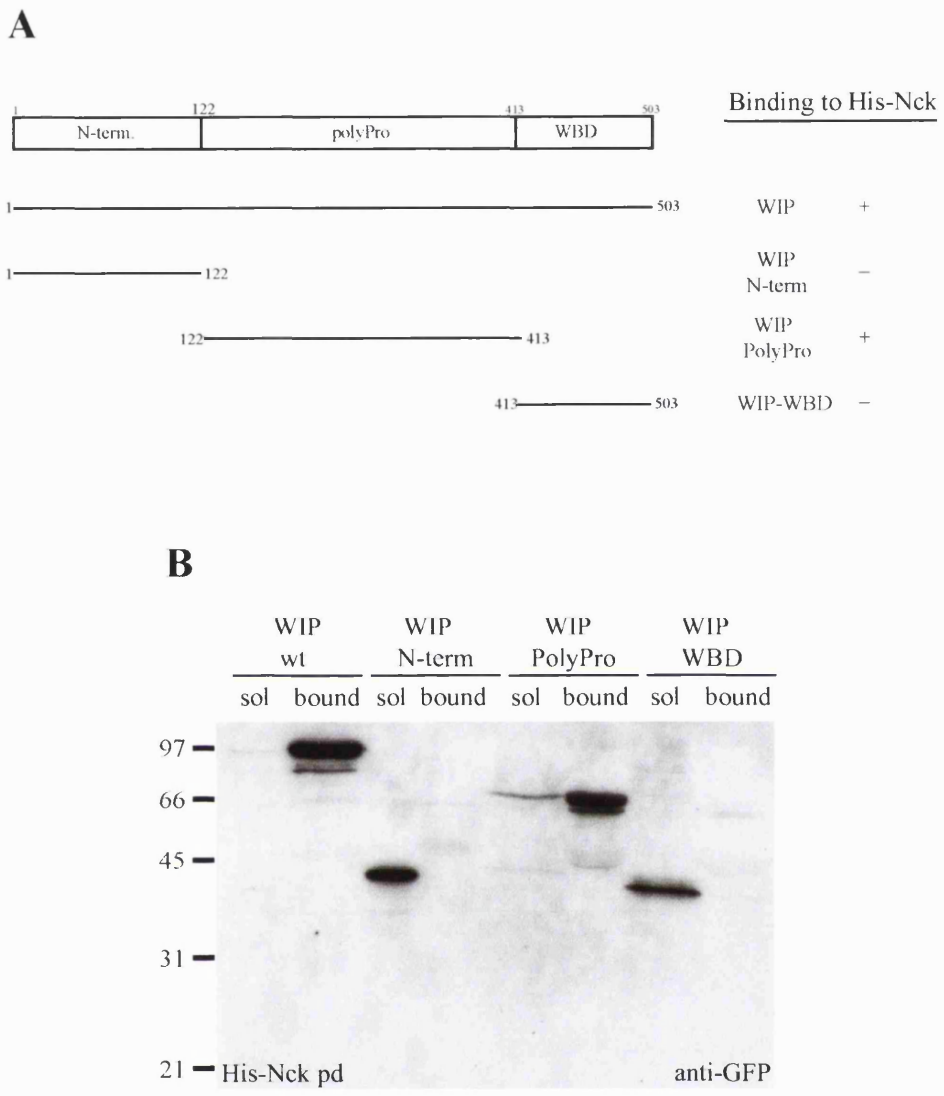
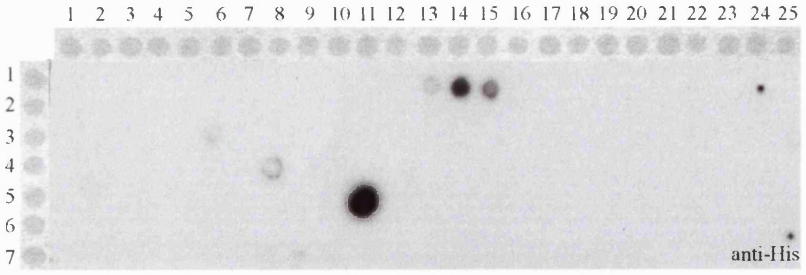


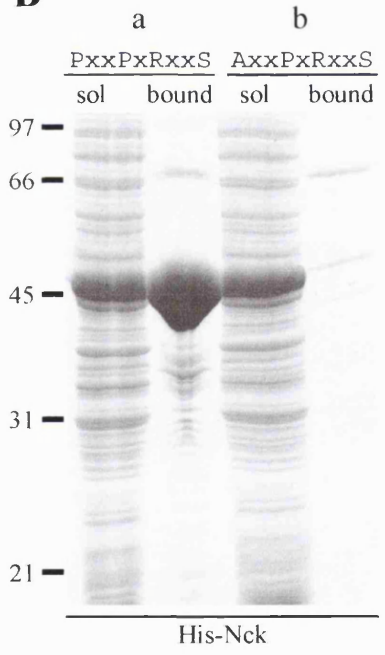
Figure 4.22. The proline rich region of WIP binds to Nck.
(A) Schematic diagram of WIP showing the constucts used to map the region of WIP that interacted with His-Nck. The numbers of the respective GFP-WIP constructs is and their ability to bind to His-Nck is indicated. **(B)** Indicated GFP tagged versions of WIP were expressed in HeLa cells and tested for their ability to bind to the recombinant expressed His-Nck. GFP-WIP constructs were detected using a GFP antibody. Full length WIP and the central proline rich domain interacted with His-Nck. The N-terminus and the C-terminal WASP binding domain of WIP (WIP-WBD) did not interact with His-Nck.

A



positive signal			
row	position	15mer WIP peptides	PXXP
1	13	$_{37}$LSDISKGKKLKKTVT$_{51}$	-
1	14	$_{40}$ISKGKKLKKTVTNDR$_{54}$	-
1	15	$_{43}$GKKLKKTVTNDRSAP$_{57}$	-
3	6	$_{166}$RNRMPPPRPDVGSKP$_{180}$	+
4	8	$_{247}$SNRPPLPPTPSRALD$_{261}$	+
5	11	$_{331}$TPRLPQRNLSLSSST$_{345}$	+

B



a $_{329}$DETPRLPQRNLSL$_{341}$

b $_{329}$DETARLPQRNLSL$_{341}$

Figure 4.23. Identification of the Nck binding site in WIP.
(A) Immobilised peptides covering the entire amino acid sequence of WIP were spotted on a nitrocellulose membrane. This membrane was incubated with purified His-Nck and subsequently detected with an anti His antibody. The WIP peptides corresponding to the positve signal are listed below the blot. Three out of six peptides, which showed a positive signal contain potential SH3 binding motifs (PXXP). The strongest signal corresponded to the predicted Nck SH3(2) binding motif with the consensus sequence PxxPxRxxS. **(B)** A peptide corresponding to the strongest Nck binding site in WIP and one in which the P332 is changed to alanine were coupled to a resin, which was subsequently used as bait for bacterially expressed His-Nck. The wild type peptide a strongly bound to His-Nck, whereas peptide b did not retain His-Nck from a soluble *E.coli* extract.

To confirm that the Nck binding motif in WIP is sufficient to confer an interaction with Nck, I performed pull down assays using peptides containing Nck binding motif in WIP. As a negative control I used the same peptide with P332 changed to alanine. I found that the wild type peptide bound endogenous Nck out of an HeLa cell extract whereas the peptide harbouring the P332A mutation did not (Figure 4.24A). This showed that the peptide encompassing the Nck binding site in WIP interacts with Nck and that the point mutation P332A was enough to disrupt this interaction. This confirmed the *in vitro* results showing that the WIP peptide/Nck interaction was mediated by a classical SH3 binding consensus motif.

Based on the intensity of the signals the results from the immobilised peptide array assay suggested that there might only be one Nck binding site in WIP. Thus I wanted to test whether full-length WIP mutants carrying a single point mutant in P332 would affect the ability of WIP to bind Nck *in vivo*. Using vaccinia virus to drive protein expression I tested His-WIP and His-WIPP332A for their capacity to interact with endogenous Nck. His-WIPP332A did bind to Nck slightly less efficiently as wild type WIP comparing the relative amounts of His-WIP bound to Nck with His-WIPP332A bound to Nck (Figure 4.24B). This result suggested that the Nck binding motif in WIP (DETPRLPQRNLSL) is involved in the WIP/Nck interaction *in vivo*. However, at this point I can not exclude the possibility that the WIP/Nck interaction is maintained via a second SH3 binding site of Nck in WIP. More likely a third protein provided an additional link, which binds Nck as well as WIP at the same time. However, using point mutations I could slightly weaken the Nck/WIP interaction *in vivo* and *in vitro*.

The task ahead was to determine whether the WIP/Nck binding motif is important to confer WIP localisation to vaccinia virus and EPEC. Therefore I expressed a GFP tagged WIP construct carrying a mutation in the Nck binding motif (P332A) and infected HeLa cells with vaccinia virus and EPEC. In addition to Nck, vaccinia virus also recruits Grb2, another SH2/SH3 containing adapter protein (Scaplehorn *et al.*, 2002). In order to avoid potential contribution of Grb2 to WIP recruitment I used a virus strain unable to recruit Grb2 (WR Y132F) to examine the localisation of GFP-WIP and GFP-WIPP332A (Scaplehorn *et al.*, 2002). The recruitment of GFP-

WIPP332A in WR Y132F and EPEC was unaffected when compared to wild type GFP-WIP (Figure 4.25). Thus weakening the WIP/Nck interaction does not interfere with its localisation to EPEC and vaccinia (see Discussion).

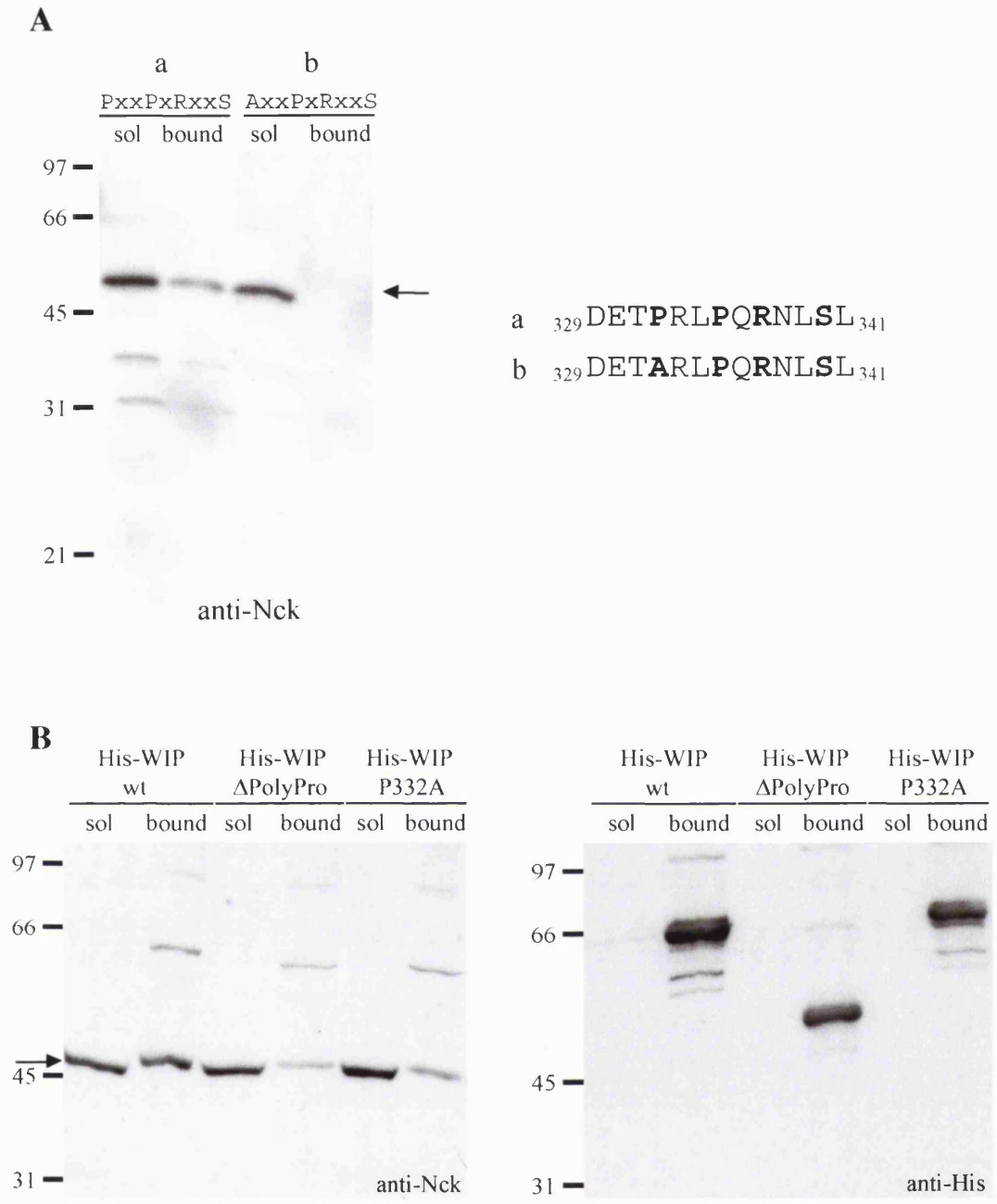


Figure 4.24. Conformation of the Nck binding site in WIP *in vivo*.
(A) Peptides a and b were coupled to resins, which were used as affinity matrixes for proteins present in HeLa cell extracts. The HeLa cell extracts were passed over the resins and resulting soluble (sol) and bound fractions subjected to western blot analysis. Endogenous Nck was detected in the bound fraction of peptide a but not peptide b, which carries a point mutation in the Nck binding site. **(B)** Indicated His-tagged WIP constructs were overexpressed in HeLa cells. A cell extract was passed over a nickel resin and soluble (sol) and bound fractions were analysed for the presence of Nck (left) and His tagged WIP constructs (right). Nck bound very strongly to His-WIP but to a weaker extent to His-WIPΔPolyPro and His-WIPP332A. The size of Nck is indicated by an arrow.

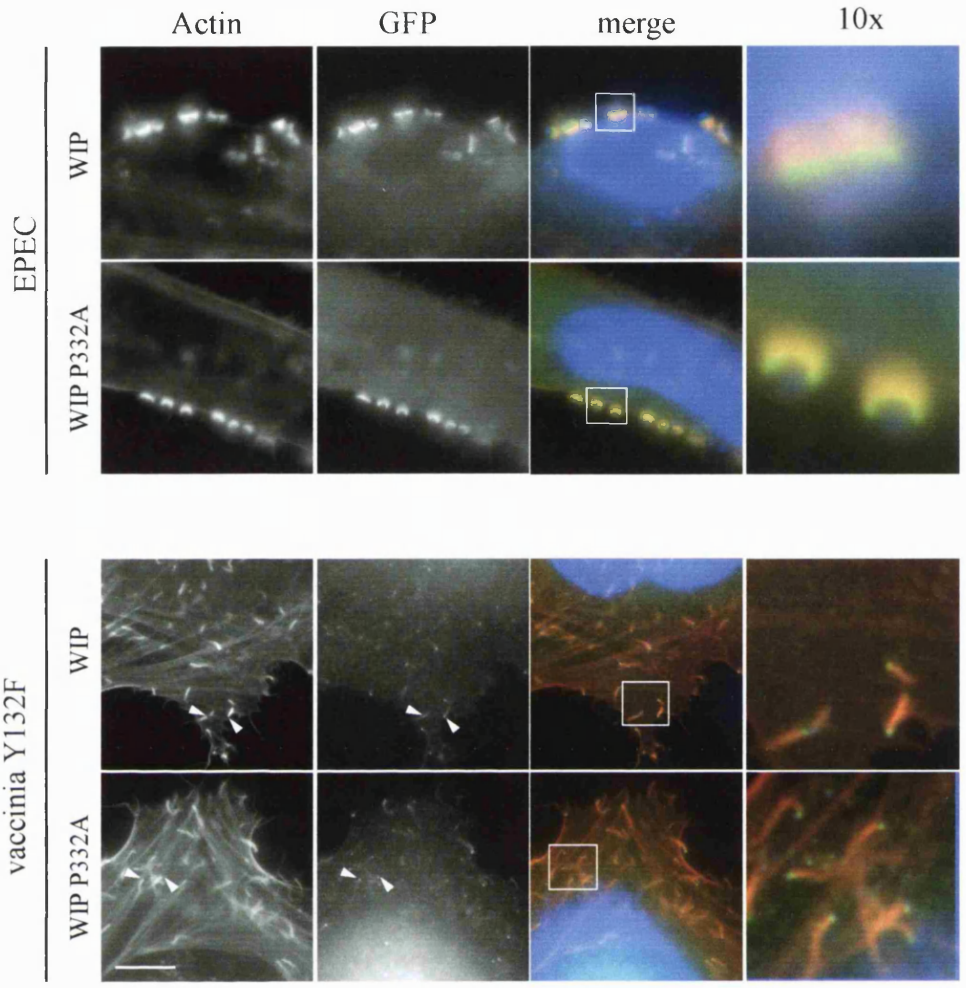


Figure 4.25. A point mutation in the Nck binding motif in WIP does not affect WIP localisation to EPEC and vaccinia virus.
(A) Immunofluorescence analysis showing that GFP-WIP and GFP-WIP P332A are recruited to the tip of EPEC pedestals.
(B) In vaccinia infected HeLa cells, GFP-WIP and GFP-WIP P332A were found at the tip of actin tails recruited to the surface of vaccinia virus. Filamentous actin was visualised with phalloidin (red), EPEC DNA was stained by DAPI (blue) and the GFP signal of the respective WIP construct is shown in green in the merged image. To show the GFP signal in relation to pathogens and their respective actin structure a 10x magnified merged image of the white box is shown to the right. Arrow heads point to the tip of actin tails and the localisation of the indicated GFP-WIP construct. Scale bar represents 10 µm.

4.3 Discussion

4.3.1 Which kinases do play a role for EPEC induced pedestal formation?

Tyrosine phosphorylation of A36R and Tir is essential for vaccinia actin tail formation and induction of EPEC actin pedestal (Frischknecht *et al.,* 1999b; Kenny, 1999). The sequence around the critical tyrosine phosphorylation sites in vaccinia and EPEC are highly identical. These sequences around Tyr 112 and Tyr 474 in vaccinia and EPEC respectively are predicted to be Src kinase substrates (Songyang and Cantley, 1995). Given vaccinia virus actin tail formation depends on Src kinases (Frischknecht *et al.,* 1999b) I investigated the possibility that Src kinases were involved in EPEC actin pedestal formation as well. Vaccinia virus induced actin tail formation is strongly inhibited by PP2 whereas EPEC pedestal formation was unaffected. Consistent with this EPEC still induces pedestals in cells lacking the three major ubiquitously expressed Src kinases Fyn, Yes and Src. These observations suggest that vaccinia and EPEC use different kinases to induce actin polymerisation and that Src kinases do not play a role in phosphorylating Tir. Although there were no apparent difference in the number of EPEC actin pedestals formed in PP2 versus PP3 treated cells, I can not rule out that Src kinases are redundant with other tyrosine kinases in the EPEC actin pedestal formation in SYF -/- cells. One way to find potential kinases, responsible for EPEC pedestal formation, is to simply look for recruitment of known tyrosine kinases and in order to filter potential candidates. Alternatively one can try to predict tyrosine kinases binding to proteins known to localise to EPEC induced pedestals. Goosney *et al.,* 2001 generated a list of host proteins recruited to EPEC pedestals. The authors provide information as to whether proteins localise at the tip or along the length of EPEC induced pedestals. Since Tir is an integral membrane protein being at the tip of the actin pedestal, one would expect that the kinase that phosphorylates Tir to be recruited to the tip of the pedestal as well. Selecting only the host proteins that localise to the tip of the pedestal one ends up with relatively few candidates (Cortactin, N-WASP, VASP, CD44 and CrkII). Furthermore the authors investigated whether these proteins are present at the tip of EHEC induced pedestals, which is formed independently of tyrosine phosphorylation. Discarding those proteins, which localise to EPEC and not to EHEC induced pedestals CrkII appears the only protein that is specifically recruited to the tips of EPEC but not to EHEC pedestals. CrkII belongs to a group of adaptor proteins that

are comprised of Src homology 2 (SH2) and Src homology 3 (SH3) domains, which interact with phosphotyrosine and proline-rich regions, respectively (Mayer *et al.*, 1988). The SH2 domain of Crk can bind to phosphotyrosine-containing proteins such as the focal adhesion components paxillin, p130 Crk-associate substrate (p130Cas) and receptors such as epidermal growth factor (EGF) and nerve growth factor (NGF) receptors (Hempstead *et al.*, 1994; Matsuda *et al.*, 1990). The N-terminal SH3 domain of Crk has been shown to bind many signalling molecules including the protein tyrosine kinase c-Abl and c-Jun N-terminal kinase (JNK) (Feller *et al.*, 1998; Girardin and Yaniv, 2001). C-Abl is a Src-like kinase with similar domain organisation and structural fold than Src kinases (Harrison, 2003). Furthermore the c-Abl minimal consensus substrate sequence (I/V/L-Y-X_{2-3}-P/F) matches with Src kinase substrate sequences found in Tir and A36R (Songyang and Cantley, 1995) (compare to Figure 4.1). Hence c-Abl might be a good candidate to be involved for the tyrosine phosphorylation of Tir and subsequent EPEC actin pedestal formation.

4.3.2 The role of Tir tyrosine phosphorylation

A recent report demonstrates that *Citrobacter rodentium* Tir is an essential virulence factor in a mouse model for EPEC infection (Deng *et al.*, 2003). *Citrobacter* Tir and EPEC Tir are functionally interchangeable and dependent on tyrosine phosphorylation to mediate actin polymerisation. EHEC Tir, which is not tyrosine phosphorylated can however substitute for *Citrobacter* Tir in the mouse model and form attaching/effacing A/E lesions (Deng *et al.*, 2003). This shows that in fact the tyrosine phosphorylation is not essential to cause A/E lesions (Deng *et al.*, 2003) *in vivo* and raises the general question what the function of tyrosine phosphorylation is.

Early results on EPEC induced pedestal formation suggested that tyrosine kinase inhibitors prevent the accumulation of phosphorylated proteins, but not actin, beneath the adherent EPEC (Rabinowitz *et al.*, 1996). This suggested that tyrosine phosphorylation was not required for EPEC pedestal formation (Rabinowitz *et al.*, 1996). However, what the authors interpreted, as EPEC pedestal was more unfocused actin accumulation than a characteristic horseshoe shaped actin pedestals. Unfocused accumulation of actin was also evident under some EPEC with Tir deletions as well as in Tir Tyr474Ser mutants, which can not form actin pedestals (Kenny, 1999; Kenny *et al.*, 1997). Thus the role of tyrosine phosphorylation of Tir could be to concentrate

initially polymerised actin into a horseshoe shaped pedestal thus forming a pedestal more efficiently.

Consistent with this suggestion, although EHEC pedestals are similar to EPEC pedestals their formation in tissue culture cells is much less efficient than those of EPEC (Cantey and Moseley, 1991). In addition there is no phosphotyrosine signal detected on EHEC induced actin pedestals (Campellone *et al.,* 2002). This is due to the fact that sequences surrounding Tir474 in EPEC Tir are missing in EHEC Tir (Campellone *et al.,* 2002). Thus EHEC pedestal formation is independent of tyrosine phosphorylation whereas tyrosine phosphorylation of EPEC Tir is essential to induced actin signalling cascades (Kenny, 1999). This suggests that EPEC have evolved a strategy to induce actin polymerisation more efficiently than EHEC, mimicking receptor tyrosine kinsae signalling cascades.

4.3.3 Does N-WASP get recruited by a GTPase to EPEC pedestals?

In many ways, EPEC pedestal formation has close parallels with vaccinia actin tail formation, being dependent on tyrosine phosphorylation of a pathogen derived membrane proteins as well as on Nck, N-WASP and the Arp2/3 complex (Frischknecht and Way, 2001). EPEC as well as vaccinia induced actin polymerisation events can be recapitulated by WASP as well as on N-WASP (Kalman *et al.,* 1999; Lommel *et al.,* 2001; Snapper *et al.,* 2001). Since WASP and N-WASP share nearly identical domain organisation, they are likely to be recruited via the same mechanism to EPEC and vaccinia virus.

Previous observations suggested that the CRIB domain of WASP is sufficient to localise WASP to EPEC, suggesting that Rho GTPases are important for EPEC actin pedestal formation (Kalman *et al.,* 1999). Despite the high degree of identity between the CRIB domain of WASP and N-WASP (60%), I found that the CRIB domain of N-WASP does not localise to pedestals. Furthermore it was demonstrated that an N-WASP constructs lacking the CRIB region (N-WASPΔCRIB) rescues EPEC pedestal formation in N-WASP deficient cells (Lommel *et al.,* 2001). The overexpression of dominant negative forms of Cdc42 and Rac as well as the Cdc42 interacting CRIB domain of N-WASP did not block EPEC pedestals (Ben-Ami *et al.,* 1998). Taken

together these observations support the idea that EPEC pedestal formation like vaccinia actin tail formation is independent of Rho GTPases. However at the moment I can not formerly rule out that the CRIB region of WASP has different targeting requirements than the CRIB region of N-WASP.

It was shown that the Rho family GTPase Chp interacts directly with WASP in its activated GTP bound form (Aronheim *et al.,* 1998). Since WASP is recruited to EPEC pedestals via its CRIB domain, Chp is considered as a potential candidate to mediate N-WASP recruitment to EPEC (Kalman *et al.,* 1999). However, EPEC pedestals are resistant to the Rho GTPase inhibitor ToxB and thus it was speculated Chp is insensitive to ToxB (Kalman *et al.,* 1999). Tox B is a large polypeptide (269 kDa) produced by many pathogenic bacteria and the causative agent of pseudomembranous colitis (Kelly and LaMont, 1998). The toxin monoglucosylates Rac and Cdc42 at threonine 35 (Just *et al.,* 1995). This modification inhibits their function preventing the binding to effector proteins, such as N-WASP. Chp is 52% identical to Cdc42. Comparing sequence alignment of Chp with Cdc42 one finds that the critical residue for glucosylation by ToxB is conserved between Cdc42 and Chp albeit at different positions in the protein (Aronheim *et al.,* 1998). The crucial threonine in Cdc 42 is at position 35 compared to position 62 in Chp. The different position in the protein could provide the basis for the speculation that Chp is ToxB resistant. However, there is no data that formally demonstrates that Chp is ToxB insensitive. Moreover currently there is no direct evidence that Chp is localised to EPEC pedestals or that overexpression of a dominant negative form of Chp blocks pedestal formation.

4.3.4 Is WIP essential for N-WASP recruitment to vaccinia and EPEC?

I found that the WH1 domain rather than the CRIB domain is required to recruit the ubiquitously expressed WASP family member N-WASP to EPEC In HeLa cells. The WH1 domain of N-WASP was efficiently targeted to EPEC pedestals. Furthermore the WH1 domain had a strong dominant negative effect due to its ability to block recruitment of endogenous N-WASP. However the WH1 domain on its own might behave completely different than the full length N-WASP molecule. Changing a single tryptophan 54 to alanine in the context of the full-length molecule abolished N-WASP recruitment to pedestals and vaccinia actin tails in infected HeLa cells. However, in N-

WASP deficient cells GFP-N-WASP (W54A) targeted and rescued EPEC pedestal as well as vaccinia actin tail formation, albeit at a lower frequency. This mutation is thought to disrupt the interaction with WIP (Moreau *et al.,* 2000).

This could suggest that WIP is not the only protein that interacts with the WH1 domain of N-WASP. WIP and WIRE belong to the same family of N-WASP binding proteins and both are able to induce filopodia formation co-operatively with N-WASP (Kato *et al.,* 2002). This suggests that their functions might as well be redundant in the actin-based motility of vaccinia and EPEC. However, I showed that both proteins share a highly conserved N-WASP binding motif. Therefore it is likely that WIP and WIRE compete for the same binding surface of the WH1 domain of N-WASP. Hence I think mutations in N-WASP affecting its interaction with WIP, like W54A, would also affect an interaction with WIRE.

Equally it is possible that the interaction of N-WASP with WIP is not completely disrupted by changing the Trp54 to Ala. It might just weaken the affinity of N-WASP to WIP so that as a consequence the mutated version of N-WASP can not compete for WIP binding with wild type N-WASP and thus is not recruited to EPEC or vaccinia in infected HeLa cells. The recently published NMR structure of the WH1 domain of N-WASP in complex with a peptide derived from WIP revealed, that the WIP/N-WASP interaction relies on an extended surface. Therefore a single point mutation in W54A in the context of the full-length molecule might not be enough to disrupt the WIP/N-WASP interaction completely. The only direct way to prove this would be to purify both proteins and test whether they can still bind to each other when W54 is mutated to alanine.

The fact that the WH1 domain was absolutely essential to rescue actin based motility of vaccinia and EPEC supports the theory that WIP or a WIP like molecule is required for N-WASP recruitment to vaccinia and EPEC. However, I can not rule out the possibility that another protein binds to the WH1 domain, which is responsible for N-WASP targeting to EPEC actin pedestals and vaccinia actin tails (see 5.1.1).

4.3.5 Are WIP/N-WASP and adaptor proteins recruited as a complex to EPEC and vaccinia?

Immunoprecipitation interaction studies have shown that 95% of WASP is complexed to WIP (Sasahara *et al.*, 2002). WIP/N-WASP complexes can also be immunoprecipitated from cell extract using antibodies against WIP (Martinez-Quiles *et al.*, 2001). Since the recruitment of WIP to vaccinia virus depends on N-WASP and visa versa it was suggested that WIP and N-WASP were recruited as a complex (Moreau *et al.*, 2000). However, both N-WASP and WIP require the WH1 and the WI-WBD respectively as well as their proline rich region in order to be localised at the tip of vaccinia induced actin tails. Since WIP and N-WASP are unlikely to interact via their respective proline rich regions (Zettl and Way, 2002) these observations support the idea that the N-WASP/WIP complex is binds an additional factor via their respective proline rich regions. Adaptor proteins such as Nck are essential for the actin tail formation of vaccinia as well as EPEC pedestal formation and are thus obvious candidates being responsible for the recruitment of the N-WASP/WIP complex. So far it was not possible to co-immunoprecipitate Nck in complex with N-WASP and WIP. However, recently published data showed that WASP forms a complex with WIP and the SH2/SH3 adapter CrkL *in vivo* suggesting the WIP/WASP complex can exist as a trimolecular complex with adaptor proteins (Sasahara *et al.*, 2002). Furthermore Sasahara *et al.*, 2002 showed that the WASP/WIP/CrkL complex was recruited to T-cell receptors upon stimulation with a ligand. TCR stimulation leads to tyrosine phosphorylation of the TCR and subsequently actin polymerisation. Vaccinia and EPEC cannot recruit WIP or Nck in the absence of N-WASP suggesting that they are recruited as a complex (Snapper *et al.*, 2001). EPEC and vaccinia virus actin polymerisation also depends on tyrosine phosphorylation and involves a similar set of proteins than TCR signalling. Therefore it is possible that N-WASP/ WIP and Nck are recruited to EPEC and vaccinia as a complex analogous to WASP/WIP/CrkL complex to TCR.

In contrast to vaccinia, the proline rich region of N-WASP was not essential for recruitment to EPEC actin pedestals. This suggested that although the proline region of N-WASP is necessary to bind Nck, N-WASP does not exploit this interaction to be recruited to EPEC induced actin pedestals. However, it turned out that N-WASP recruitment to EPEC was solely dependent on its WH1 domain, suggesting that WIP

might be important in recruitment of N-WASP to EPEC. The recruitment of WIP to vaccinia virus and to the tip of EPEC pedestals depended on the interaction with N-WASP as well as its proline rich domain. Since Nck can bind to the proline rich region of WIP (Anton *et al.,* 1998) and is essential for the vaccinia actin tail formation and EPEC pedestal formation (Frischknecht *et al.,* 1999b; Gruenheid *et al.,* 2001) it is a good candidate protein responsible for recruitment of WIP to the respective pathogen. Taken together this data is compatible with a model in which N-WASP/WIP and Nck are recruited to the surface of EPEC and vaccinia virus as a complex. However, the main difference between vaccinia and EPEC is that N-WASP does not need its proline rich region in order to be recruited to EPEC, which is not the case for vaccinia. Although the proline rich region of N-WASP is not absolutely required for EPEC pedestal formation, the rescue efficiencies with an N-WASP lacking the proline rich region were significantly lower in N-WASP deficient cells. This suggests that the proline rich region plays a secondary role in targeting N-WASP to EPEC and/or activating the Arp2/3 complex mediated N-WASP stimulated actin polymerisation on the surface of the bacterium. Thus although vaccinia virus and EPEC induced actin polymerisation appear to use the same signalling components the interaction necessary for recruitment of N-WASP appear to different. This suggests that vaccinia and EPEC might use different proteins to induce actin tails or pedestals.

4.3.6 Is the interaction of WIP and Nck important for recruitment of WIP to vaccinia and EPEC?

I have identified the Nck binding motif in WIP *in vitro* and confirmed that WIP/Nck interaction appears to be reduced. In addition WIP recruitment depended on its proline rich domain, which binds Nck (Anton *et al.,* 1998). Using point mutants in the Nck binding site of WIP in the context of the full-length molecule (WIP P332A), I could not detect an altered localisation of WIP P332A to EPEC or vaccinia virus. One could explain this observation invoking that Nck binds to N-WASP and WIP simultaneously using their respective proline rich regions as docking platforms. Thus a complex of N-WASP/WIP/Nck would still be maintained by the N-WASP/WIP interaction even though the Nck binding site is disrupted in WIP. N-WASP binding to Nck and WIP might therefore explain why WIP P332A is still recruited to vaccinia and EPEC. In order to demonstrate that sequences around P332 in WIP are the only Nck binding site

one could think of using N-WASP deficient fibroblast and test the interaction between WIP and Nck independently of N-WASP.

In addition to Nck, EPEC recruits other adaptor proteins like CrkII, Shc and Grb2 (Goosney *et al.,* 2001), which could potentially account for WIP recruitment to EPEC. Investigating the potential molecular interactions between these adaptor proteins and WIP might give more insights how WIP is recruited to EPEC. However, since WIP and N-WASP are important signalling components for EPEC I would not be surprised if EPEC developed a back up system consisting of several adaptor proteins that bind to WIP in order to ensure N-WASP recruitment.

Chapter 5

5 Discussion

5.1 What is the function of WIP?

A very powerful method to gain insights into the function of a protein is to generate loss of function mutants for the gene of interest. For WIP this has been done in *Saccharomyces cerevisiae* and mouse (Anton *et al.*, 2002; Vaduva *et al.*, 1997). In both cases the resulting phenotypes indicate that WIP functions to regulate the actin cytoskeleton although exactly how remains to established.

5.1.1 WIP deficiency point to function in cytoskeleton

In yeast the functional WIP homologue verprolin plays a critical role in processes such as polarity, cell growth, endocytosis and cytoskeletal organisation defects (Vaduva *et al.*, 1997). Defects in these processes, which occur in the absence of verprolin, can be rescued by human WIP (Vaduva *et al.*, 1999). This strongly suggests that WIP functions to regulate the actin cytoskeleton. Furthermore it has also became clear that WIP appears to work in connection with N-WASP (Las17/Bee1 in yeast), which has emerged as a central regulator of actin polymerisation via activating the Arp2/3 complex

Verprolin null and temperature sensitive mutants are inviable at 37 °C and have partially depolarised cortical actin patches. The loss of viability at 37 °C is attributed to a block in cytokinesis (Naqvi *et al.*, 2001). F-actin patches localise to the neck region in mitosis where they are thought to function in cytokinesis the process, which creates two daughter cells with a complete set of chromosomes and cytoplasmic organelles. (Winsor and Schiebel, 1997). During division cells display an invagination in their membrane, which grows inward due to its association with an underlying actomyosin-based structure called the contractile ring (Glotzer, 2001). Constriction of the contractile ring is driven by the pulling force generated by myosin II on actin filaments and is also often accompanied by the fusion of membrane vesicles with the invaginating

membrane (Glotzer, 2001). In *S. cerevisiae* however cell division does not absolutely require the function of actomyosin ring (Bi *et al.,* 1998; Lippincott and Li, 1998). Mutants that lack an actomyosin ring (*myo1*Δ) or have defects in constriction (*bni1*Δ) still divide (Bi *et al.,* 1998; Vallen *et al.,* 2000). A popular hypothesis is that a redundant pathway involving secretion to the bud neck mediates septation. This alternative pathway relies on Hof1p function (the homologue of the mammalian proline serine threonine phosphatase-interacting protein-PSTPIP), since loss of both the actomyosin ring and Hof1p function leads to inviability (Vallen *et al.,* 2000). Verprolin deletion leads to defects in both actomyosin constriction and Hof1p localisation, both of which can be rescued by verprolin (Thanabalu and Munn, 2001). The molecular mechanism by which verprolin influences these two pathways are not fully understood. However, since verprolin binds to Las17 (N-WASP homologue) and type 1 myosins, both of which stimulate the Arp2/3 complex (Evangelista *et al.,* 2000), it is possible that actin polymerisation plays a role in the constriction and the formation of the actin myosin ring. Alternatively verprolin might be an adaptor protein for myosins and Hof1p at the same time. Verprolin deficiency also causes defects in endocytosis and disturbs the polarised morphology of the actin cytoskeleton as a consequence of which *S.cerevisiae* can not grow anymore at 37°C. This suggests that the function of verpolin, which can be rescued by human WIP (Vaduva *et al.,* 1999), is essential for the normal formation of the actin cytoskeleton at multiple levels in baker's yeast.

Considering the pleiotropic effects of verprolin deficiency in *S.cerevisiae* and the broad expression pattern of WIP (heart, brain, spleen, lung, liver, placenta, kidney, pancreas, thymus, prostate, testis and ovaries) (Ramesh *et al.,* 1997; Vetterkind *et al.,* 2002), it is surprising that WIP deficient mice do not display any obvious differences from wild type littermates in growth, weight, or health (Anton *et al.,* 2002). While careful investigations of WIP -/- mice is required, to date the only defects are found in the immune system (Anton *et al.,* 2002). WIP-/- mice show normal B and T-lymphocyte development, but their T-cells fail to proliferate as judged by radioactive thymidine incorporation upon exposure to proliferative stimuli like CD3 (Anton *et al.,* 2002). CD3 binds to the T-cell receptor (TCR) and stimulates an increase in cellular F-actin and consequently the secretion of IL-2. Dynamic rearrangements of the actin cytoskeleton at the TCR are important for T- cells to establish a continuous contact with antigen presenting cells (APC), which is required for full T-cell activation (Penninger

and Crabtree, 1999). WIP-/- mice do not increase their F-actin content and are deficient in IL-2 secretion upon CD3 stimulation when compared with wild type mice. The phenotype reported in T-cells derived from WIP-/- cells is very similar to defects in T-cells lacking WASP (Derry *et al.,* 1994). T-cells lacking WASP also fail to secret IL-2 and do not increase their F-actin content (Derry *et al.,* 1994). Consistent with a role in actin polymerisation electron microscopy studies on B and T-cells from WIP -/- mice show that actin filaments fail to form after stimulation with CD3 (Anton *et al.,* 2002). B-cells from WIP-/- mice in contrast to T-cells from these mice do hyperproliferate and show normal B-cell receptor function. These differences suggest that the function of WIP is different in T- and B-cells but is always related to the actin cytoskeleton. Furthermore, given the similarity in phenotype in WIP-/- and WASP-/- mice in respect to the immune system suggest that both proteins function as unit as suggested for filopodia formation and pathogens (Martinez-Quiles *et al.,* 2001; Moreau *et al.,* 2000).

The observation that deletion of the functional *S.cerevisiae* homologue of WIP verprolin causes inviability and that there is no severe phenotype of WIP deficient mice also suggest that there are redundant homologues of WIP in mammals (e.g. WIRE, CR16). Consistent with this hypothesis the results presented in this thesis suggest that WIP might not be essential for the actin-based motility of vaccinia and EPEC (see 4.3.4). Data presented in this thesis show that the WH1 binding motif (ESRFYFHPISD) is conserved throughout the WIP family of N-WASP binding proteins (Figure 3.2). So far this family consists of four proteins, which have been reported to bind to N-WASP/WASP, the yeast homologue of WIP, verprolin, a brain specific homologue of WIP CR16, and another WIP like molecule called WIRE (also named WICH). All proteins interact with the WH1 domain of N-WASP consistent with the high degree of identity in the WASP binding region (see Figure 3.2, 3.5). WIP, CR16 and WIRE induce filopodia and localise at the tip of these thin actin projection (Aspenstrom, 2002; Ho *et al.,* 2001; Martinez-Quiles *et al.,* 2001). Apart form their function in filopodia formation not much is known about the different mammalian WIP homologues, although there are some suggestions that they might serve different functions in regard to N-WASP. For instance CR16 does not interfere with N-WASP stimulation of actin polymerisation by the Arp2/3 complex *in vitro* (Ho *et al.,* 2001). In contrast WIP inhibits Cdc42 stimulated N-WASP activation of the Arp2/3 complex (Martinez-Quiles *et al.,* 2001). Functional differences in mammalian WIP family

proteins would explain the overlapping expression patterns of WIP, WIRE and CR16. WIP and WIRE seem to be expressed in most tissues including brain, whereas CR16 appears to be brain specific (Ho *et al.,* 2001; Kato *et al.,* 2002; Vetterkind *et al.,* 2002). This raises the question whether they all bind to N-WASP when expressed in the same tissue. One possible insight might come from the observation that peptides derived from the same corresponding regions of WIP, CR16 and WIRE interacted with the WH1 domain with different affinities (Figure 3.5). Another way to regulate, which WIP-like molecule would bind to N-WASP at any one time, is to adjust the relative level of each protein in a given cell. Alternatively it might possible that the WH1 domain offers docking surfaces for more than one WIP -like molecule simultaneously, which is unlikely given their conserved N-WASP binding domain. More detailed biochemical binding studies will help to clarify whether WIP-like molecules interact with WASP/N-WASP using the same binding surfaces.

Since all WIP family proteins interact with the WH1 domain of N-WASP this suggest that the WIP (WIRE/CR16)/N-WASP complex are formed in the same way (Ho *et al.,* 2001; Kato *et al.,* 2002; Moreau *et al.,* 2000). The WH1 domain of N-WASP is absolutely required for vaccinia actin tail and EPEC pedestal formation (Figure 4.9, 4.11). The only known function of the WH1 domain of N-WASP is to bind WIP family protein, which is consistent with the idea that WIP plays an important role in the actin based motility of vaccina and EPEC. It will be interesting to see whether fibroblasts derived from WIP-/- mice are able to induce actin polymerisation upon vaccinia or EPEC infections. Given the fact that WIP belongs to a family of possibly partial redundant proteins it would not be surprising to find that other WIP like proteins might be involved in the actin based motility of vaccinia and EPEC.

5.1.2 WIP is an adaptor protein for N-WASP

Numerous reports have shown that N-WASP and WIP function in the same pathways as a functional unit. WIP and N-WASP induce filopodia (Martinez-Quiles *et al.,* 2001), are found to play a role in T-cell receptor activation (Sasahara *et al.,* 2002) and are functionally important for the actin based motility of vaccinia and EPEC (Moreau *et al.,* 2000)(Chapter 4). The existence of a N-WASP (WASP)/WIP complex *in vivo* has been demonstrated in NIH3T3 and T-cells (Martinez-Quiles *et al.,* 2001) (Sasahara *et al.,*

2002). The structure of the WH1 domain of N-WASP in complex with a WIP derived peptide showed that the WH1 domain of N-WASP adopts the same fold as EVH1 domains found in Ena, Mena and VASP family proteins (Fedorov *et al.,* 1999; Volkman *et al.,* 2002). Unlike EVH1 domains, which bind small peptide ligands, the WH1 domain interacts with WIP exploiting a combination between extended surface as well as short peptide ligands found in WIP. This mechanism offers a potential explanation for the observation that 95% of WASP is complexed to WIP in lymphocytes (Sasahara *et al.,* 2002) suggesting that WIP and N-WASP/WASP form a constitutive unit.

Since it is not entirely clear whether WASP/N-WASP is always complexed to WIP, it will be important to see if WASP localisation to T-cell receptors in WIP-/- T-cells is disrupted and visa versa. If WASP or WIP localisations are affected in WIP-/- and WASP-/- cells respectively, it would confirm that their localisation is dependent on each other suggesting that WIP and WASP are recruited as a complex to T-cell receptors. This situation would resemble the one found for WIP/N-WASP recruitment to vaccinia, EPEC and *Shigella* (Moreau *et al.,* 2000; Snapper *et al.,* 2001). However, the molecular mechanism how WIP is recruited to activated T-cell receptor and affects actin polymerisation awaits closer examination. Experiments done with vaccinia virus and *Shigella* show that WIP exploits its WASP binding domain in order to be recruited to these pathogens indicating its dependence to interact with N-WASP to be recruited to those pathogens (Moreau *et al.,* 2000).

The complex between N-WASP and WIP is maintained via a direct interaction between the WASP binding domain of WIP (WIP-WBD) and the WH1 domain of N-WASP (Moreau *et al.,* 2000; Zettl and Way, 2002). Overexpression of the WIP-WBD is therefore thought to sequester N-WASP and hence dominant negative for vaccinia actin tail formation (Moreau *et al.,* 2000). However overexpression of the WIP-WBD has no effect on *Shigella* actin tail formation. Furthermore WIP-WBD by itself is recruited to *Shigella* but not to vaccinia (Moreau *et al.,* 2000). To explain these observations it has been proposed that the N-WASP/WIP complex can be recruited in different orientations to vaccinia and *Shigella* (Figure 1.11, 1.16). In vaccinia WIP is suggested to be upstream of N-WASP and downstream of N-WASP in *Shigella* (Moreau *et al.,* 2000). It is possible to block recruitment of endogenous N-WASP by saturating its binding sites on vaccinia virus with a construct encompassing the WH1 and the CRIB domain

(Moreau *et al.,* 2000). In this situation vaccinia virus can not form actin tails due to the absence of N-WASP and consequently the Arp2/3 complex on the virus surface but is still able to recruit WIP supporting the idea that WIP is upstream of N-WASP in vaccinia (Moreau *et al.,* 2000). For *Shigella* it has been shown that N-WASP directly interacts with the *Shigella* surface protein IcsA (Egile *et al.,* 1999) and therefore overexpression of the WIP-WBD might be able to disrupt recruitment of endogenous WIP but not IcsA mediated N-WASP localisation to *Shigella*. Does *Shigella* recruit WIP just because it is always complexed to N-WASP or is it an indication that WIP might be more than a simple adaptor protein for N-WASP?

5.1.3 WIP keeps N-WASP inactive – two ways out of the inhibition.

WASP family proteins have emerged to be important activators of the Arp2/3 complex and therefore actin polymerisation (Higgs and Pollard, 1999). Thus the activity of WASP family proteins has to be tightly controlled in order to achieve spatial and temporal actin polymerisation. WASP/N-WASP can be activated by several stimuli (see 1.10.3.2). Consequently mechanisms may exist to ensure that WASP family proteins are kept in an inactive state to prevent random actin polymerisation.

In addition to intramolecular inhibitory interactions of N-WASP, WIP has been proposed to negatively regulate the activity of N-WASP *in vitro* (Martinez-Quiles *et al.,* 2001). Martinez-Quiles *et al.,* 2001 show that WIP reduces the stimulatory effect of Cdc42 on Arp2/3 complex mediated N-WASP stimulated actin polymerisation. Interestingly the binding sites of WIP and Cdc42 do not overlap. However, if N-WASP is stimulated by Cdc 42 and PIP_2 or PIP_2 alone, WIP is no longer able to inhibit N-WASP mediated Arp2/3 dependent actin polymerisation *in vitro* (Martinez-Quiles *et al.,* 2001). It will be interesting to see what other factors that activate N-WASP (tyrosine phosphorylation, SH2/SH3 adaptor proteins, IcsA) are able to overcome the inhibitory effect of WIP on N-WASP. This observation seems to be puzzling since WIP and N-WASP act as a unit to stimulate filopodia and colocalise at the tip of vaccinia actin tails and EPEC pedestals. It is therefore likely that WIP functions as an N-WASP inhibitor keeping N-WASP in its inactive closed conformation until N-WASP is stimulated by two different stimuli at the same time (see 1.10.3.3). Filopodia are enriched in Cdc42 and PIP_2 and thus provide local stimuli to fully activate N-WASP (Ward *et al.,* 2004). For pathogens such vaccinia the stimulation of N-WASP might be achieved via

SH2/SH3 adaptor proteins such as Nck and Grb2 (see 1.10.3.4). Since vaccina virus actin tail formation depends on tyrosine phosphorylation it is also possible that N-WASP is also tyrosine phosphorylated, which might contribute to its activation (see 1.10.3.5).

More recently a complex of WASP/WIP together with the SH2/SH3 containing adaptor protein CrkL has been found to be targeted to clustered T-cell receptors (TCR) upon antigen stimulation (Sasahara *et al.,* 2002). Sasahara *et al.,* 2002 propose a model in which WIP dissociates from WASP upon stimulation of T-cell receptors thereby allowing Cdc42 to activate N-WASP to stimulate actin polymerisation via the Arpp2/3 complex. Furthermore, the authors showed that WIP is phosphorylated by PKCθ. However, the evidence showing WIP phosphorylation is indirect based on the loss of reactivity of a peptide antibody, which was raised against a short unphosphorylated peptide (483-ESRSGSNRRERGGAP-496) containing a consensus PKC phosphorylation motif (RxxS/TxR). The loss of reactivity of this antibody was taken to indicate phosphorylation of WIP, which was reversed by treatment of cell lysates from antigen stimulated T-cells with phosphatase suggesting the loss of reactivity is due to phosphorylation (Sasahara *et al.,* 2002). Furthermore Sasahara *et al.,* 2002 showed that after antigen stimulation WIP dissociates from WASP. This effect is reversible by treatment with phosphatase and PKCθ inhibitors suggesting that PKCθ phosphorylation causes disruption of the WIP/WASP complex (Sasahara *et al.,* 2002). PKCθ is essential for F-actin accumulation as a result of antigen stimulation, which stabilises the T-cell-antigen presenting cell (APC) interface (Sasahara *et al.,* 2002). Inhibition of PKCθ thus blocked actin accumulation following antigen stimulation but had no affect on WIP or WASP localisation to T-cell-APC interface. This supports the idea that when WIP cannot be phosphorylated it remains bound to WASP. This interaction inhibits the stimulatory effect of WASP on the Arp2/3 complex mediated actin polymerisation when activated by Cdc42 (Sasahara *et al.,* 2002). Ser488 in WIP is the only residue that falls within a consensus motif of PKC phopshorylation. The authors show that mutating the Ser488 in the WIP-WBD to Asp, mimicking the negative charge introduced by phosphorylation, severely reduced the capacity of the WIP-WBD to bind directly to WASP (Sasahara *et al.,* 2002). Taken together their observations suggested that a complex of WIP and WASP can be disrupted by serine phosphorylation of WIP

(Sasahara *et al.*, 2002). However their work does not contain direct evidence that serine phosphorylation of WIP takes place *in vivo* or whether the dissociation of the WIP/WASP complex is directly related to WIP phosphorylation. Since Cdc42 localises to T-cell receptors (Bromley *et al.*, 2001) the authors suggested that upon WIP dissociation from WASP, the Rho GTPase would locally activate WASP, thereby stimulating the Arp2/3 complex. Consequently, the activation of the Arp2/3 complex would lead to localised actin polymerisation, which is ensures a tight contact of T-cells with antigen presenting cells.

Overall there are probably two mechanisms how WIP inhibition of N-WASP can be overcome. First WIP inhibition of N-WASP can be released by changing the conformation of N-WASP into an active form upon simultaneous binding of the right stimuli (e.g. PIP_2 and Cdc42). Secondly the N-WASP/WIP complex could be physically disrupted by serine phosphorylation of WIP or indirectly via a second protein that when serine phosphorylated disrupts the WIP/WASP complex. Therefore WIP is used as an adaptor, which at the same time negatively regulates the function of N-WASP rendering this protein complex into a signalling platform able to co-ordinate multiple signals ensuring regulated localised actin polymerisation.

5.2 N-WASP independent functions of WIP

If all WIP functions would be related to WASP/N-WASP and if WIP inhibits N-WASP stimulatory effect on Arp2/3 complex mediated actin polymerisation one would expect decreased levels of F-actin upon overexpression of WIP. However, ectopic expression of WIP increases the F-actin content of both B-cells and NIH 3T3 fibroblasts (Martinez-Quiles *et al.*, 2001; Ramesh *et al.*, 1997). The N-terminus of WIP (amino acids 1-127) has the capacity to interact with G-actin and F-actin (Martinez-Quiles *et al.*, 2001). The increase in levels of F-actin might therefore be due to WIP directly and could be due to increased filament formation, stabilisation of actin filaments or both. WIP has been found to prevent actin filaments from disassembly *in vitro* in a concentration dependent manner, which is mediated via its F-actin binding capacity (Martinez-Quiles *et al.*, 2001). Thus WIP may increase cellular F-actin content by virtue of its ability to stabilise F-actin. Consistent with this observation WIP colocalises with filopodia and stress fibres *in vivo* (Martinez-Quiles *et al.*, 2001; Vetterkind *et al.*, 2002). The effect of

WIP may be similar to that of several actin filament-binding proteins, such as tropomyosin, θ-actinin and caldesmon, that have been implicated in stabilising actin filaments against depolymerisation and severing (Cano *et al.,* 1992; Hitchcock-DeGregori *et al.,* 1988; Lehman *et al.,* 1997).

Alternatively WIP may activate actin polymerisation through pathways that include binding partners such as type I myosins, profilin, G-actin and cortactin (Evangelista *et al.,* 2000; Ramesh, *et al.,* 1997; Kinley *et al.,* 2003) (Martinez-Quiles *et al.,* 2001). In fact, it was reported that the yeast homologue of WIP, verprolin, binds to Myo3p and Myo5p which are critical for induction of actin polymerisation as these proteins are also able to stimulate the Arp2/3 complex (see 1.9).

Profilin promotes the formation of ATP-actin and binds to the barbed ends of actin filaments complexed with ATP-actin. Simultaneous binding of profilin and G-actin by WIP may promote actin polymerisation by increasing the local concentration of actin monomers at the surface of pathogens for instance. WIP contains two putative profilin binding motifs (APPPPP) identified in Mena and VASP (Gertler *et al.,* 1996). The second APPPP motif of WIP is conserved in the *S.cerivisiae* homologue of WIP verprolin (Vaduva *et al.,* 1999). The severe growth defects seen in the absence of verprolin can be rescued by human WIP but not by a WIP mutant carrying point mutations in the APPPP motif (APAPAPA). Although there is no biochemical evidence that these mutations affect the ability of WIP to interact with profilin interaction these results suggest that this motif is important for full activity of WIP *in vivo*.

Cortactin is a Src substrate, which binds to F-actin and is associated with sites of dynamic actin assembly at the leading edge of migrating cells (Kaksonen *et al.,* 2000b. Cortactin has been shown to be recruited to cortical actin in response to a wide range of stimuli including growth-factor stimulation, cellular stress and bacterial invasion of Shigella {Weed et al., 2000; Wu and Parsons, 1993). Consistent with a possible role of cortactin in cell motility cortactin overexpression results in increased cell motility, whereas expression of a phosphorylation deficient mutant impairs cell migration (Huang *et al.,* 1998; Patel *et al.,* 1998). More recent evidence provides evidence that cortactin interacts directly with the Arp2/3 complex through a 84-residue N-terminal

(NTA) domain(Weed *et al.,* 2000). Due to a conserved DDW or DEW motif in the NTA domain, which is also found in WASP-family proteins as well as ActA (Weed *et al.,* 2000). Because of this interaction cortactin is able to stimulate the actin nucleation activity of the Arp2/3 complex (Uruno *et al.,* 2001). However, the efficiency of cortactin stimulated Arp2/3 complex mediated actin polymerisation is much lower when compared to the WA domain of N-WASP (Uruno *et al.,* 2001). Direct visualisation of *in vitro* actin polymerisation by the Arp2/3 complex together with the WA domain and cortactin reveals that more actin filament branches are formed when both protein are combined and that these branches are more stable against debranching (Weaver *et al.,* 2001). These observations together suggested that cortactin modulates actin filament dynamics by activating the Arp2/3 complex as well as by stabilising branched filament network. Recent observation have extended our understanding of cortactin identifying WIP as a binding partner by yeast two hybrid (Kinley *et al.,* 2003). Cortactin binds to residues 110-170 of WIP via its SH3 domain *in vivo* as shown by immunoprecipitation using FLAG tagged WIP constructs binding to endogenous cortactin (Kinley *et al.,* 2003). Furthermore endogenous cortactin and overexpressed FLAG tagged WIP colocalise at the cell periphery depending on whether WIP contains the cortactin binding site (amino acids 110-170). Furthermore WIP increases the efficiency of cortactin-mediated activation of Arp2/3 complex *in vitro* albeit at lower levels than the isolated WA domain of N-WASP (Kinley *et al.,* 2003). Co-expression of cortactin and WIP stimulates membrane protrusions in a manner dependent on an intact cortactin SH3 domain, which is responsible for binding the proline rich region of WIP (residues 110-170). Thus it was suggested that the interaction between cortactin and WIP serves to regulate cortical actin dynamics and lamellipodia protrusion (Kinley *et al.,* 2003).

Given the observations that WIP overexpression lead to an increase in F-actin content, interacts with profilin and is able to stimulate actin polymerisation via cortactin it is possible that in contrast to its inhibitory effect on N-WASP, WIP stimulates actin polymerisation via the above mentioned mechanisms.

5.3 Does the WH1 domain of WASP/N-WASP only bind to WIP?

The importance of the WH1 domain was underlined by observations that >85% of the mutations causing Wiskott Aldrich Syndrome were found in the N-terminus of WASP

(Schindelhauer *et al.,* 1996). The WH1 domain shows weak sequence identity with Plekstrin homology regions, which bind to PIP_2 (Harlan *et al.,* 1994). Following this prediction N-WASP was initially shown to bind to PIP_2, which consequently lead the authors to speculate that the WH1 domain is responsible for membrane retention of N-WASP (Miki *et al.,* 1996). Since N-WASP transmits signals to the actin cytoskeleton N-WASP binding to membrane produced PIP_2 provided at the time a hypothesis of how the actin cytoskeleton is connected to signalling events at membranes. PIP_2 is indeed an activator of N-WASP stimulated Arp2/3 complex dependent actin polymerisation (Rohatgi *et al.,* 2001). In contrast to previous results (Miki *et al.,* 1996) direct binding assays of the WH1 domain to PIP_2 containing vesicles demonstrate that there is no interaction between PIP_2 and the WH1 (Volkman *et al.,* 2002). However, PIP_2 has been demonstrated to bind a basic region (N-WASP residues 178-196) adjacent to the WH1 domain (residues 1-129 used by Miki *et al.,* 1996) (Prehoda *et al.,* 2000).

Confusingly the WH1 domain of N-WASP was also predicted to have a calmodulin binding motif and calmodulin has been demonstrated to bind to N-WASP *in vivo* and *in vitro* (Miki *et al.,* 1996). Calmodulin is a small Ca^{2+} binding protein that binds to θ-helical ~25 amino acids long binding motifs which contain the consensus sequence IQxxxRGxxxR (Bahler and Rhoads, 2002). This suggested that N-WASP could also be linked to Ca^{2+} signalling. However, to date there is no functional data related with calmodulin binding motif in N-WASP. Recent observations start to shine some light on the importance of this motif. The calmodulin binding motif is a lysine rich θ-helix, which is also a hallmark of nuclear localisation signals (NLS) (Chook and Blobel, 2001). N-WASP is localised in the nucleus, which is dependent on its NLS containing WH1 domain (Vetterkind *et al.,* 2002). Deletion of the NLS (amino acids 126-145) of N-WASP, results in a predominantly cytoplasmic localisation of N-WASP (Suetsugu and Takenawa, 2003). Since WIP has not been detected in the nucleus it will be interesting to investigate how the N-WASP/WIP complex is disassembled and how the N-WASP shuttles back and forth in the nucleus. There is some evidence that N-WASP regulates transcription in the nucleus but a more extensive analysis is needed to achieve a complete picture (Suetsugu and Takenawa, 2003). One of the functions of WIP might be to keep N-WASP cytoplasmic to function in processes like filopodia formation (Vetterkind *et al.,* 2002).

Evidence supporting that the WH1 domain binds to more than one protein at the time comes from the *Shigella flexneri*, whose actin based motility is strictly dependent on N-WASP (Snapper *et al.*, 2001). It has been suggested that the bacterial surface protein IcsA activates N-WASP stimulated Arp2/3 dependent actin polymerisation similar to Cdc42 (Egile *et al.*, 1999). Surprisingly however the recruitment of N-WASP to *Shigella* is strictly dependent on the WH1 domain of N-WASP and not on the CRIB domain of N-WASP as one would predict for a mechanism in which the *Shigella* surface protein IcsA mimics Cdc42 (Suzuki *et al.*, 2002). It has been demonstrated that the interaction of N-WASP and IcsA is direct and dependent on the glycine rich region of IcsA (Egile *et al.*, 1999; Suzuki *et al.*, 1998). Furthermore pull down experiments showed that the WH1 domain of N-WASP is required to bind to IcsA (Suzuki *et al.*, 2002). Thus taken together it is likely that the WH1 domain binds directly to glycine rich region of IcsA. Since one can find WIP and N-WASP at the surface of *Shigella*, the IcsA-N-WASP interaction does not disrupt the N-WASP/WIP complex (Moreau *et al.*, 2000). The binding of IcsA to N-WASP might cause a conformational change within the WIP/N-WASP complex that releases the inhibitory interaction between the WA domain and the CRIB domain of N-WASP. WIP was suggested keep N-WASP in an inactive conformation (Martinez-Quiles *et al.*, 2001) and because WIP and IcsA bind to the WH1 domain, it is also possible that IcsA modulates the inhibitory effect WIP exerts on N-WASP. Hence, the WH1 domain binds to IcsA and WIP simultaneously. In summary the WH1 domain of N-WASP might not only be a protein-protein interaction module but also function as a platform that releases N-WASP inhibition.

The GST tagged N-terminus (residues 1-276), including the WH1 domain of N-WASP sediments with pure filamentous actin stabilising F-actin filaments (Egile *et al.*, 1999). Egile *et al.*, 1999, speculate that the F-actin binding site resides in the WH1 domain. However, the authors do not provide a good negative control such as deletion mutants in the N-terminal fragment. Since N-WASP is concentrated on one pole of *Shigella flexneri* the authors hypothesise that *in vivo* one function of the WH1 domain is to focus F-actin filaments to the surface of the *gram*-negative pathogen. It would be interesting to see where F-actin binds in N-WASP and whether mutations in this binding site have any effect on the actin based motility of *Shigella* or vaccinia.

5.3.1 The WH1 domain of N-WASP binds to tyrosine kinases.

Pull down experiments using GST tagged SH3 domains of tyrosine kinases or overexpression of tagged WASP and tyrosine kinase constructs showed that WASP binds several tyrosine kinases including Fyn, Tec, Itk and Btk (Banin *et al.,* 1996; Bunnell *et al.,* 1996; Cory *et al.,* 1996). In these studies WASP has been shown to bind to the SH3 domains of the respective kinases via its proline rich region. The SH3 domain of Src kinases can undergo intramolecular binding, which inhibits their kinase activity (Thomas and Brugge, 1997). According to this model WASP binding to the respective Src kinase could disrupt the SH3 mediated inhibition of the kinase. A recent study by Schulte *et al.,* 2003, shows that WASP binds to Src and to the closely related Abl kinase. Surprisingly the authors map the region responsible for WASP/Src (Abl) interaction to the WH1 domain of WASP. The SH3 domains of Src and Abl are dispensable to maintain this interaction as is the proline rich domain of WASP (Schulte and Sefton, 2003). *In vitro* kinase assays performed with immuno complexes of WASP/Src and WASP/Abl show severely reduced kinase activity compared to the kinases alone. The authors show that amino acids 81-93 of WASP are important in mediating the interaction of WASP with Src and Abl. Furthermore, a peptide derived of WASP, spanning residue 81-93 of WASP, has been shown to inhibit Src and Abl activity compared to a scrambled peptide using the amino acids 81-93 of WASP. At the moment it is however a matter of speculation whether WASP interacts with Src and or Abl *in vivo* as all the experiments done by Schulte *et al.,* 2003 are based on co-expression of tagged WASP and Src or Abl constructs. Therefore it remains to be shown that the reported interactions are of any relevance in a cellular context. Additionally, the mechanism how the WH1 domain inhibits Src is not clear. Furthermore it is not clear whether WIP and Src can bind simultaneously to the WH1 domain. However, given that Src and WIP binding sites in the WH1 domain only partially overlap (Volkman *et al.,* 2002) it is a possibility that Src binding to the WH1 domain also contributes to modulate the WIP/WASP interaction to activate WASP to stimulate the Arp2/3 complex.

5.4 Future aspects

Most of the studies performed in order to investigate the activity of N-WASP are done *in vitro* using Arp2/3 complex dependent actin polymerisation as a read out. *In vivo* read outs for N-WASP activation mostly rely on indirect observations such as inhibiting actin polymerisation or recruitment of the Arp2/3 complex by overexpressing the WA domain of N-WASP or constructs lacking the WA domain. WASP proteins exist in an autoinhibited conformation in which the WA domain is bound to the CRIB domain. Since the structure of the autoinhibited and the activated conformation is known (Kim *et al.,* 2000), it should be possible to design antibodies directed against an epitope that is masked by intramolecular interactions in the autoinhibited conformation and thus specifically recognising active WASP. A recent report shows that the Tec family kinase Itk is important to activate Cdc42 and WASP at the activated TCR (Labno *et al.,* 2003). The authors monitor the activation of WASP with an antibody raised against a buried epitope of WASP, which recognises the activated form of WASP. However, in this report no characterisation of this important reagent is provided.

It would be interesting to see whether conformational changes in WASP can also be monitored by fluorescence energy transfer (FRET). FRET is a distance dependent physical process by which energy is transferred from an excited fluorophore (the donor) to another fluorophore (the acceptor) if they have overlapping emission and excitation spectra respectively. FRET is a way of measuring molecular proximity because its efficiency decreases by the inverse sixth power of intermolecular separation (dos Remedios *et al.,* 1987). For WASP/N-WASP one could tag the N- and C-terminus with suitable fluorophores for FRET such as CFP (donor) and YFP (acceptor) for instance. Theoretical one would should be able to measure FRET in the inactive closed conformation reflecting the close proximity of the N- and C-terminus. Activation of WASP/N-WASP would lead to conformational changes separating the WA domain from the CRIB region thereby increasing the distance between the two fluorophores attached to the ends of WASP. Since FRET only occurs when the two fluorophores are in close proximity (typically 3-6nm), FRET would be lost or decreased when WASP/N-WASP is activated. A recent report showed that an N-terminally YFP tagged and C-terminally CFP tagged N-WASP construct upon stimulation by PIP_2 and Cdc42 in cell extracts displayed synergistically decreased FRET efficiency as shown by measuring

emitted light intensities of CFP and YFP (Ward *et al.*, 2004). These results reflect the activity changes of N-WASP in Arp2/3 complex dependent actin polymerisation *in vitro*. Furthermore photobleaching the acceptor fluorophore (YFP) increased the intensity of the donor (CFP) in cells suggesting that the N-terminus and the C-terminus were in close proximity to each other and FRET occurred reflecting the inactivated state of N-WASP. Upon expression of Cdc42 FRET efficiency is significantly lower suggesting that N-WASP was activated by Cdc42 *in vivo* (Ward *et al.*, 2004). Monitoring both YFP and CFP at the same time in cells stimulated with EGF, which is known to activate cytoskeletal rearrangements in an N-WASP dependent manner (Miki *et al.*, 1996), showed that upon EGF stimulation CFP emission increased indicating a loss of FRET and N-WASP activation (Ward *et al.*, 2004). The same technique was applied to filopodia stimulated by EGF where CFP/YFP ratios are found to be significantly higher than in the cytoplasm suggesting that N-WASP is activated in filopodia (Miki *et al.*, 1996).

Also the generation of an *in vivo* sensor for N-WASP activation will an important tool to study WASP/N-WASP proteins in the context of pathogens as well. One would predict that a FRET sensor as described above would lose FRET efficiency when localised to vaccinia virus, *Shigella* or EPEC induced actin structures. Furthermore, since the actin based motility of these pathogens is dependent on several proteins including adaptor proteins such as Nck and the activity of Src kinases for vaccinia it would be of great interest to develop a temporal spatial picture of *in vivo* interactions using FRET. Tagging N-WASP and WIP or Nck and N-WASP we could start understand whether these proteins are complexed all the time or whether complex formation coincides with actin polymerisation.

Since we are beginning to understand the structural mechanisms of N-WASP activation and inhibition it will be interesting to see how the wide range of activators and inhibitors influence the structure of WASP proteins. WASP proteins are mostly complexed to WIP, which appears to inhibit WASP and thus it would be very interesting to see how WIP achieves this inhibition. Clearly a structure of the WASP/WIP complex will give valuable insights as to how WIP manages to interfere with N-WASP function. Since WIP binds *in trans* to the WH1 domain, it might reveal yet another mechanism as to how to keep WASP proteins inactivated.

Furthermore, very little is known about the function of WIP family proteins such WIRE and CR16. Based on current knowledge it seems that all WIP like proteins act in conjunction with N-WASP/WASP however, it is not clear whether these proteins have independent functions or whether their effect on WASP/N-WASP is the same. Clearly pathogens such vaccinia, *Shigella* and EPEC will give important insights into the molecular details of their function. Also the generation of WIRE and CR16 knock mice and a combination of WIP/WIRE/CR16 deficiencies are of particular interest to determine the full spectrum of WIP like molecules in mammals. Cell lines derived from these mice will be instrumental to address the question whether WIP like molecules are essential for the actin based motility of pathogens.

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