Effects of invasin and YopH of *Yersinia pseudotuberculosis* on host cell signaling

by

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Front cover: The GD25 fibroblast-like cell line derive from β1-integrin deficient stem cells that were stably transfected with β1B-integrins (GD25β1B). These cells were spread on 10 µg/ml of the high-affinity ligand of β1-integrin, invasin, for 3 h. The cell membrane (red), F-actin (green) or phosphotyrosine (blue) were detected by immunofluorescence staining with WGA, phalloidin or antibodies to phosphotyrosine (PPY) followed by secondary labeling with AMCA-conjugated donkey-anti-rabbit antibodies. The images were captured by immunofluorescence microscopy (Zeiss axioskope 50) and a CCD camera (ORCA, Hamamatsu) and processed using Adobe software (Adobe).

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# Table of Contents

**ABSTRACT** ................................................................................................................................. 5
**SAMMANFATTNING PÅ SVENSKA** .......................................................................................... 6
**PAPERS IN THIS THESIS** ...................................................................................................... 8
**ABBREVIATIONS** ..................................................................................................................... 9
**PROTEIN-PROTEIN INTERACTION MODULES** ..................................................................... 10

## INTRODUCTION

The eukaryotic cell .................................................................................................................. 11

*Integrins* ..................................................................................................................................... 12
  - The integrin structure, activation and ligand binding ......................................................... 13
  - The integrin cytoplasmic tail ............................................................................................. 13
  - β1-integrins ...................................................................................................................... 14

*Cell-matrix adhesions* ........................................................................................................... 15

*Cell-matrix adhesion proteins* ............................................................................................. 17
  - Non-receptor protein tyrosine kinases ............................................................................. 17
    - Src family kinases ....................................................................................................... 17
    - FAK ............................................................................................................................ 17
    - Etk/Bmx ...................................................................................................................... 18
    - Abl .............................................................................................................................. 18
  - Adaptor and docking proteins .......................................................................................... 18
    - p130Cas ...................................................................................................................... 18
    - Crk .............................................................................................................................. 19
    - Nck .............................................................................................................................. 19
    - ADAP (FYB/SLAP-130) .............................................................................................. 19
    - SKAP-55 ..................................................................................................................... 20
    - Talin and PI(4,5)P2 ...................................................................................................... 20
    - Vinculin ...................................................................................................................... 21
    - Paxillin-α ................................................................................................................... 21

*The Cytoskeleton* ...................................................................................................................... 21
  - Actin ................................................................................................................................. 21
    - Actin binding proteins ................................................................................................. 22
    - Actin-based motors ...................................................................................................... 23
    - Actin polymerization ................................................................................................. 23
      - WASP ..................................................................................................................... 23
      - WAVEs ................................................................................................................... 24
      - Ena/VASP ............................................................................................................... 24
  - Rho GTPases ..................................................................................................................... 25
    - Rac and lamellipodia ................................................................................................. 25
    - Cdc42 and filopodia ................................................................................................. 26
    - RhoA and stress fibers .............................................................................................. 26
    - The Rac-GEF Dock180 .............................................................................................. 27

*Cell spreading* ......................................................................................................................... 27

*Cell migration* .......................................................................................................................... 27
  - “Polarized migration” ..................................................................................................... 27
  - Round morphology migration ...................................................................................... 28
### Phagocytosis

Yersinia .................................................................................................................................................. 29

### Pathogenesis of Yersinia infections

- Plague ................................................................................................................................................. 30
- Yersinosis ............................................................................................................................................ 30

### Bacterial adhesion to the host

- YadA .................................................................................................................................................... 31
- Invasin .................................................................................................................................................. 31
- pH6 Antigen ......................................................................................................................................... 32
- Ail ....................................................................................................................................................... 32

### Yersinia weaponry and administration of these

- The virulence plasmids of human pathogenic Yersinia ....................................................................... 32
- The type III secretion system .............................................................................................................. 33
- Translocation ...................................................................................................................................... 33
- Regulation of secretion and translocation ........................................................................................... 34
- Chaperones ......................................................................................................................................... 34

### Virulence effectors

- YopH .................................................................................................................................................. 35
- YopE .................................................................................................................................................. 35
- YopT .................................................................................................................................................. 36
- YpkA .................................................................................................................................................. 36
- YopM .................................................................................................................................................. 37
- YopJ .................................................................................................................................................. 37

### AIM

AIM .......................................................................................................................................................... 38

### RESULTS AND DISCUSSION

- What are the targets for YopH in macrophages (paper I) ................................................................. 39
- Which parts of the cytoplasmic tail of β1-integrins are important for uptake of Yersinia and spreading on invasin (paper II) .................................................................................................................. 40
- Which proteins are important for filopodia formation (paper II and III) ........................................ 41
- The role of myosin X (paper IV) .......................................................................................................... 42
- Is VASP involved in the formation of filopodia .................................................................................. 42
- Mechanism behind invasin-induced filopodia formation in GD25β1B cells .................................. 44
- Upstream of p130Cas ......................................................................................................................... 45

### CONCLUSIONS

CONCLUSIONS ...................................................................................................................................... 48

### ACKNOWLEDGEMENTS

ACKNOWLEDGEMENTS ...................................................................................................................... 49

### REFERENCES

REFERENCES .......................................................................................................................................... 50
Abstract

Integrins are a large family of membrane-spanning heterodimeric (αβ) receptors that bind to ligands on other cells or to extracellular matrix (ECM) proteins. These receptors mediate bidirectional signaling over the cell membrane to induce signaling cascades mediating functions as cell adhesion, spreading and migration. This signaling takes place at cell-matrix adhesions, which are sites where clustered and ligand-bound integrins connect to and mediate stabilization of the actin cytoskeleton, and induce signaling cascades. Integrins have a short cytoplasmic tail that is crucial for the bidirectional signaling, and the β1-integrin subunit exists in five splice variants only differing in the membrane-distal part of the cytoplasmic tail. This region of the almost ubiquitously expressed β1-integrin, β1A, contains two protein tyrosine motifs (NPXYs) interspaced with a threonine-rich region, while this region of the β1B splice variant is completely different and lacks known motifs. In contrast to the β1A-integrin, the β1B variant cannot mediate cell-matrix adhesion formation following binding to ECM ligands.

The enteropathogenic bacterium Yersinia pseudotuberculosis binds to β1-integrins on the host cell with invasin, and this stimulates uptake of the bacterium. However, upon binding to the host cell, pathogenic Yersinia strains inject virulence effectors that block uptake. One effector responsible for the blocking is a tyrosine phosphatase, YopH. We identified the targets for this effector in the macrophage-like cell line J774A.1, which represent a professional phagocyte and thus is the likely target cell for the antiphagocytic effect of Yersinia. Two YopH target proteins were p130Cas and ADAP, of which the latter interestingly is an adapter protein specifically expressed in hematopoietic cells. ADAP has previously been implicated to participate in Fc-receptor-mediated phagocytosis and in communication between T-cell receptors and integrins.

We also studied the importance of the cytoplasmic tail of β1-integrin for uptake of Yersinia. The GD25 cell line, which is a fibroblast-like cell line that lacks endogenous β1-integrins, was used together with GD25 cells transfected with β1B, β1A or cytoplasmic tail mutants of β1A. These studies revealed that β1B-integrins could bind to invasin but not mediate uptake of Yersinia, while β1A both bound to invasin and mediated uptake. The first NPXY motif (unphosphorylated) and the double-threonines of the unique part of β1A were important for the ability of integrin to mediate uptake of Yersinia. These studies lead to the interesting finding that, when these cells were allowed to spread on invasin, those that expressed β1A spread as normal fibroblasts while for β1B-integrin-expressing cells, only finger-like protrusions of filopodia were formed. This provided us with a tool to study formation of filopodia without interference of the tightly linked process of lamellipodia formation. Initially, proteins that localized to the tip complex of these filopodia were identified. These were talin, VASP and interestingly the p130Cas-Crk-DOCK180 scaffold, while FAK, paxillin and vinculin were absent. In addition, VASP, p130Cas and Crk were shown to be important for the filopodia formation in GD25β1B. Further, the role of the actin motor myosin X, which previously has been implicated in formation of filopodia, was studied in the GD25β1B cells and it was shown that myosin X not was important for filopodia formation, but that it recruited FAK and vinculin to the tip complexes of filopodia.
Sammanfattning på Svenska

**Effekter av proteinerna Invasin och YopH från bakterien *Yersinia pseudotuberculosis* på värdcellen**

Celler binder till proteiner som finns i omgivningen (s.k. extracellulärt matrix, ECM) med hjälp av en receptor ("mottagare"), integrin (består av två hopsittande proteiner, α och β), i en process som kallas adhesion (Figur P1, P2). Detta leder till att integrinerna klunpar ihop sig och ändrar form så att signalproteiner i cellen kan binda och aktiveras av integrinerna samt vidarebefordra signaler som är viktiga för cellens överlevnad och delning. Det är många proteiner som binder till eller aktiveras av integriner, vilket gör att det bildas en förtätning av proteiner nära integrinerna i en struktur som kallas för cell-matrix adhesioner. Integriner aktiverar också cellen att sprida ut sig över matrixen (Figur P2), och tillsammans med signaler från andra receptorer stimulerar cellen att röra på sig, migrera. Förståelsen för cell adhesion, spridning och migration är viktiga eftersom dessa processer är centraala under fosterutveckling, för försvar mot mikroorganismer och för sårhälsa, men också för spridningen av cancer (bildandet av metastaser).

För att celler ska kunna sprida ut sig och migrera använder de sig av ett flexibelt skelett som består av olika sorts "proteintrådar" uppbyggda av små runda proteiner, G-aktin. Dessa trådar kan snabbt byggas ihop med hjälp av andra proteiner som aktiveras när integriner binder till ECM, vilket leder till bildande av två olika strukturer uppbyggda med aktintrådar, filopodier och lamellipodier, som trycker ut cellmembranet och därmed sprider ut cellen över en större yta (Figur P2). Filopodier är tunna fingerliknande utskott från cellen och består av flera parallella aktinkablar som har små bindningspunkter till integriner längst ut i tippen. Lamellipodier är breda utåtskjutande delar av cellen där de förgrenade aktintrådarna bildar ett brett nätverk med inkorporering av G-aktin i den främre delen och nedbrytning av aktintrådarna i den bakre delen.


I den första artikeln studerade vi vilka målproteiner ett av gifterna, YopH, har i makrofager, vilket är en sorts vit blodkropp som är proffs på att fagocytera bakterier. Tigardare studier i vår forskargrupp har visat att när *Yersinia* injicerar YopH i cellen förstör cell-matrix adhesionerna genom att YopH inaktiverar två proteiner som är viktiga i uppbyggnaden av dessa adhesioner, FAK och p130Cas, och detta leder till att cellen inte kan äta upp bakterien. Om *Yersinia* däremot saknar YopH, kommer bakterien att ätas upp (figur P3 t.h.). Dessa tidigare studier gjordes i en "amatör"-cell linje vad gäller fagocytos och eftersom...
olika celltyper har olika funktioner och också uttrycker olika proteiner kan en och samma process ske på olika sätt i olika celltyper. Vi visade att YopH förstör cell-matrix adhesioner även i makrofager och att YopH inaktiverar p130Cas och ADAP i makrofager för att åstadkomma denna effekt (Figur P3, bild t.v.). p130Cas är ett protein som aktiveras av integriner och har visats vara ett nyckel-protein för att inducera cell migration, vilket gör den till ett strategiskt mål för YopH och antifagocytos. ADAP är ett protein som bara uttrycks i vita blodkroppar men funktionen av proteinet i makrofager är i stort sett okänt i dagsläget. Däremot är ADAP inblandat i samspelet mellan integriner, aktin och ev. i fagocytos av bakterier som känts igen av antikroppar (binder och tas upp via en annan receptor än integrin), vilket indikerar att också ADAP är ett strategiskt mål för YopH.

I de följande studierna fokuserade vi på β1-integrinens roll i bindning och upptag av *Yersinia*. Integriner har en stor domän utanför cellen som bl.a. binder till ligand, en del som spänner cellmembranen och en kort cytoplasmatic del (Figur P4). Det finns flera olika varianter av β1-integriner, där den normala varianten, som uttrycks i nästan alla sorters celler, kallas β1A medan en variant som är betydligt ovanligare, och endast uttrycks i ett fåtal celler, kallas β1B. β1A har en längre cytoplasmatic del än β1B och kan också binda till fler proteiner i cell-matrix adhesioner med olika protein-bindande domäner som β1B saknar (Figur P4). Vi såg att både β1A och β1B kunde binda till *Yersinia* men det var endast β1A som kunde stimulera cellen att ta upp *Yersinia*, vilket visar att den del av β1A som β1B saknar är viktig för upptag. Dessutom visade vi att två av dessa protein-bindande domäner som β1A har var viktiga för upptag eftersom mutationer i dessa gjorde att cellen inte kunde ta upp bakterien.

Papers in this thesis

This thesis is based on the following articles referred to in the text by their roman numerals (I-IV)

**Paper I**

**Paper II**

**Paper III**
Gustavsson A, Yuan M and Fällman M. **Temporal dissection of β1-integrin signaling indicates a role for p130Cas in filopodia formation.** Submitted manuscript.

**Paper IV**
Gustavsson A and Fällman M. **Myosin X recruits FAK and vinculin to the tip complexes of filopodia.** Manuscript.

* Fyn-binding protein (FYB) is an old name for this protein and the new name ADAP is used in this thesis.
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>ADAP</td>
<td>adhesion and degranulation promoting adapter protein</td>
</tr>
<tr>
<td>ail</td>
<td>accessory invasion locus</td>
</tr>
<tr>
<td>Btk</td>
<td>Bruton’s tyrosine kinase</td>
</tr>
<tr>
<td>CR</td>
<td>complement receptor</td>
</tr>
<tr>
<td>CRIB</td>
<td>Cdc42 and Rac interactive binding domain</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>Etk</td>
<td>epithelial and endothelial tyrosine kinase</td>
</tr>
<tr>
<td>F-actin</td>
<td>filamentous actin</td>
</tr>
<tr>
<td>FAT</td>
<td>focal adhesion targeting</td>
</tr>
<tr>
<td>FcR</td>
<td>Fc receptor</td>
</tr>
<tr>
<td>G-actin</td>
<td>globular actin</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase activating protein</td>
</tr>
<tr>
<td>GDI</td>
<td>guanine nucleotide dissociation inhibitor factor</td>
</tr>
<tr>
<td>GEF</td>
<td>guanine nucleotide exchange factor</td>
</tr>
<tr>
<td>ICAP-1</td>
<td>integrin cytoplasmic domain-associated protein</td>
</tr>
<tr>
<td>ILK</td>
<td>integrin linked kinase</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>LCR</td>
<td>low calcium response</td>
</tr>
<tr>
<td>LIM</td>
<td>Lin-11, Isl-1, Mec-3</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MLCK</td>
<td>myosin light chain kinase</td>
</tr>
<tr>
<td>MYM</td>
<td>multiple yop mutant strain</td>
</tr>
<tr>
<td>NRPTK</td>
<td>non-receptor protein tyrosine kinase</td>
</tr>
<tr>
<td>pH6Ag</td>
<td>pH6 antigen</td>
</tr>
<tr>
<td>PI(4,5)P₂</td>
<td>phosphatidylinositol-4,5-bisphosphate</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphatidylinositol 3 kinase</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PRR</td>
<td>proline-rich region</td>
</tr>
<tr>
<td>PTB</td>
<td>Phosphotyrosine binding domain</td>
</tr>
<tr>
<td>PTPase</td>
<td>protein tyrosine phosphatase</td>
</tr>
<tr>
<td>SKAP55HOM</td>
<td>Src kinase associated protein of 55-kDa homologue</td>
</tr>
<tr>
<td>TcR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TTSS</td>
<td>type III secretion system</td>
</tr>
<tr>
<td>VASP</td>
<td>vasodilator-stimulated phosphoprotein</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>vascular cellular adhesion molecule 1</td>
</tr>
<tr>
<td>WASP</td>
<td>Wiscott-Aldrich syndrome protein</td>
</tr>
<tr>
<td>WAVE1/Scar</td>
<td>WASP family verprolin homologous 1/suppressor of cAR</td>
</tr>
<tr>
<td>WHD/SHD</td>
<td>WAVE-homology/SCAR-homology domain</td>
</tr>
<tr>
<td>ysc</td>
<td>Yop secretion</td>
</tr>
</tbody>
</table>
Protein-protein interaction modules

Src homology 2 (SH2) domains function in protein-protein interactions where they bind to phosphorytosine-containing sequences with the p-YYXq consensus, where different groups of SH2s prefer different compositions of this sequence (Schlessinger and Lemmon, 2003; Songyang et al., 1993).

Phosphotyrosine binding domains (PTB) have similar functions to SH2 domains and fall into two groups. Group I PTBs bind to ligands containing NPXp-Y cores, where the tyrosine is phosphorylated. Group II PTBs bind to ligands with NPXY cores where tyrosine not necessarily is phosphorylated (Schlessinger and Lemmon, 2003).

SH3 domains recognize ligands that are proline-rich, PXXP, and adopt a left-handed polyproline-II helix conformation. The consensus for SH3 domain ligands is: K/RXPqXPq (class I site) and qPXqPXR/K (class II site) however, there are also some unconventional ligands that lack the PXXP core (Zarrinpar et al., 2003). The PXXP motif is usually hidden, and becomes exposed following phosphorylation/dephosphorylation of SH2-interacting domains or when the proteins are recruited to the correct site following “activation”.

WW domains recognize proline-rich regions in ligands. These proline-rich regions include PPXY, PPLP, PR-repeats and S/TP-repeats where the S/T is phosphorylated (Zarrinpar et al., 2003). However, no consensus sequence has been identified for the ligand.

Ena/VASP homology 1 (EVH1) domains recognize proline-rich regions in ligands. These proline-rich regions are FPXqP for class I ligands (vinculin, zyxin, actA, ADAP, robo) and XPPXXF for class II ligands (shank, ip3r, mglur, ry; Ball et al., 2002).

Band 4.1/ezrin/radixin/moesin (FERM) domains are around 400 amino acid long domains that are involved in localizing proteins to the plasma membrane. In addition, the FERM domain can interact with Rho GDI, PI(4,5)P2, phosphatidylerine, calmodulin and p53 in vitro (Chishti et al., 1998).

Pleckstrin homology (PH) domains are classically considered to bind to phosphoinositides and this interaction brings the proteins to membranes. However, around 90% of the PH domains bind to phosphoinositides with very low affinity and thus need to cooperate with other PH domains to bring the protein or protein complex to the cell membrane (Lemmon et al., 2002).

Lin-11, Isl-1. mec-3 (LIM) domains are zinc-binding cysteine-rich modules, which are involved in protein-protein interactions. There is no consensus LIM-binding motif identified but LIM domains can bind to each other, helix-loop-helix domains, SH3 domains, ankyrin repeats, spectrin repeats, PDZ domains and tyrosine-containing tight turns (Khurana et al., 2002).

Helix-loop-helix (HLH) domains usually mediate homo- or heterodimerization of proteins. Basic HLH domains bind to DNA and regulate gene transcriptions with the basic amino acid region. (Id)HLH domains, which lack the basic region, inhibit the bHLH from binding to DNA by binding to the HLH of bHLH proteins (Norton, 2000).

Abbreviations: p-Y = phosphorylated tyrosine; X = any amino acid; q = hydrophobic residue; P = proline; R = arginine; K = lysine; L = leucine; S = serine; T = threonine; F = phenylalanine; W = tryptophane
Introduction

Pathogens are constantly attacking us and to defend ourself from the danger we have a complex and efficient defense system consisting of both an innate and an adaptive immune system working in our bodies. The innate immune system is the first line of defense that includes neutrophils, natural killer cells and macrophages that circulate the body looking for virulent bacteria, which they ingest and degrade. Further, they present the bacterial remnants to the adaptive immune system, which among others includes B- and T-cells, leading to activation of this system. In response to priming, the adaptive immune system learns to recognize, kill and remember a specific enemy (Janeway et al., 2001).

Bacteria that want to survive in this hostile environment have to find ways to circumvent these defenses. This can occur in several different ways. Some bacteria cover themselves to hide from the immune system while others willingly are ingested, but then takes mean to prevent the host cell from degrading them (Pieters, 2001; Wurzner, 1999). The pathogen discussed in this thesis, *Yersinia pseudotuberculosis*, utilizes a third way to stay alive. It binds to a host cell receptor, β1-integrin, and uses a type III secretion system to “inject” virulence effector proteins, yops, into the host cell. Three of these virulence effectors, YopH, YopE and YopT destroy the initial host cell signaling machinery needed for the host cell to ingest the bacterium. Hence, the bacteria will remain alive, bound to the outside of the host cell. Thus, understanding of the host targets of these effectors provide useful knowledge in how phagocytosis through β1-integrins occur, which was studied in paper I for YopH in macrophages. However, the bacterium will be ingested if YopH, YopE and YopT are absent or mutated. This knowledge was used to study the role of the host cell receptor, β1-integrin, in bacterial uptake (paper II).

Bacterial uptake through β1-integrins, cell spreading and migration triggered by binding to extracellular matrix proteins through β1-integrins partially occur through the same signaling pathways. Thus, infection with *Yersinia* or binding to invasin is a good tool for studies on regulation of “normally occurring” integrin-mediated cellular events. The events of cell binding and migration are tightly regulated and errors in this control can be fatal or lead to body malformations or cancer. However, this is a complex process and only a small fraction is understood. The processes of cell spreading and migration overlaps and both involve lots of intercalating signaling pathways and proteins. In paper II, we show that normal cells that bind to invasin also spread on invasin. However, in our work using different β1-integrin mutants we found that a splice variant that lacked most of the intracellular part still bound to invasin but the spreading was strongly impaired and only what is likely to be an early step in cell spreading could be seen (filopodia). We have utilized this observation to study pathways that are involved in this early form of spreading (paper III-) and this also provides us the potential to dissect the pathways needed for further spreading.

The eukaryotic cell

Monocytes derive from granulocyte-monocyte progenitor cells, which are stem cells in the bone marrow. The monocytes migrate from the bone marrow and circulate the blood stream for 1-3 days until they reach a tissue that send out local factors that promotes differentiation of monocytes into macrophages, which mainly occur in response to infection/inflammation (Shepard and Zon, 2000). The macrophages are efficient “eaters” of particles (a process denoted phagocytosis) including bacteria and cellular debris. Macrophages express several receptors that recognize and trigger phagocytosis including Fc receptors (FcR) that recognize the Fc portion of antibodies and Complement receptors (CR) that recognize particles labeled with certain complement residues (Aderem and Underhill, 1999). Both antibodies and complement residues are used to tag (opsonize) bacteria for recognition by macrophages and other professional phagocytes (Aderem and Underhill, 1999).
Fibroblasts are flat, irregularly shaped connective tissue cells that secrete components of the extracellular matrix. They are not professional phagocytes (Lawerence, 1995), but have the ability to phagocytose particles or bacteria if these particles express ligands that are recognized by the cell.

Both macrophages and fibroblasts express β1-integrins and recognize invasin (a protein exposed on the surface of Yersinia pseudotuberculosis, see below) and thus, both cell types can phagocytose Yersinia. Since fibroblasts lack specialized phagocytic receptors, they offer an “easier” environment to study β1-integrin mediated effects and hence, a fibroblast-like cell line was used (paper II-) for these studies. This cell line, GD25, was derived from β1-integrin knockout murine embryonic stem cells that were treated with DMSO to induce differentiation followed by immortalized with SV-40 large T (Fässler et al., 1995). GD25 was further stably transfected with wild type β1A-integrin, β1B-integrin or cytoplasmic mutants of β1 A-integrin (Armulik et al., 2000; Sakai et al., 1998; Wennerberg et al., 2000; Wennerberg et al., 1998; Wennerberg et al., 1996; paper II). However, cells differ in their intracellular protein composition, signaling pathways and hence phagocytose bacteria by different mechanism. Thus, studies of phagocytosis in macrophages has to be performed to understand the molecular mechanisms behind how these cells phagocytose Yersinia both through FcR, CR and β1-integrins. In paper I, the molecular targets for YopH was elucidated in the macrophage-like cell line J774A.1.

**Integrins**

Integrins are a large family of eukaryotic heterodimeric α-β receptors, which consist of at least 18 α and 8 β integrin subunits (the human genome project suggested that there may be 24 α and 9 β subunits (Venter et al., 2001)) making up more than 24 receptors. These receptors, depending on heterodimers, mediate interactions between cells, cell-soluble protein and cell- extracellular matrix (ECM). Ligand binding leads to integrin clustering, recruitment of signaling proteins and indirect connection of the actin cytoskeleton to the integrin-cytoplasmic domain at sites denoted cell-matrix adhesions (se below; Hynes, 2002).

Integrins are essential in the regulation of several biological processes, including cell survival (anchorage dependence), cell growth, differentiation, immune responses and morphological changes. In many of these events, integrins crosstalk with other receptors on the cell, including T cell receptors (TcR), B cell receptors (BcR) and different growth factor receptors. This crosstalk modulates cell signaling in different ways depending on which receptor that is involved (Miranti and Brugge, 2002). Thus, outside-in signaling from integrins and other receptors integrate and leads to a very complex web of protein-protein interactions within the cell, with the role to adapt the cell to its surroundings. In addition, the binding affinity of integrins is regulated from within the cell in a response known as inside-out signaling. To date, it is known that integrins stimulate activation of many signaling pathways including the Ras-MAPK (mitogen-activated protein kinase) cascade, JNK (c-Jun N-terminal kinase), actin-regulating proteins, protein kinase C (PKC), Src, FAK, p130Cas, phosphoinositols and many more (Brakebusch and Fässler, 2003; Miranti and Brugge, 2002), but less is known about the exact mechanisms how integrins activate these signaling cascades.

With the aspect that at least 23 integrin receptors are involved in modulating the actin cytoskeleton (α6β4 modulates the intermediate filaments not actin), what is the specific role of each receptor. The expression pattern of integrins differs between cell types where for instance β1-integrins are ubiquitously expressed while the expression of β2-integrins are restricted to hematopoietic cells. Knockout studies in mice have further shown that several of the subunits are essential where the knockouts died before birth or at birth (α3, α4, α5, α6, α8, α9 αv, β1, β4, β8). Others knockouts showed severe phenotypes still allowing survival (α1, α7, αL, αM, αE, α1IB, β2, β3, β6, β7), and only a few (α10 and β5) showed no apparent phenotype (Bouvard et al., 2001; Reynolds et al., 2002). Thus, most if not all integrin receptors have their specific role(s) throughout life.
The integrin structure, activation and ligand binding

Recently the αVβ3-integrin was crystallized, both in inactive and active binding states (Xiong et al., 2001; Xiong et al., 2002). The extracellular domains of α- and β-subunits interact and form a head domain that sits on two legs (one from each subunit; figure 1) that span the cell membrane region and continue as short intracellular regions of less than 100 amino acids (except the β4 subunit which has a 1000 amino acid long intracellular region). The ligands for integrins bind to the head where both the α and β subunit are involved in ligand-binding specificity (Hynes, 2002). The legs contain a flexible knee domain and can bend so that the head domain is close to the cell membrane and this bent conformation was shown to be a conformation with low-affinity for ligand (figure 1, middle). The high-affinity conformation of integrins is in contrast standing up from the cell membrane with separated legs and an open headpiece (figure 1, left) while there also is a straight conformation of intermediate affinity where the legs are “crossed-over” and the headpiece is in a closed conformation (Takagi et al., 2002).

Figure 1: The crystal structure of the extracellular domain of αvβ3-integrin in the extended conformation with open headpiece (left) and the bent conformation (middle), respectively. The motifs are illustrated in the bent conformation (right). The figures are adapted from Takagi et al. (2002) and Xiong et al. (2001). The abbreviations of the β subunit stands for: Hy–hybrid domain, E- EGF domains, βT – β-tail domain.

The regulation of these affinities occur by divalent cations in the head and through modulation of the integrin from within the cell, inside-out signaling, which apart from changing the integrin conformation also allows clustering of integrins (Ginsberg et al., 1992; Hughes and Pfaff, 1998; Hynes, 2002; Shattil and Ginsberg, 1997). The membrane-proximal cytoplasmic tails of inactive integrins interact with a “weak handshake” and mutations that disrupt this interaction make integrins constitutively active. Further, the binding between β-integrin and a known integrin activator, talin, unclasps this interaction to activate the integrin, which probably is due to that the “legs” of the integrins are distanced from each other and thus stimulate an opening of the integrin to the active extended shape (Tadokoro et al., 2003; Vinogradova et al., 2002).

The integrin cytoplasmic tail

β1-, β2- and β3-integrins that lack the cytoplasmic tails, fail to localize to cell-matrix adhesions, show reduced ligand binding and cannot mediate downstream signaling (Liu et al., 2000), showing that the β cytoplasmic tail is essential for the function of integrin. These tails are well conserved between species and there are strong similarities between β1A-, β1D-, β2-, β3A-, β5A-, β6- and β7-integrins, indicating that they share functional properties. These integrins contain the membrane-proximal cyto-1 domain and one or two PTB ligand-binding motifs, NPXY or NXXY, interspaced by some threonines (not β1D, figure 2).

The NPXY and NXXY motifs are implicated in several integrin functions including cell-matrix contact localization, cytoskeletal association and cell adhesion (LaFlamme et al., 1997). Some signaling proteins have been shown to bind to the NPXY motifs including talin, Dab1, Eps8, tensin and filamin (Calderwood et al., 2003; Kääpä et al., 1999; Liu et al., 2000). The tyrosines of NPXY and NXXY can be phosphorylated, which occurs at least for β1 and β3, and phosphorylation is important for the β1-integrin-directed cell motility, but does not seem to affect other roles as attachment and localization (Calderwood et al., 2003; Sakai et al., 1998). Phosphorylation of the NPIY tyrosine inhibits at least talin binding to β1 in v-Src transformed cells, indicating that phosphorylation also could regulate integrins in a negative manner (Tapley et al., 1989).
The threonine sites are only starting to be studied. Mutation in β1A of the threonines to alanines shifted the extracellular conformation of the integrin toward the inactive state resulting in reduced adhesion (Wennerberg et al., 1998). These threonines bind ICAP-1, which is a protein implicated in negative regulation of β1-integrin avidity (Brakebusch and Fässler, 2003). Recently, it was shown that these threonines become phosphorylated during the G2/M phase of the cell cycle leading to reduced linkage to actin and reduced cell adhesion (Suzuki and Takahashi, 2003). Thus, these results imply that the threonines are involved in regulating the ligand-binding affinity of integrin.

The cyto-1 region is implicated in binding to signaling proteins, including FAK, paxillin, skelemin, melusin and α-actinin. Mutations in this region alter formation of cell-matrix adhesions and stress fiber assembly (Liu et al., 2000). In addition, integrin-linked kinase (ILK) binds to the cytoplasmic tails of β1, β2 and β3, but it is not known where ILK binds (Liu et al., 2000).

Less is known about the role of α cytoplasmic tails. These tails have a common KXGFFKR membrane-proximal sequence that is important for integrin inactivation and mutations herein leads to constitutive activation of integrins (Liu et al., 2000). In addition, this sequence bind calreticulin (a calcium-binding protein) and calreticulin-deficient cells show severe defects in integrin-mediated cell adhesions. The rest of the tail differ markedly between the α subunits but each α subunit is highly conserved between species indicating that the α subunits are important for integrin functions and gives specificity to the heterodimeric couples (Liu et al., 2000).

β1-integrins

The β1-integrin subunit dimerizes with several of the α subunits (α1-9, α11 and αv) and the resulting dimers bind to many ligands of the extracellular matrix, to other cells and to soluble ligands, but also to ligands from viruses and bacteria (table 1). The most studied ligand is fibronectin, which also interacts with other integrins including αvβ3 and αIIbβ3 (Pankov and Yamada, 2002). There are five splice variants of β1-integrins, β1A, β1B, β1C-1, β1C-2 and β1D. These splice variants only differ in the end of the cytoplasmic region while the 26 membrane proximal amino acids of the cytoplasmic region and the extracellular and transmembrane region is conserved (figure 2), and they bind to the same α subunits and ligands, but the cellular response differs markedly (Armulik, 2002; de Melker and Sonnenberg, 1999).

The β1A splice variant is expressed in almost all cells with the exception of skeletal and cardiac muscles where β1D is expressed instead (Armulik, 2002). The β1A splice variant is the most studied β1-integrin and all work where it only says β1 refer to this splice variant (also in this thesis). The roles and function of β1A will be described in appropriate sections of this thesis and here I will focus the discussion to the other splice variants.
Table I: Extracellular ligands for β1-integrins. Adapted from Plow et al. (2000) and van der Flier and Sonnenberg (2001).

<table>
<thead>
<tr>
<th>Integrin</th>
<th>Ligand</th>
</tr>
</thead>
<tbody>
<tr>
<td>α1β1</td>
<td>Collagens, Laminin</td>
</tr>
<tr>
<td>α2β1</td>
<td>Collagens, Echovirus 1, Laminin, chondroadherin, rotavirus, leech</td>
</tr>
<tr>
<td>α3β1</td>
<td>Epiligrin, Fibronectin, reelin, Invasin, Thrombospondin, Laminins</td>
</tr>
<tr>
<td>α4β1</td>
<td>Fibronectin, osteopontin, Invasin, VCAM-1, prepro Von Willebrands factor, coagulation factor XIII, angiostatin, tissue transglutaminase, rotavirus</td>
</tr>
<tr>
<td>α5β1</td>
<td>Fibronectin, Invasin, osteopontin, Collagens, Echovirus 1, Laminin, chondroadherin, rotavirus, leech</td>
</tr>
<tr>
<td>α6β1</td>
<td>Epiligrin, Fibronectin, reelin, Invasin, Thrombospondin, Laminins</td>
</tr>
<tr>
<td>α7β1</td>
<td>Laminin</td>
</tr>
<tr>
<td>α8β1</td>
<td>Cytotactin/tenascin-D, Fibronectin, nephronectin, TGFβ latency-associated peptide</td>
</tr>
<tr>
<td>α9β1</td>
<td>Cytotactin/tenascin-D, osteopontin, collagen, laminin, prepro Von Willebrands factor, coagulation factor XIII, angiostatin, tissue transglutaminase, VCAM-1, ADAM-12, ADAM-15</td>
</tr>
<tr>
<td>α10β1</td>
<td>Collagens</td>
</tr>
<tr>
<td>α11β1</td>
<td>Collagens</td>
</tr>
<tr>
<td>αvβ1</td>
<td>Fibronectin, Invasin, Vitronectin, TGFβ latency-associated peptide, Parechovirus 1</td>
</tr>
</tbody>
</table>

The β1B, β1C-1 and β1C-2 splice variants have only been found in humans. β1B is expressed at detectable levels in keratinocytes and hepatocytes even though low levels of mRNA has been detected in all human tissues and cell lines (Armulik, 2002). The cytoplasmic region of β1B lacks known motifs and the proposed role for this splice variant in vivo is to have a regulatory role of adhesion-mediated signaling. However, it could also be a splice variant that occur as a mistake and thus lacks real functions. In cell line studies, however, it has been shown that this splice variant act dominant negative on the function of β1A, do not localize to focal complexes, cannot mediate phosphorylation of FAK or bind to filamin or talin in vitro (de Melker and Sonnenberg, 1999). The β1C-1 and β1C-2 splice variants only differ with six amino acids and both are expressed in a broad range of cell lines and tissues but at a very low frequency compared to β1A. β1C inhibits cell proliferation, causes growth arrest in late G1 of the cell cycle and has been ascribed to downregulate neoplasticity (Armulik, 2002).

The muscle-specific β1D variant can localize to focal adhesions and trigger FAK and the MAPK pathway when expressed in GD25 cells. This variant adhered stronger to fibronectin than β1A, showed a stronger association with the actin cytoskeleton and bound talin and filamin with higher affinity than β1A, suggesting that the role of β1D is to strengthen the cytoskeletal-matrix link in muscles. However, the lack of β1D in transgenic mice only caused a mild ventricular dysfunction but did not affect muscle formation. In addition, β1D cannot replace β1A since mice where β1A was replaced with β1D died in uteri due to several developmental defects (Armulik, 2002).

Cell-matrix adhesions

Cell-matrix adhesions are sites close to the cellular membrane where proteins inside the cell are connected to the extracellular matrix/substratum through integrins, which is the receptor forming the adhesion sites (figure 3). At least 50 proteins are associated with these structures either transiently or stably. These proteins anchor and stabilize the actin cytoskeleton connected to the integrins and participate in intracellular signaling and in modulation of the integrin activation state. Proteins found at these sites include vinculin, zyxin, paxillin, talin, filamin, α-actinin, tensin, FAK, p130Cas, Src, Crk and F-actin. Many of these proteins harbor multiple binding sites for other proteins making up a complicated signaling web, nicely illustrated by Zamir and Geiger (2001b).

The cell-matrix adhesions are divided into several subtypes based on their appearance and molecular composition. To date, most studies on cell-matrix adhesions have been made on cells adhering to surfaces...
coated with matrix proteins, i.e. two-dimensional adhesions. In this situation, there are two types of small cell-matrix adhesions, podosomes and focal complexes (Zamir and Geiger, 2001b). The podosomes are cylindrical structures containing an actin core, phosphorylated proteins, vinculin, talin and other yet uncharacterized proteins. These adhesions are found in a variety of cells including monocytes, macrophages and osteoclasts. The small, dot-like adhesions of focal complexes are present at the tips of filopodia or at the edges of lamellipodia. These adhesions bind rather weakly to the substrate in stationary cells but apply strong traction forces to the substrate during cell migration (Beningo et al., 2001; Hall, 1998; Zamir et al., 1999).

The larger adhesions observed on two-dimensional adhesion substrates have been characterized and denoted focal adhesions, which further are divided into several classes, defined by (Zamir et al., 1999):

I) Classical focal contacts, which are arrowhead shaped, mainly located at cell periphery and contains relatively high levels of zyxin, vinculin, paxillin α-actinin, FAK, F-actin and αvβ3. These contacts have a high content of tyrosine phosphorylation but low levels of tensin, are devoid of fibronectin and have “needle eye” patterns of α5β1 in the periphery (Katz et al., 2000; Zamir et al., 1999). They are associated with the termini of actin stress fibers and depend on tension upheld by actomyosin contractility (Katz et al., 2000). II) Fibrillar adhesions, which are elongated or beaded in shape, locates mainly centrally in cells and contains high levels of fibronectin, tensin, parvin/actopaxin, F-actin and α5β1 but has a low phosphotyrosine content and little FAK, vinculin, paxillin or α-actinin. These fibrils are distinct from stress fibers (see below; Katz et al., 2000; Olski et al., 2001; Zamir et al., 1999). In contrast to classical focal contacts, these assemblies may not need actomyosin contractility for maintenance (Zamir et al., 1999).

III) Mosaic focal contacts have a general appearance of classical focal contacts but with a non-uniform internal molecular structure (Zamir et al., 1999).

Recently, studies with more in vivo-like situations have been performed where the ligands are presented in 3D matrixes, illustrated either with a collagen-gel or when the cells are allowed to build up their own “natural” matrix. These 3D-matrix adhesions differ in many aspects to the 2D focal adhesions in that they adhere with stronger affinity, have faster proliferation, FAK is present but not autophosphorylated, paxillin, tensin, talin, vinculin and phosphotyrosine-proteins are also present (Yamada et al., 2003).

Cell-matrix adhesions are dynamic, a focal complex can rapidly (a few seconds to a few minutes) mature into a focal contact (marked by recruitment of zyxin (Zaidel-Bar et al., 2003)), and focal contacts can turn into focal complexes (de-adhesion). In addition, fibrillar adhesions are assembled at focal contacts and are transported in an actomyosin-dependent manner towards the center of the cell (Zamir et al., 2000). This process is regulated, and depends on signals from matrix proteins, Rho GTPases (see below) and other factors (Clark et al., 1998; Riveline et al., 2001; Rottner et al., 1999b). The regulation and roles of the cell-matrix adhesions will be discussed further in appropriate sections of this thesis. Due to this dynamicity, there will also be intermediates between these distinct classes and without labeling techniques and a very trained eye it is tedious to determine exactly which adhesion that is found at a certain situation and in this thesis and accompanying papers we only distinguish between focal complexes and focal adhesions/focal contacts. In addition, the adhesions in our studies are 2D.

Figure 3: Compositions of cell-matrix adhesions are diverse and a few proteins that typically are found in these sites are illustrated in this image.
Cell-matrix adhesion proteins

Due to the innumerable proteins that are found in the cell-matrix adhesions, I will only describe the structure and function of the main proteins that are considered in this thesis. The protein structure and some of the interacting proteins for each protein will be illustrated in appropriate places. Abbreviations used in these figures are: SH - Src homology, PRR - proline rich region, PH - pleckstrin homology, NLS - nuclear localization signal, FAT - focal adhesion targeting, H1-5 - histone-like motifs, LD - leucine-aspartic acid rich motifs, LIM - Lin-11, Isl-1 mec-3 domain, FERM - Band 4.1/ezrin/radixin/moesin domain, HLH - helix-loop-helix domain, NES - nuclear export sequence, HL - DNA-binding region and Y (or YXXX where X stands for any amino acid), S and T are potential or known sites for phosphorylation at tyrosine, serine or threonine, respectively. Arrows indicates autoinhibiting interactions or autophosphorylations, respectively.

Non-receptor protein tyrosine kinases

Src family kinases

Src family kinases (SFKs) are non-receptor protein tyrosine kinases (NRPTKs) that have an overall similar structure (figure). SFKs can interact with and phosphorylate several proteins including FAK and p130Cas (Schwartzberg, 1998; Scott et al., 2002). The SFKs include nine proteins, Hck, Fgr, Blk and Lck, which are expressed in hematopoietic cells, Lyn and Yrk, which are expressed in neurons and hematopoietic cells and Src, Yes and Fyn, which are ubiquitously expressed (Brown and Cooper, 1996; Lowell and Soriano, 1996; Thomas and Brugge, 1997). Src, Yes and Fyn (SYF)-deficiency is lethal and SYF− fibroblasts isolated from these mouse embryos exhibit reduced motility on fibronectin and almost eliminated tyrosine kinase activity upon integrin-mediated binding (Klinghoffer et al., 1999), hence indicating that SFKs are involved in integrin-mediated signaling. Src is autoinhibited by two molecular interactions (depicted by arrows in figure) and is opened up by proteins that interact with the SH2 domain, or by dephosphorylation of the very C-terminal tyrosine (Bjorge et al., 2000; Brandt et al., 2003; Ling et al., 2003; Schwartzberg, 1998; Wang et al., 2003b). In addition, SFKs autophosphorylates the tyrosine in the kinase domain following SFK activation, which allow the kinase access to the substrate (Bjorge et al., 2000).

FAK

The Focal Adhesion Kinase (FAK) protein is a 125-kDa cytosolic NRPTK and docking protein that is widely expressed during development, in adult tissues and in many cell lines, however not at all or only slightly in monocytes/macrophages (De Nichilo and Yamada, 1996; Hsia et al., 2003; Schaller, 2001a; Zhai et al., 2003). Several stimuli induce FAK kinase activity and tyrosine phosphorylation, including reagents that stimulate G-protein coupled receptors, growth factors and neuropeptides (Rodriguez-Fernandez, 1999). However, the main way to activate FAK is through integrin-dependent adhesion to the ECM. The active FAK localizes to cell-matrix adhesions and/or lamellipodia (Hsia et al., 2003; Schaller, 2001a) where the localization to cell-matrix adhesions is determined by the C-terminal focal adhesion targeting (FAT) domain (Schaller, 2001a).

Activation of FAK leads to autophosphorylation of a tyrosine sitting just before the kinase domain of FAK, which allows SFKs to bind and further phosphorylate FAK at other YXXq sites leading to enhanced kinase activity of FAK and allowing interaction with downstream proteins (figure; Schaller, 2001a). FAK-deficient mice die early at day 8.5 days post coitum (dpc) due to mesodermal defects similar to fibronectin-deficiency, indicating that FAK is an important mediator of fibronectin-integrin
interactions at this stage of development (Ilic et al., 1995). Fibroblast-like cells isolated from these mice have a round morphology, elevated numbers of cell-matrix adhesions and defects in cell migration (Ilic et al., 1995). In addition to its role in cell-matrix adhesion turnover and cell migration, FAK is also involved in cell cycle progression and cell survival (Parsons, 2003).

**Etk/Bmx**

The Etk (epithelial and endothelial tyrosine kinase)/Bmx protein belongs to the Btk (Bruton’s tyrosine kinase) family of non-receptor tyrosine kinases, which mainly are of hematopoietic origin. However, Etk/Bmx is expressed in a variety of tissues and cell types including hematopoietic, epithelial, endothelial, lung and prostate cells (Qiu and Kung, 2000). The structure and known binding partners are illustrated in the figure. Etk/Bmx is activated by the interaction with the protein tyrosine phosphatase (PTPase) PTPD1 or by FAK. It is involved in several biological events including IL-6-induced differentiation of prostate cancer cells, Gtx12/13-induced activation of serum response factor in fibroblasts, neuroendocrine differentiation, transformation, antiapoptosis, activation of p21-activated kinase (Pak1) causing anchorage-independent and tumorigenic growth and promotion of cell migration upon integrin stimulation (Bagheri-Yarmand et al., 2001; Qiu and Kung, 2000). Independently of RhoA GTP/GDP status, Etk/Bmx binds to and activates RhoA, and disrupts the interaction between RhoA and Rho-GDI (Kim et al., 2002).

**Abl**

The Abl family of NRPTKs consists of Abl of *Drosophila*, *C. elegans* and vertebrates and the Abl-related gene (Arg; Hernández et al., 2004). The ubiquitously expressed Abl kinase localizes to both nucleus and the cytoplasm and contains several known protein-protein interaction motifs as well as DNA-binding domains (figure). It responds to extracellular signals (growth factors, cell adhesion and cytokines) and internal signals (DNA damage, oxidative stress) and is strictly regulated by both auto-inhibition and co-inhibition (Wang, 2004). Upon adhesion to fibronectin, it transits from the nucleus to the cytoplasm, where it localizes to F-actin bundles and cell-matrix adhesions (Hernández et al., 2004). The cytoplasmic Abl regulates F-actin dependent processes while the nuclear Abl regulate cell-cycle progression and cellular responses to genotoxic stress (Hernández et al., 2004). Overexpression of Abl promotes protrusive membrane ruffling and the formation of filopodia-like microspikes, but inhibits cell migration (Hernández et al., 2004). Constitutively activated Abl is the most common cause of chronic myelogenous leukemia (CML) and the oncogene Bcr-Abl cause upregulation of mitogenic and antiapoptotic pathways, increased cell migration, membrane ruffling and filopodia extension, causing the premature release of CML cells from the bone marrow and hence, cancer (Hernández et al., 2004).

**Adaptor and docking proteins**

**p130Cas**

p130Cas was identified as a tyrosine phosphorylated protein in v-Src and v-Crk transformed cells, it was shown to be a docking protein with
several protein-protein interaction sites (figure; Kanner et al., 1991; Law et al., 1999; Nakamoto et al., 1996; Sakai et al., 1994). This docking protein is involved in the regulation of cell motility (Panetti, 2002), integrin-mediated cell-matrix adhesion formation (Honda et al., 1998; Nojima et al., 1995) and JNK activation (Dolfi et al., 1998; Oktay et al., 1999). Mouse embryos deficient of p130Cas dies 11.5-12.5 dpc showing disorganized myofibrils and Z-discs in the heart and abnormal blood vessels (Honda et al., 1998). Fibroblasts isolated from these embryos exhibit changed cellular morphology, changed distribution and organization of the actin cytoskeleton with thin, short and irregular actin filaments at the cell periphery (Honda et al., 1998).

p130Cas is activated upon integrin-mediated cell adhesion, EGFR engagement, insulin-like growth factor I and by 12-O-tetradecanoylphorbol-13-acetate (PMA; Casamassima and Rozengurt, 1998; Fagerström et al., 1998; Nojima et al., 1995; Ojaniemi and Vuori, 1997). Activated p130Cas localize to focal adhesions and this localization depends on both the SH3 domain and the C-terminus of p130Cas (Harte et al., 2000). It has been suggested that activated FAK binds to p130Cas and phosphorylates the YDYVHL motif, which allows Src to bind to p130Cas to phosphorylate the other tyrosines of p130Cas in the substrate domain (Harte et al., 1996; Petch et al., 1995; Tachibana et al., 1997). In addition, Bmx/Etk was recently shown to interact with the YDYVHL domain of p130Cas and phosphorylate p130Cas in a similar manner as Src (Abassi et al., 2003). p130Cas can also be phosphorylated independent of FAK (see results and discussion).

Crk

Crk proteins are SH2-SH3 containing adaptors with several interacting proteins (figure). They are implicated in many signaling pathways downstream of integrins, insulin receptor, platelet derived growth factor αP, BcR and TcR (Feller, 2001). There are at least four mammalian Crk proteins, CrkI, CrkII, CrkL and CrkIII. CrkI contains one SH2 and one SH3 domain while the rest have two SH3 domains, as CrkII (Feller, 2001; Prosser et al., 2003). CrkI is always in an open conformation while CrkII has been suggested to be autoinhibited by an interaction between the SH2 domain and a tyrosine situated in between the two SH3 domains (Feller, 2001). Upon integrin stimulation, the complex of p130Cas and Crk acts as a molecular switch that induces cell migration (Klemke et al., 1998; see result and discussion). In addition, CrkII potentially acts as a determinant of apoptosis since it stimulates apoptosis when being in the nucleus but prevents apoptosis when it localizes to cell-matrix adhesions (Smith et al., 2002; Cho, 2000).

Nck

Nck-1/Nckα and Nck-2/Grb4/Nckβ are adapter proteins that are activated downstream of several receptors (growth factor receptors, integrins, TcR, insulin receptors), interacts with several proteins and undergoes substantial phosphorylation on tyrosine, threonine and serine residues in response to growth factor stimulation and Src transformation (figure; Buday et al., 2002; Li et al., 2001). The main function of Nck seem to be to link the cell-surface receptors to the actin cytoskeleton, either by direct interaction with the receptor or interactions downstream of the receptor (Buday et al., 2002; Li et al., 2001).

ADAP (FYB/SLAP-130)

Adhesion and Degranulation promoting Adapter Protein (ADAP) formerly known as p120/130 or Fyn-binding protein (FYB) or SLP-76-associated protein of 130 kDa (SLAP-130) is an adapter protein (figure) that is expressed in mononuclear cells of hematopoietic origin, however not in B-cells (da Silva et al., 1997a; Fujii et al., 2003; Krause et
ADAP exists in two splice variants, 120 and 130 kDa where the larger variant contains a 46 amino acid insert between two tyrosine based motifs (Veale et al., 1999). Most studies on ADAP concern its role in T-cells where ADAP become tyrosine phosphorylated upon T cell receptor activation as well as through activation of \( \alpha 4\beta 1 \)-integrin (da Silva et al., 1993; da Silva et al., 1997a; da Silva et al., 1997b; Hunter et al., 2000; Musci et al., 1997). ADAP act as a linker between the upstream adaptor SLP-76 and VASP during TCR-stimulation and Fc\( \gamma \)R-mediated phagocytosis and this complex is suggested to be involved in \( \beta 1 \)- and \( \beta 2 \)-integrin clustering, upregulation of integrin-fibronectin binding and integrin-stimulated cellular activation (Coppolino et al., 2001; Peterson, 2003). In addition, ADAP upregulates Fc\( \varepsilon \)R-dependent degranulation release in mast cells (Geng et al., 2001).

ADAP-null mouse show impaired T-cell development and function, a modest thrombocytopenia but spreading or aggregation of platelets is unaffected. Further, myeloid development is undisturbed; macrophages can phagocytose antibody-coated red blood cells and \textit{Listeria monocytogenes} \textit{in vitro}. This might however be due to functional redundancy with ADAP homologs, such as PML-RAR regulated adapter molecule-1. T-cells deficient of ADAP shows impairment in TCR-stimulated adhesions to ligands for \( \beta 1 \)- and \( \beta 2 \)-integrins and impaired proliferation (Peterson, 2003).

**SKAP-55**

SKAP-55 and SKAP-HOM/ SKAP55R are two structurally related adaptor proteins that interact with ADAP and some other proteins (figure). SKAP-55 is only expressed in T-cells while SKAP-HOM is ubiquitously expressed (Geng and Rudd, 2002; Timms et al., 1999). SKAPP-55 couples to the transmembrane PTPase, CD45, which activates Fyn by dephosphorylation and thus lead to TcR activation (Wu et al., 2002a). Fyn also phosphorylates SKAP-55, which is suggested to be involved in MAPK activation downstream of the TcR (Wu et al., 2002b). SKAP-HOM is tyrosine phosphorylated by SFKs and upon integrin-mediated adhesion in macrophages and it localizes to membrane ruffles, but the function is still unknown (Black et al., 2000; Timms et al., 1999).

**Talin and PI(4,5)P\(_2\)**

Talin is a large cell-matrix adhesion-localized protein of 235 kDa consisting of a 47-kDa N-terminal head domain and a C-terminal 190-kDa tail domain that interacts with several proteins (figure; Brakebusch and Fässler, 2003; Rees et al., 1990). It is tightly coupled to integrin function and talin knockouts show similar phenotypes to integrin knockouts (Brown et al., 2002; Monkley et al., 2000). Talin binds directly to integrins and is important for increased ligand affinity of integrins and is a direct link to F-actin (Calderwood et al., 2002; Calderwood et al., 1999; Vinogradova et al., 2002).

In addition, talin was recently shown to be crucial for the localized production of phosphatidylinositol-4,5-bisphosphate (PI(4,5)P\(_2\)) at newly engaged integrins by recruiting one splice isoform of type 1 phosphatidylinositol-4-phosphate 5-kinase (PIP5K) to these sites (Di Paolo et al., 2002; Ling et al., 2002). PI(4,5)P\(_2\) strengthen the interaction between talin and \( \beta \)-integrin, regulates the interaction between vinculin and talin and modulates the activity of other cytoskeletal proteins at the plasma membrane promoting their binding to the plasma membrane and actin filament assembly (Brakebusch and Fässler, 2003).
Vinculin

Vinculin is a 117-kDa protein that localizes to cell-matrix adhesions where it is involved in actin regulation (figure; Critchley, 2000; Xu et al., 1998). It is autoinhibited and become activated by P(4,5)P2 following talin activation (Critchley, 2000). Vinculin-deficient mice die at day E8-E10 due to heart and brain defects and the embryos were smaller (Xu et al., 1998). Fibroblasts isolated from knockout mice show reduced spreading, a higher migration rate and a decreased mechanical stiffness of the integrin-cytoskeleton linkage (Coll et al., 1995; Goldmann et al., 1998; Xu et al., 1998).

Paxillin-α

Paxillin-α is a 68-70 kDa docking protein that primarily localize to focal adhesions, but is also found in the nucleus (Schaller, 2001b; Wang and Gilmore, 2003). It interacts with a wide number of proteins using its protein-binding modules (figure; Schaller, 2001b; Turner, 2000a; Turner, 2000b). Paxillin knockout studies reveal that it is essential for embryonic development and for cell spreading and motility of fibroblasts (Hagel et al., 2002). In addition to paxillin-α, two splice isoforms with more restricted expression have been found, paxillin-β and -γ. Moreover, paxillin has two additional family members Hic-5 and leupaxin, which are very similar in domain organization as paxillin. However, these proteins cannot functionally replace paxillin (Schaller, 2001b).

The Cytoskeleton

The cytoskeleton is composed of protein filaments that build up the structural framework of a cell. The main groups of protein filaments are the microtubular network, the actin filaments and the intermediate filaments. These fibers all have their important roles in the cell and as the name reflects, can be rigid fibers that uphold a specific cell structure such as neural axons, the muscle filaments or protect the cell against mechanical stress. However, in most situations, the actin and microtubule filaments are dynamic, they grow and shrink in response to cell signaling to change the morphology of cells or parts of a cell. In addition, they are used as molecular highways to transport organelles and proteins within the cell (Goode et al., 2000). In this thesis, the focus lies on the actin filaments, since they are involved in the uptake of bacteria, in the control of cell spreading and are tightly linked to integrins. Microtubules are also important for *Yersinia* uptake and in cell spreading (McGee et al., 2003; Small and Kaverina, 2003), while the roles for intermediate filaments in these processes are less clear.

Actin

Actin is only found in eukaryotes and there are three classes of actin proteins in higher mammals, α-, β- and γ-isoforms where the α-form is found in muscle cells while the β- and γ-forms are the principal constituents of non-muscle cells (Alberts et al., 2002). Stabilized filaments of actin form the core of microvilli and are critical components of the contractile apparatus of muscle cells. Actin filaments are also important in stabilizing transient structures during phagocytosis and cell movements, including filopodia, lamellipodia and stress fibers (see below).

Actin monomers (also known as G-actin) are two-domain globular proteins with an ATP/ADP-binding site in the center of the molecules. Binding of ATP or ADP to G-actin stabilizes the structure (Pollard and Earnshaw, 2002). The monomers dimerize or form trimers with the help of nucleation factors...
(see below), which is followed by extension of this core by further incorporation of G-actins to form actin filaments (F-actin). Polymerization of actin is a rapid process that requires K\(^+\), Mg\(^{2+}\) and ATP-bound G-actin. The ATP of G-actin is hydrolyzed into ADP soon after polymerization, which leads to decreased binding affinity of this subunit to neighboring subunits and to destabilization of the filament (Alberts et al., 2002).

The F-actin structure can be considered as a two-stranded helical polymer of actin monomers that are assembled in a head to tail manner. This manner of assembly makes the filaments polarized where the G-actin incorporation mainly occurs at the plus or barbed end while the minus or pointed end may loose actin monomers. In some situations the actin filaments can grow at the barbed end and loose actin molecules at the pointed end at the same rate; this dynamic behavior is denoted treadmilling (figure 4; Alberts et al., 2002). A living cell has to have a tight control of the actin cytoskeleton to shape the cell in the correct manner. For this purpose, at least 60 classes of known actin-binding proteins are involved in the regulation of actin-assembly, stabilization and disassembly (Pollard, 1999; Raab et al., 1997).

Figure 4: Model for Arp2/3 branching/ debranching and actin dynamics at a leading edge of a cell. Modified from Pollard et al. (2000). 1) WASP binds to ATP-bound G-actin and ATP-bound Arp2/3 upon activation from extracellular signal. 2) This complex incorporates into an existing filament thus forming a branching point where this G-actin sits at the mother filament and the ATPs of the Arp2/3 complex allow incorporation of G-actins in the daughter filament. 3) The ATP-ADP hydrolyzation of the G-actin incorporated into the mother filament leads to detachment of the WA domain (of WASP) thus allowing extension of the mother filament. 4) The ATP of Arp2 becomes hydrolyzed after some time and this hydrolyzation destabilizes the branch and leads to debranching possibly leaving the Arp2/3 complex (with ADP-Arp2) on the mother branch. 5) The depolymerization of the F-actin (from the pointed end) relieve the Arp2/3 complex and at this stage the ADP of the Arp2 can be exchanged with an ATP to allow this complex to participate in another cycle of branching.

Actin binding proteins

The wide variety of actin binding proteins can be grouped into functional families, which briefly will be described in this section. Capping proteins as CapZ, CapG, tensin and fragmin cap the barbed end of actin filaments, which leads to inhibition of actin polymerization. In contrast, tropomodulin and the Arp2/3 complex (see below) cap the pointed end, which leads to a stabilization of F-actin (Pollard, 1999). Severing proteins cut F-actin, which creates a free barbed end of F-actin. The gelsolin superfamily is a group of severing proteins but they also caps actin filaments following severing (Pollard, 1999; Raab et al., 1997). Another family of severing proteins, which in contrast to gelsolin creates free barbed ends of F-actin, are the ADF/Cofilins, which also are actin depolymerizing factors that increase the off rate at the pointed ends of filaments and binds to ADP-actin and inhibit the ADP to ATP exchange (Pollard, 1999). The F-actins are also cross-linked into bundles or networks, which is mediated by actin cross-linking proteins including fascins, spectrins (as α-actinin), filamins and dystrophins (Brakebusch
and Fässler, 2003; Kureishy et al., 2002; Roberts, 2001). In addition, F-actins are stabilized by proteins that bind along the side of actin filaments, including tropomyosin, nebulin and caldesmon, and some of these proteins control the interaction between actin and the actin-based motors, myosins (Pollard and Earnshaw, 2002). G-actin binding proteins have several functions. Profilin bind with high-affinity to monomeric actin and i) catalyze the ADP to ATP exchange of actin monomers, ii) inhibits hydrolysis of ATP bound to G-actin, iii) transports “active” G-actins to sites of actin elongation by interacting with several actin nucleating and elongation factors, such as WASP- and VASP-family proteins, and iv) enhance cofilin-induced filament turnover (Pollard, 1999). Another ATP-G-actin binding protein, thymosine inhibits polymerization of actin (Pollard and Earnshaw, 2002).

Actin-based motors

Myosins are actin-based motors that play fundamental roles in many forms of eukaryotic motility such as muscle contraction, cell crawling, phagocytosis, growth cone extension, maintenance of cell shape and organelle/particle trafficking (Ruegg et al., 2002; Tuxworth and Titus, 2000). The myosin family is very large and there are around 40 myosin genes in the human genome, which are subdivided into at least 18 classes where myosins belonging to the myosin II class is denoted the conventional myosins and the others are called unconventional myosins (Tuxworth and Titus, 2000). These proteins consist of a heavy chain and two light chains. The heavy chain contains a head domain, which binds to F-actin and generates movement along actin filaments in an ATP-dependent manner, a flexible neck region, which interacts with the myosin light chains and a tail domain that varies significantly between the myosin classes (Alberts et al., 2002). Myosins are activated by a conformational change of the heavy chain in response to phosphorylation of one of the light chains by myosin light chain kinase (MLCK; Alberts et al., 2002).

The myosin II class of actin motors are involved in contraction and have to multimerize since each myosin only contains one F-actin binding domain. This class of myosins exerts fundamental contraction of actin cables in processes as muscle contraction, formation of contractile rings, cytokinesis, epithelial wound healing and tension of stress fibers (Alberts et al., 2002; Bement, 2002). Unconventional myosins have diverse functions, including phagocytosis (myosin VII), organelle and protein transport (myosin V), intrafilopodial motility (myosin X; see paper IV) and are divided into different classes depending on the motifs located in their tail domains (Berg et al., 2001).

Actin polymerization

Actin elongation occurs at free barbed ends, accomplished by uncappping, severing of existing filaments or de novo creation (Condeelis, 2001). De novo nucleation is an unfavorable event compared with elongation of already existing filaments, which means that actin monomers do not spontaneously form F-actins, instead nucleation factors are involved (Pollard and Cooper, 1986). The seven-subunit Arp2/3 complex is the best-characterized cellular initiator of de novo actin filament nucleation, but it can also bind to pre-existing actin filaments and form 70° angle Y-shaped branches (figure 4), which for instance occur at the leading edges of cells (Kelleher et al., 1995; Kiehart and Franke, 2002; Le Clainche et al., 2003; Machesky and Insall, 1998). The Arp2/3 complex contains two actin-related proteins Arp2 and Arp3, which both bind ATP, and five novel proteins (p40, p35, p19, p18 and p14). The complex is intrinsically inactive and activation is dependent on nucleation promoting factors including WASP, WAVE, ActA of Listeria monocytogenes, myosin I, cortactin and Abp1p (Higgs and Pollard, 2001). In addition to Arp2/3, formins, ActA of Listeria monocytogenes and possibly zyxin and Ena/VASP can nucleate actin independent of Arp2/3 (Evangelista et al., 2003; Fradelizi et al., 2001; Huttelmaier et al., 1999; Krause et al., 2002; Walders-Harbeck et al., 2002).

WASP

The ubiquitously expressed N-WASP (Neural-Wiscotts Aldrich syndrome protein) and the hematopoietic-specific WASP are Arp2/3-
inducing factors involved in formation of filopodia, T-cell activation, phagocytosis of macrophages, actin comet formation by *Shigella flexneri*, *Listeria monocytogenes* and *Vaccinia virus* (Takenawa and Miki, 2001). These proteins are very similar in structure and known binding partners (figure; Badour et al., 2003; Cory et al., 2003; Kato et al., 2002; Panchal et al., 2003). Both proteins are autoinhibited by an intramolecular interaction, which can be released by Cdc42 and PI(4,5)P₂ leading to activation toward Arp2/3 (Caron, 2002). SFKs can activate N-WASP when N-WASP is phosphorylated at the tyrosine sitting in between the CRIB (Cdc42/Rac interactive) and PRR (proline-rich region) domain since this phosphorylation weakens the autoinhibitory interaction. However, this site can only be reached for phosphorylation when N-WASP is in the active conformation, indicating that SFKs only can activate N-WASP that already has been activated by Cdc42 or other activators at an earlier stage (Cory et al., 2002; Suetsugu et al., 2002; Torres and Rosen, 2003). In addition, other proteins including WASP-interacting SH3 protein (WISH), Nck, Grb-2 and Abl also activate N-WASP, either alone or in conjunction with PIP₂ but independent of Cdc42 (Carlier et al., 2000; Fukuoka et al., 2001; Rohatgi et al., 2001; Suetsugu et al., 2002). It is however not elucidated whether these proteins can activate unphosphorylated N-WASP or not.

Abbreviations in figure: PH - pleckstrin homology, IQ- IQ motif, WH1 - WASP homology 1, CRIB - Cdc42 and Rac1 interactive binding domain, V - verprolin-homology, C - central domain, A - acidic domain, WA - WASP homology 2 and acidic domain, Y and S indicates potential or known sites for phosphorylation at tyrosine or serine, respectively. Arrows indicates autoinhibiting interaction.

**WAVEs**

WAVE/Scars (the WASP family verprolin homologous 1/suppressor of cAR) belong to the WASP family of Arp2/3-inducing factors and consist of three proteins, WAVE1/Scar and WAVE3, which predominantly are expressed in neuronal tissues and WAVE2 that is ubiquitously expressed. The C-terminus of these proteins is similar to WASP, while the N-terminus contains a WAVE-homology/SCAR-homology (WHD/SHD) domain followed by a basic region, but lacks the CRIB domain and cannot bind Rac or Cdc42 (Caron, 2002). Overexpression of WAVE1 induces lamellipodia formation and this protein localizes to lamellipodia and membrane ruffles. WAVE2 and WAVE3 also localize to the initiation sites and the tips of filopodia indicating that they are involved in filopodia formation (Miki et al., 1998; Nakagawa et al., 2003; Nozumi et al., 2003). WAVE1-deficient mice show sensorimotor retardation and defects in learning and memory, while WAVE2-deficient mice died, where the fetuses displayed growth retardation and morphological abnormalities (Miki and Takenawa, 2003; Yan et al., 2003). Fibroblasts derived from WAVE−/− stem cells were defective in cell motility, lamellipodium formation and Rac-mediated actin polymerization (Yan et al., 2003) and recent data show that Rac indirectly activate WAVEs by different mechanisms (Eden et al., 2002; Krugmann et al., 2001; Takenawa and Miki, 2001).

**Ena/VASP**

The Ena/VASP family consists of four proteins, the *Drosophila* Ena (enabled), and the three mammalian proteins VASP (Vasodilator-stimulated phosphoprotein), Evl (Ena/VASP-like protein) and Mena (mammalian Ena) that all have a similar structure (figure; Kwikatowski et al., 2003). Members of this protein family localize to cell-matrix adhesions, cell-cell adherence junctions, along stress fibers, at the rim of lamellipodia, membrane ruffles and to tips of filopodia by interacting with (F/L/W/Y)PPP-containing proteins (Dutarte et al., 1996; Haffner et al., 1995; Renfranz and Beckerle, 2002). They are involved in many actin-dependent cell functions including axonal guidance, neural tube closure, cell-cell adhesion, phagocytosis, T-cell activation and intracellular movement of the bacteria *Listeria monocytogenes* (Kwikatowski et al., 2003). The function of VASP has been debated for a long time and recent data suggest that VASP decreases F-actin branching and increase the rate of actin polymerization, which leads to formation of long non-branched actin filaments (Bear et al., 2002;
Samarin et al., 2003). However, VASP reduces the persistence of the protrusion, which causes a slowing down in the rate of cell motility (Bear et al., 2002).

Abbreviations in figure: EVH - Ena/VASP homology domain, PRR - proline rich region.

**Rho GTPases**

Rho GTPases are members of the Ras family of small GTP-binding proteins. Among other functions, they participate in the regulation of the actin and microtubule cytoskeletons in processes such as cell adhesion, cell motility, endocytosis/phagocytosis, cytokinesis, smooth muscle contraction, cell polarity and morphogenesis (Johnson, 1999; Kaibuchi et al., 1999; Ridley, 2001a; Ridley, 2001b; Takai et al., 2001). Most Rho GTPases are posttranslationally modified by (iso)prenylation at a cystein in an N-terminal CAAX (cystein-aliphatic amino acid-aliphatic amino acid-any amino acid) motif and exert their function associated to the cell membrane (Clarke, 1992). The Ras family of GTPases cycles between GDP-bound inactive and GTP-bound active states. These transitions are enhanced by three classes of proteins: i) guanine nucleotide exchange factors (GEFs) that catalyze the exchange of GDP to GTP (Cherfils and Chardin, 1999), ii) GTPase activating proteins (GAPs) that promotes the intrinsic GTP hydrolysis of Rho family proteins (Bernards, 2003) and iii) guanine nucleotide dissociation inhibitor factors (GDIs) that bind to GDP-bound GTPases and prevent nucleotide release and the GDIs also masks the CAAX motif to inhibit membrane localization of the Rho GTPases (figure 5; Olofsson, 1999). In addition, many of the Rho GTPases can be point-mutated to either lock the proteins in a GTP-bound state, which cause a constitutively active protein, or in a GDP-bound state, which for most Ras family proteins seems to make the construct dominant negative. These types of mutants have been widely used to study the role of individual Rho family members where most studies so far have focused on the functions of the family members Cdc42, Rac1 and RhoA. Other members of the Rho family are RhoB-E, RhoG, RhoH/TTF, Rac2-3, Rnd1-2 and TC10 (Takai et al., 2001).

**Figure 5:** The regulation of small GTPases and models of RhoA-, Rac1- and Cdc42-induced formation of stress fibers, lamellipodia and filopodia, respectively.

**Rac and lamellipodia**

Activated Rac1 play a central role in cell migration by promoting lamellipodia formation and membrane ruffling. Lamellipodia are broad, flat protrusions consisting of a branched network of filamentous actin, which move forward by a treadmilling mechanism (figure 4), whereas membrane ruffles essentially are lamellipodia that are curled up away from the substratum (Small et al., 2002). In addition, Rac1 induces the formation of focal complexes by an unknown mechanism (Hall, 1998; Ridley, 1999). Rac1 is activated downstream of β1-integrins through at least two pathways, either through the
p130Cas-Crk-DOCK180 scaffold or through the paxillin-PKL-PIX scaffold where DOCK180 and PIX are Rac-GEFs (DeMali and Burridge, 2003). In addition, insulin and growth factors activate Rac1 through PI3K (phosphatidyl-inositol 3 kinase) and possibly SFKs and this leads to the activation of GEFs including VAV, Tiam1 and Sos (Kjoller and Hall, 1999; Welch et al., 2003). The Ras-family proteins can also crosstalk where activation of Cdc42, Ras and RhoG leads to activation of Rac1 (Kjoller and Hall, 1999).

The activated Rac1 in its turn activate several downstream effectors including PAKs, PI4P5K, insulin receptor tyrosine kinase substrate p53 (IRSp53), which all are involved in actin rearrangements. PAKs are serine/threonine kinases that among others phosphorylate and thus inhibit MLCK, which leads to reduced actomyosin assembly, they phosphorylate LIMK, which inhibits coflin activity, and relocalize cortactin to membrane ruffles, leading to stabilization of F-actin (Bishop and Hall, 2000; Vidal et al., 2002). IRSp53 links Rac1 activation to WAVE2 to induce the Arp2/3 complex, while PI(4)P5K makes PI(4,5)P2 leading to local actin filament uncapping to allow further elongation of actin (Ridley, 2001a).

Cdc42 and filopodia
Injection of active Cdc42 into NIH3T3 cells induces formation of focal complexes and the formation of filopodia, which are long, thin, parallel, tight bundles of F-actin that protrude the cell membranes (figure 5; Hall, 1998; Wood and Martin, 2002). Filopodia are sensors that lead the cell into the right direction in response to cell migration (see below) or guides the growth cones of axons to their targets (Koleske, 2003; Robles et al., 2003).

The group of Borisy has recently made great advances in understanding the formation of filopodia in vitro and the initiation of filopodia in vivo. They managed to induce filopodia formation in vitro on WASP-coated beads with the addition of diluted cell lysates. The actin structures close to the beads resembled lamellipodia and depended on WASP and Arp2/3 for actin nucleation. Structures further away showed the typical parallel actin bundles of filopodia, was stabilized by fascin and incorporated actin at the tips of uncapped barbed ends (Vignjevic et al., 2003). They also showed that the filopodial bundles in B16F1 melanoma cells are initiated by a gradual reorganization of the long actin filaments in the lamellipodial dendritic network to form Λ-shaped precursors at the edge of the lamellipodium. These Λ-precursors merged with other Λ-precursors that matured into filopodia (Svitkina et al., 2003). The filopodia and particularly the tip complex inicial adhesions of the filopodium is enriched with fascin that bundles actin, N-WASP, talin, Ena/VASP, myosin X, Syndapin I, VAV, Abl and Abl interacting proteins (Abi), but the individual roles of these proteins have not been fully determined (Bartles, 2000; Berg and Cheney, 2002; DePasquale and Izzard, 1991; Ho et al., 2001; Kranewitter et al., 2001; Kureishy et al., 2002; Lanier et al., 1999; Qualmann and Kelly, 2000; Rottner et al., 1999a; Stradal et al., 2001). Thus, the Cdc42-dependent initiation of filopodia is a WASP- and Arp2/3-dependent process and this is followed by extension of F-actin in the tip to form filopodia. In addition to Cdc42, other small GTPases also induce filopodia in certain situations. These GTPases include TC10, Chp, Wrch-1, RhoD and Rif (Aronheim et al., 1998; Aspenström et al., 2004; Ellis and Mellor, 2000; Neudauer et al., 1998; Tanabe et al., 2000; Tao et al., 2001).

RhoA and stress fibers
In contrast to Rac and Cdc42 that induces new actin polymerization and are involved in protrusive events, RhoA is downregulated in the protrusive elements (Jaffe and Hall, 2003) and only causes reorganization of existing filaments associated with retractive events (Machesky and Hall, 1997). Active RhoA induces focal adhesions and the assembly of stress fibers (figure 5), which are antiparallel bundles of F-actin that can contract with a similar mechanism as muscles (Hall, 1998; Ridley, 1997). RhoA activate the downstream effectors mouse Diaphanous (mDia), Rho kinase and LIM kinase, where Rho kinase stimulates the myosin contractility, mDia effect the formation of actin bundles and LIM kinase
inhibits coflin-induced degradation of F-actin (Geiger and Bershadsky, 2001; Kaverina et al., 2002; Maekawa et al., 1999; Pruyne et al., 2002; Watanabe et al., 1999).

**The Rac-GEF Dock180**

Mammalian Dock180 was identified as a protein that, when targeted to cell membranes, induces cell spreading and is involved in phagocytosis of apoptotic cells. Dock180 contains an unconventional Rac-GEF motif and activates Rac following integrin activation by binding to Crk, which binds to p130Cas, and Dock180, and thus links the migration promoting p130Cas-Crk scaffold to Rac activation (Brugnera et al., 2002; Cote and Vuori, 2002; Kiyokawa et al., 1998a; Kiyokawa et al., 1998b). Dock180 contains a basic region that interacts with PI(3,4,5)P3, which recruits Dock180 to the cell membrane in a PI3K-dependent manner (Kobayashi et al., 2001). In addition, Nck binds to Dock180 in yeast-two hybrid system (Tu et al., 2001). Another Dock180-interacting protein, Elmo, has been shown to enhance the Dock180-mediated Rac activation by an upstream binding to activated RhoG (Katoh and Negishi, 2003).

**Cell spreading**

Cells adhere and spread in two dimensions on a surface coated with integrin-binding proteins as fibronectin or vitronectin. This is to increase the adhesive strength to the matrix and to cover large areas with only a few cells. These cells show filopodia and lamellipodia protrusions all around the cell, which act to protrude the cell membrane away from the nucleus. The tips of filopodia and the rim of lamellipodia contain integrins that anchor the cell to the matrix by the formation of initial adhesions and focal complexes, respectively. During further spreading, these adhesion points are pulled toward the cell center due to the formation of contractile actin cables that associate to opposing matrix adhesions and pull them toward each other (Smilenov et al., 1999). In contrast, migrating cells usually have stable strong focal adhesions in the leading edge, toward which the contractile stress fibers pull the rest of the cell (see below; Smilenov et al., 1999).

**Cell migration**

Migration of cells is essential to life since cells have to migrate during development, but also in adults for repair of damage and for defense. However, cell migration is bad in some situations, including cancer (metastasis), atherosclerosis and chronic inflammatory diseases. Thus, understanding the complex cell migration process could lead to identification of new targets to treat these diseases. Many studies concerning migration have been made in Boyden chambers, which consists of a two-chamber unit (upper and lower chamber) separated by a porous membrane (Wuyts et al., 2003). In this assay, cells moving from the upper chamber through the membrane, in response to attractants in the lower chamber or attractants coated on the lower side of the membrane, are scored as chemotactically or haptotactically migrating cells, respectively. The migration of cells can also be directly monitored using another two-chamber assay system, the Dunn chamber (Zicha et al., 1997). In addition, scratch wound assays are used where cells are allowed to grow to confluence on a 2D matrix followed by removal of some cells by scratching them off the substrate to mimic a wound. The cells in the edge of the wound are then studied for migration into the wound area. To date two forms of migration have been observed, polarized migration and round morphology migration.

**“Polarized migration”**

The migration of a polarized cell is divided into five steps: i) polarization of the cell, ii) protrusion of the front by formation of filopodia and lamellipodia with adhesion to integrins and the formation of focal complexes in the leading edge of the cell, iii) firm adhesion to the substratum with stationary focal adhesions, which are developed from the focal complexes, iv) traction where the body of the cell moves...
forward toward the focal adhesions with the help of contractile stress fibers and v) release of the rear end (Hall and Nobes, 2000; Holt and Koffer, 2001; Webb et al., 2002).

The ability of a cell to decide where to migrate (directional cell migration) is as critical as its ability to migrate since random migration rarely serves a purpose. The initial visible stage of directional cell migration is the formation of cell polarity. Polarized cells are cells that have a lamellipodia- and filopodia-rich protrusion in the front, the golgi and the MTOC (microtubule organizing center) directed towards the leading edge, a pool of stable microtubules, which are oriented towards the leading edge, and a rear end that is marked by large focal adhesions (Palazzo et al., 2001). The mechanisms behind cell polarization differs markedly between different kinds of cells, but Cdc42 is usually involved in this process (Small and Kaverina, 2003).

The second step of migration is generally considered to be regulated by Rac1, which activates lamellipodia formation and form focal complexes. In some cells, filopodia initiated by Cdc42 is seen, and these structures are considered as sensors for migration (see Cdc42 and filopodia section). At this stage, Rho is suppressed by PKC and Src-mediated tyrosine phosphorylation of p190RhoGAP and/or degraded in response to ubiquitination by Smurf1 (Arthur and Burridge, 2001; Hall, 1998; Nobes and Hall, 1999; Troller et al., 2004; Wang et al., 2003a). The dense F-actin network of the lamellipodium is protruding forward by the F-actin treadmilling process (figure 4; Small et al., 2002). Some F-acts remain after depolymerization in the rear of lamellipodia and these contribute to the contractile stress fibers, which together with the formation of focal adhesions, forms in response to upregulation of RhoA, potentially mediated by FAK (Small et al., 2002; Zhai et al., 2003). Stress fibers contract the cell body and move the whole body in the direction of the leading edge, and the front of the cells have to adhere to the substratum to allow this contraction (Ridley, 2001a).

The cells need a way to disassemble the focal complexes in the back of the leading edge and the focal adhesions in the rear in order to allow migration and the mechanism behind this is not clearly elucidated. One way to disassemble focal adhesions seems to be to guide dynamic microtubules to a focal adhesion, which leads to turnover of that specific focal adhesion possibly by microtubule-assisted local delivery of a relaxing factor that dissolve the focal adhesion (Kaverina et al., 1999). In addition, Rac1, RhoA, H-Ras, FAK, Src, calpain, SHP-2, PTP-PEST, growth factor receptor activation and ICAP-1α have all been implicated in the disassembly process (Bouvard et al., 2003; Nobes and Hall, 1999; Schoenwaelder and Burridge, 1999; Webb et al., 2002). The detachment of the rear end of a cell can occur by calpain cleavage of rear focal adhesions particularly in slow moving cells and the other factors mentioned in the process of focal adhesion disassembly could also be involved in this process (Ridley, 2001a).

**Round morphology migration**

Interestingly, Sahai and Marshall (2003) recently identified another mode of migration by tumor cells, where the cells migrated with a round morphology without cell polarity and independent of Rac. Instead, this migration depended on RhoA, Rho kinase and ezrin. The cells that moved with round morphology still moved toward a chemoattractant, thus, knew in what direction to migrate (Sahai and Marshall, 2003). Hence, it appear that cells can adapt their migratory behavior depending on the environment.

**Phagocytosis**

Phagocytosis is a process to ingest and clear large particles (>0.5 µm), including bacteria, senescent cells and cellular debris. This is associated with food uptake in lower unicellular organisms, with tissue homeostasis and remodeling in metazoan while in higher eukaryotic organisms such as mammals phagocytosis is associated with host defense by allowing clearance of infectious agents and by participating in the immune and inflammatory responses (Chavrier, 2001).
Cells that have the capacity to phagocytose particles are denoted phagocytes and can be divided in non-professional, paraprofessional and professional phagocytes, depending in their efficiency to phagocytose. The professional phagocytes, which usually are the cells denoted phagocytes, including monocytes/macrophages, neutrophils and dendritic cells, are equipped with an array of specialized phagocytic receptors and are very efficient in phagocytizing particles. Non-professional and paraprofessional phagocytes, which virtually can be any kind of cell, also have the capability to ingest particles, however, less efficiently since they only have a limited amount of devoted receptors (Vieira et al., 2002).

Professional phagocytes have opsonic phagocytic receptors, which include the Fc receptors (FctrR, FceR and FcgR) and complement receptors (CR1 and CR3). These receptors bind to opsonized (host-derived proteins that coat the surface of a particle to make the particle susceptible for phagocytosis) particles. There are also receptors that bind directly to ligands naturally expressed by the particle, these includes the β1-, β3- and β5-integrins, CR3, endotoxin receptors, mannose receptor, galactose receptor, scavenger receptors and β-glucan receptor (Tjelle et al., 2000) and these receptors are found in professional phagocytes, but some as integrins are also found in other cells.

The start of phagocytosis occurs by the interaction of these specific surface receptors of the phagocyte with ligands on the particle. The mechanism and structures involved in the initial phagocytic process depends on the receptor involved, but common to the initial stage of internalization through all receptors is that it requires polymerization of actin, kinase activation, alteration in phospholipid metabolism and acceleration of membrane production (Underhill and Ozinsky, 2002; Vieira et al., 2002).

This initial stage of phagocytosis is followed by formation of a cup-like pseudopod around the particle, which becomes included into the cup, which is sealed by cell membrane fusion to form a closed phagosome. In addition, other mechanisms exist, where the cell ingests the bacteria without extending pseudopods. The closed phagosome then buds off from the membrane and the phagosome mature into a phagolysosome after undertaking several events of fusion and fission to other vesicles to modify the composition of their limiting membrane and of their content in a very complex process involving around 600 proteins (Desjardins, 2003; Tjelle et al., 2000). The phagolysosomes possess a number of degradative properties including a very low pH, hydrolytic enzymes, defensins and the ability to generate toxic oxidative compounds and thus is well equipped to clear bacteria (Hampton et al., 1998; Tapper, 1996; Tjelle et al., 2000).

**Yersinia**

There are three human pathogenic *Yersinia* species, *Yersinia pestis*, *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* and at least eight more that not are considered to be human pathogens but could be opportunistic or pathogenic to other species (Smego et al., 1999; Sulakvelidze, 2000). The *Yersinia* species belong to the Enterobacteriaceae family, are gram-negative facultative anaerobic non-sporing rods or coccobacilli and can grow in temperatures from 4°C to 40°C (Nihlén, 1969). *Yersinia sp.* grow on McConkey agar plates, are catalase positive, oxidase negative and ferment glucose with the production of acid (Smego et al., 1999). *Yersinia pestis* forms an envelope at 37°C, is non-motile and does not survive freely in nature since it is unable to synthesize several vitamins, amino acids and intermediary metabolism enzymes (Brubaker, 1991). *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* are flagellated and motile at 25°C, but become non-motile at 37°C in the host (Boyce, 1985).
Pathogenesis of *Yersinia* infections

**Plague**

*Yersinia pestis* is the causative agent of bubonic plague, being responsible for the death of millions of people over the years, particularly during three pandemics: i) the Julian plague 541-750 AD, ii) the Black Death 1347-1351 which lasted well into 17th century in Europe and iii) the pandemic that started in China 1855 and lasted to around year 1940 (Perry and Fetherston, 1997). Sporadic outbreaks of plague around the world still occur today, mainly in the southeastern region of Africa and Madagascar, but also in Asia and the Americas (Perry and Fetherston, 1997). 1894, during a plague attack in Hong Kong, Alexandre Yersin isolated and identified the bacteria causing plague in humans and rats, and this bacterium was later given the name *Yersinia pestis* (Perry and Fetherston, 1997; Yersin, 1894).

*Yersinia pestis* is a zoonosis that is maintained in infected wild rodents and is transmitted among animals and occasionally to humans by the bite of an infected flea or by direct contact with infected animals. When a flea has ingested a blood meal from an infected animal, the bacteria induce clotting of the blood in the flea’s foregut, preventing ingestion of subsequent blood meals. Bacteria multiplying in the foregut can then easily infect a new host as the flea tries in vain to feed (Perry and Fetherston, 1997). When the population of the natural flea host has diminished, the flea will look for other hosts, such as humans, to feed. This will lead to the onset of bubonic plague. The first signs of bubonic plague are typically onset of fever, chills, headache and weakness, followed by swelling and tenderness of lymph nodes (buboes), which occurs during the time the bacteria colonize the lymph nodes close to the injection site. Later some bacteria evade the lymph nodes and spread through the blood stream to deeper organs such as liver, spleen and lungs. Bacteria in the blood stream will cause sepsis, while the bacteria in the lung results in severe pneumonia. Patients with pneumonic plague are highly contagious and spread the bacteria through aerosols, which cause primary pneumonic plague in the recipient. Both forms of plague are severe systemic diseases where the bubonic plague has a lethality of 60-80% in untreated otherwise healthy individuals while the pneumonic form is almost 100% lethal unless treated within the first day post-infection (Smego et al., 1999).

**Yersinosis**

*Yersinia enterocolitica* and *Yersinia pseudotuberculosis* cause an enteric infection in humans, called yersinosis. This is a zoonotic disease, which transmits to human by infected beverages and food or by direct contact with infected mammals where pigs are the major reservoir (Bottone, 1999; Smego et al., 1999). The disease occurs mainly in regions with temperate climates especially during winter months rather than tropical or sub-tropical climates (Smego et al., 1999).

The infection route of non-plague *Yersinia* occurs through the ileal mucosa in the gastrointestinal tract where they are taken up into the lymphoid follicles through M-cells. These specialized epithelial cells cover the lymphoid follicles of Peyer’s patches and engulf bacteria in a way that resembles active phagocytosis (Grassl et al., 2003). The bacteria multiply within the Peyer’s patches, which are intestinal lymph glands, and then drain to mesenteric lymph nodes. The clinical manifestations of yersinosis occurs 3 to 7 days after infection and is characterized by a variety of gastrointestinal symptoms ranging from diarrhea, abdominal pain and fever to more severe mesenteric lymphadenitis lasting for 5-14 days as the disease is usually self-limiting (Smego et al., 1999). In some cases, sequelae such as reactive arthritis (Reiter’s syndrome; about 10 to 30% of diagnosed adult cases of yersinosis in Scandinavia) and uveitis can occur. This risk is enhanced in individuals having the HLA-B27 tissue antigen (Appel et al., 1999).

Although nonplague yersinosis rarely becomes systemic/septicemic, this form of the disease is highly mortal despite treatment and may include abscess formation in liver and spleen, pneumonia, meningitis, septic arthritis and other life-threatening symptoms (Smego et al., 1999). A suggested model for how yersinosis can become systemic has recently been presented. It suggests that the bacteria, following uptake through M-cells, infect epithelial cells from the basal side, triggering the host to secrete...
proinflammatory cytokines that recruit immune cells including neutrophils, macrophages and T-cells to the infection site (Grassl et al., 2003). However, since Yersinia are probably already primed to induce the Yop weaponry (see below), these bacteria would be resistant to immune cell phagocytosis causing suppression of the immune system (Orth, 2002; Rosqvist et al., 1988). In the event that the host is unable to kill the bacteria, the latter could hitchhike with the immune cells to disseminate to other parts of the body, such as the liver and spleen. In most cases, however, humans are sufficiently equipped to kill the bacteria before they spread from the ileal tract (Grassl et al., 2003). On the other hand, mice are readily susceptible to Yersinia yersiniosis, since the disease is systemic (Heesemann et al., 1993).

**Bacterial adhesion to the host**

The essential, initial stage of a Yersinia infection is adherence and penetration of the epithelial barrier of the ileum. The enteropathogenic Yersinia species have three or four proteins with the ability to adhere to host tissues, including YadA, invasin, pH6Ag and ail, where invasin and YadA can trigger phagocytosis of the bacterium.

**YadA**

YadA is a protein expressed on the virulence plasmid by Yersinia enterocolitica and Yersinia pseudotuberculosis, but not in Yersinia pestis. The YadA protein is produced at 37°C independent of calcium and makes up a homotrimeric fibril with a “lolli-pop”-like shape forming a fibrillar matrix covering the bacterium (El Tahir and Skurnik, 2001). This dense matrix increases the surface hydrophobicity of the bacterium, is involved in autoagglutination, contributes to resistance of complement-mediated killing by serum, and in the inhibition of the anti-invasive phenotype of interferon (El Tahir and Skurnik, 2001). The protein binds to mucus, β1-integrins, laminin, collagen and fibronectin (El Tahir and Skurnik, 2001). YadA has been implicated in numerous biological activities including the formation of fibrillae (Kapperud et al., 1987), bacterial adhesion to cultured epithelial cells (Heesemann and Grüter, 1987) and bacterial attachment and invasion of host tissues (Eitel and Dersch, 2002; Kapperud et al., 1987). However, while it is required for virulence of Yersinia enterocolitica, it is not required by Yersinia pseudotuberculosis (El Tahir and Skurnik, 2001).

**Invasin**

Invasin is a 103-kDa chromosomally encoded outer membrane protein expressed by Yersinia enterocolitica and Yersinia pseudotuberculosis, but not Yersinia pestis (Rosqvist et al., 1988). It binds to β1-integrins (α3β1-, α4β1-, α5β1- and αvβ1-integrins) of the host cell and triggers uptake of the bacterium if there is a high density of invasin (Isberg et al., 2000; Isberg and Leong, 1990; Isberg and Tran Van Nhieu, 1994; Isberg et al., 1987; Plow et al., 2000). The maximal expression of invasin occurs at 26°C at a pH of 8.0 and in early stationary phase, which is a plausible environment for meat or beverages from wherein the bacteria spread. The expression is also high at 37°C in pH 5.5, implying that invasin is expressed in the stomach and intestine of the host. Invasin plays a role in the early stages of infection by enhancing uptake through intestinal M-cells, allowing the bacteria to colonize the Peyer’s patches (Isberg et al., 2000). Invasin expression is reduced at 37°C at a neutral pH, implying that invasin may not be needed during

![Figure 6: Crystal structure of the integrin-binding domains of invasin and fibronectin.](image)
later stages of infection (Isberg et al., 1988; Pepe et al., 1994).

Invasin has an outer membrane region in the first ~500 N-terminal amino acids making up a β-barrel while the C-terminal 497 amino acids makes up the extracellular region, which contains five tandem domains (D1-D5, where D1 is at the N-terminal end; Hamburger et al., 1999; Leong et al., 1990). D1 to D4 have the folding structure resembling the eukaryotic immunoglobulin superfamily while the D5 has a folding topology related to that of C-type lectin-like domains (Hamburger et al., 1999). D2 contains an invasin cross-linking domain allowing invasin to form homomultimers, which makes invasin more efficient in mediating internalization (Dersch and Isberg, 1999). D4 and D5 correspond to the C-terminal 192 amino acids, which makes up the integrin-binding region and are sufficient to promote uptake (Leong et al., 1990; Rankin et al., 1992).

As invasin, fibronectin bind to integrins with a two-domain structure, Fibronectin type III repeats 9 and 10 (D9 and D10). Fibronectin and invasin binds to the same or overlapping regions of β1-integrin where invasin binds with high affinity while fibronectin binds with low affinity (100-fold lower than invasin). The integrin-binding domains of fibronectin and invasin show a similarity of the relative position of several residues implicated in integrin interactions including two aspartates and an arginine (D811, R883 and D911 of invasin or D1373, R1379 and D1495 of fibronectin; figure 6; Hamburger et al., 1999; Leong et al., 1995; Saltman et al., 1996). However, the overall folding topology and surface structures of the integrin-binding regions of invasin and fibronectin differ markedly. The D4 and D5 of invasin interacts extensively and create a rigid, rather flat domain that has a large integrin-binding surface, while the D9 and D10 of fibronectin contain a cleft, is flexible and only has a small integrin-binding surface (figure 6; Hamburger et al., 1999; Leahy et al., 1996; Rankin et al., 1992; Tran Van Nhieu and Isberg, 1993; Van Nhieu and Isberg, 1991; Van Nhieu et al., 1996). This could explain the difference in integrin-binding affinity.

**pH6 Antigen**

pH6 antigen (pH6 Ag) is a flexible fimbriae that originally was identified as an antigen produced only when *Y. pestis* was cultured at temperatures above 36°C and in a pH lower that 6.7, and this protein is also expressed in *Y. pseudotuberculosis* and *Y. enterocolitica* (Ben-Efraim et al., 1961; Lindler et al., 1990). This antigen causes haemagglutination, promotes adhesion to mammalian cells, is induced by intracellular adhesion with macrophages and bind to Fc-receptors of macrophages (Bichowsky-Slomnicki and Ben-Efraim, 1963; Lindler and Tall, 1993; Yang et al., 1996; Zav’yalov et al., 1996). pH6Ag mutants of *Y. pestis* show reduced virulence in a murine infection model (Lindler et al., 1990) but the role of pH6Ag in *Y. pseudotuberculosis* and *Y. enterocolitica* is still unclear.

**Ail**

Accessory invasion locus (Ail) was identified as a 17-kDa protein found in pathogenic *Yersinia* species (Miller et al., 1989). It promotes tissue culture adherence and invasion into several cell lines and contributes to serum resistance at 37°C in *Yersinia enterocolitica* (Pierson and Falkow, 1993). The expression of Ail is greater at 37°C compared to ambient temperature (Bliska and Falkow, 1992). However, Ail is not involved in virulence in a murine infection model (Wachtel and Miller, 1995). Moreover, Ail of *Yersinia pseudotuberculosis*, did not exhibit any adhesive activity, but still contributed to serum resistance (Yang et al., 1996).

**Yersinia weaponry and administration of these**

*The virulence plasmids of human pathogenic Yersinia*

Human pathogenic *Yersinia* species contain a ~70-kb plasmid that is necessary for virulence (Portnoy et al., 1981). This 70-kb virulence plasmid encodes: a type three secretion system (TTSS), a
translocation system, at least six virulence effector proteins and regulators of the secretion and translocation apparatus (figure 7). *Y. pestis* has two additional plasmids that contribute to the increased virulence and unique infection route of this species, one plasmid of 9.5 kb, which encodes the Pla protease and the other of 110 kb, which encodes the murine toxin and the fraction 1 (F1) capsule-like antigen (Ferber and Brubaker, 1981). Apart of the virulence proteins associated with the plasmids, *Yersinia* also harbors potential virulence genes on the chromosome, such as the genes encoding for invasin, RovA, pH6Ag and ail (Revell and Miller, 2001).

### The type III secretion system

The term secretion is used to describe the transport of proteins from the bacterial cytoplasm to the extracellular space and the secretion through TTSS occurs in a continuous process without the presence of distinct periplasmic intermediates. Some proteins secreted by the type III secretion system (TTSS) are also dedicated to the further transport of anti-host effectors into the cytosol of eukaryotic cells.

The TTSS is a secretion system that is found in many bacterial pathogens of animals and plants, including human pathogenic *Yersinia* spp., *Shigella* sp., *Salmonella* typhimurium, enteropathogenic *Escherichia coli* (EPEC), *Pseudomonas aeruginosa*, *Xanthomonas campestris*, *Erwinia* spp. (Hueck, 1998). The secretion system is comprised of approximately 20 proteins, including a cytoplasmic ATPase, several proteins located in the inner and outer membranes, those which span the periplasmic space in a composition that resembles the flagellar biosynthesis apparatus and an outer membrane protein that resembles the secretin of type II secretion systems (Hueck, 1998). Proteins secreted by the TTSS are not subjected to amino-terminal processing during secretion. In fact, the secretion signal remains unclear, but resides within the first 15 to 20 amino acids of the secreted proteins or in their 5’-mRNA. The TTSS system in *Yersinia*, which was the first to be identified visually, consists of a basal body-like structure, reminiscent of the bacterial extracellular flagellum and is topped by an extracellular needle-like appendage that protrudes outside the bacterium (Cornelis, 2002). The components of the TTSS apparatus in *Yersinia* are encoded by three operons: yscA to yscL, yscN to yscU (Yop secretion) and lcrD (low calcium response D; Hueck, 1998) and will not be discussed further here.

### Translocation

Tightly linked to the TTSS of *Yersinia* is the translocation system, which couples bacterial secretion to the transport of proteins from the bacterium into the cytoplasm of the host cell. The translocation of Yops is polarized, implying that protein delivery occurs in the direction of the zone of contact between bacteria and the host cell. Thus, no proteins are released into the surrounding extracellular medium and no Yops are actively translocated when the bacterium is internalized (Håkansson et al., 1996b; Persson et al., 1995; Rosqvist et al., 1994; Sory et al., 1995; Sory and Cornelis, 1994). The translocation system depends upon the function of YopB, YopD and LcrV (Håkansson et al., 1996b; Neyt and Cornelis, 1999; Pettersson et al., 1999). LcrV is surface located before target cell contact and antibodies to LcrV block the translocation of Yop effectors into the target cell, indicating that LcrV plays a critical role in the delivery of Yops to the host (Pettersson et al., 1999). Recently it was shown that LcrV has the ability to form pores of defined size in lipid bilayers (Bröms et al., 2003; Holmström et al., 2001). In addition, YopB and YopD may also contribute to pore-formation (Håkansson et al., 1996b; Neyt and Cornelis, 1999; Tardy et al., 1999), since they have domains typical for membrane-spanning proteins (Håkansson et al., 1993). YopK play a modulatory role in translocation by negatively affecting the pore size of the target plasma membrane (Holmström et al., 1997). *Yersinia* that lack YopK due to mutation hypertranslocates Yops,
causing a more rapid cytotoxic response upon infection of HeLa cells, although this mutant is avirulent in mice infection models (Holmström et al., 1997; Holmström et al., 1995a; Holmström et al., 1995b).

**Regulation of secretion and translocation**

*Yersinia* tightly regulates the expression of genes involved in type III secretion, translocation and the production of Yop effectors. At least *in vitro*, this regulatory response is known as the low calcium response (LCR; Hueck, 1998), which is characterized by repression of the type III secretion machinery at 26°C. Moreover, at 37°C in the presence of Ca²⁺, Yop synthesis is also repressed. However, at 37°C in the absence of Ca²⁺, when the TTSS is fully formed, Yop synthesis is heavily induced. Linked to this regulatory response, is the observation that the bacteria are unable to grow when producing Yops (i.e. at 37°C in Ca²⁺-deprived media).

The expression of the TTSS, translocation system and virulence factors are positively regulated by an increase in temperature from 26°C to 37°C. The upregulation of the TTSS depends on the AraC-like transcriptional activator LcrF (VirF in *Yersinia enterocolitica*). It binds to multiple sites along the promoter regions of several genes involved in the TTSS (Cornelis et al., 1989; Cornelis, 1993). The cofactor, YmoA also contributes to TTSS regulation by altering the DNA conformation to facilitate LcrF binding (Cornelis, 1993). The negative regulation of Yop synthesis by Ca²⁺ occurs through a negative feedback mechanism that ensures the production of Yops when the TTSS is completely functional. This is controlled by LcrQ. In high Ca²⁺ conditions, the intracellular concentration of LcrQ is also high and acts negatively on Yop transcription. However in low Ca²⁺ conditions, LcrQ is rapidly secreted through a functional TTSS, which de-represses yop transcription. However, the precise mechanism of this apparent LcrF/LcrQ antagonism is not known. This low calcium-response is only seen *in vitro*. *In vivo* the calcium response is mimicked by the binding of the bacterium to the host cell, which also induces the TTSS and translocation apparatus to allow LcrQ to exit the bacterium (Pettersson et al., 1996).

In addition, other proteins are involved in the LCR. Although their roles are less clear, presumably they are important for fine-tuning. Mutants of *yopN*, *tyeA* and *lcrG* secrete Yops even in the presence of calcium, showing that they are involved in the calcium regulation by providing a gate-keeping function by localizing at the inner and outer extremities of the secretion channel in high calcium-like conditions (Cheng et al., 2001; Cornelis, 2000; Cornelis, 2002; Matson and Nilles, 2001). Hence, only in low Ca²⁺ conditions are the “gates” presumed to be opened to allow secretion. In addition SycH, LcrH/SycD, YscY and YopD are also involved in the LCR (Cambronne et al., 2000; Day and Plano, 2000; Francis et al., 2001; Frithz-Lindsten, 2000; Iriarte and Cornelis, 1999).

**Chaperones**

Some of the Yops have their own specific cytoplasmic chaperone that is important for efficient secretion of the cognate Yop. The Yops that have chaperones and the names of their dedicated chaperones are YopH-SycH, YopE-YerA/SycE, YopT-SycT, YopN-SycN, YopD-LcrH/SycD and YopB-LcrH/SycD (Hueck, 1998). These chaperones are small (15 to 20 kDa), acidic (pI around 4.5) proteins with a potential C-terminal α-helix region, but lack any ATP-binding domain, which usually is a feature of cytoplasmic chaperones (Hueck, 1998). Interestingly, despite their common functions, the chaperones are not extensively similar at the genetic level.

The specific function of these chaperones does not seem to be to pilot the Yops to the secretion machinery (Frithz-Lindsten et al., 1995; Schesser et al., 1996; Sory et al., 1995). Rather, SycE stabilizes YopE prior to secretion and the chaperones seem to play a role in anti-folding of the Yops to maintain them in a conformation that is adequate for secretion. This is consistent with that the chaperone-binding site of YopE and YopH is overlapping with the region of Yops known to be essential for their translocation (Frithz-Lindsten et al., 1995; Schesser et al., 1996; Wattiau et al., 1994; Wattiau and Cornelis, 1993; Woestyn et al., 1996). Interestingly, the translocated Yop effectors that utilize chaperones all are important at the early stages of infection, while the Yop effectors that lack chaperones are involved
in immunomodulatory functions. This suggests that the chaperones regulate the order of secretion and/or translocation (Lloyd et al., 2002).

**Virulence effectors**

**YopH**

YopH is a protein tyrosine phosphatase of 51 kDa that is essential for virulence in mice (Bölin and Wolf-Watz, 1988; Fällman et al., 2002). YopH has an N-terminal region (amino acid 1-17) important for secretion followed by a translocation and SycH binding region (within amino acid 20-70) and the C-terminal PTPase domain (residues 206-468; Guan and Dixon, 1990; Sory et al., 1995; Woestyn et al., 1996). The PTPase activity of YopH is the highest of any PTPase identified to date (Zhang et al., 1992) and a critical residue for the PTPase activity of YopH is Cysteine 403, which is situated in the P-loop of the PTPase domain. Mutation of this cysteine to alanine or serine completely disrupts the PTPase activity (Guan and Dixon, 1990).

The translocation of YopH following infection of host cells leads to a specific and rapid (1-2 minutes) dephosphorylation of target phosphotyrosine proteins of approximately 60 and 120-130 kDa, as well as blocking early Ca²⁺-release and phagocytosis (Fällman et al., 2002). In the later stages of infection (h), the activity of YopH leads to a complete dephosphorylation of almost all tyrosine-phosphorylated proteins in the cell. However, this late response is not important for the anti-phagocytic response, since phagocytosis is a rapid process and the real targets of YopH are thus likely to be the proteins that are rapidly dephosphorylated (Fällman et al., 2002).

To identify these target proteins, a “substrate-trapping” mutant of YopH was used where the cysteine residue of the PTP catalytic signature motif HCXXGXXR was replaced with alanine or serine. This mutant (YopHC403A/S) retains the ability to bind substrate but is completely abrogated in enzyme activity and thus recognizes and form stable complexes with its target substrates. These complexes can further be immunoprecipitated with anti-YopH antibodies followed by recognition of associated proteins by Western blot (Fällman et al., 2002; Tonks and Neel, 2001). In addition, YopH localizes to cell-matrix adhesions (Black and Bliska, 1997; Bliska et al., 1992; Persson et al., 1997). This hinted that YopH most likely had cell-matrix adhesion proteins as main targets and that the C403A mutant could be used to co-immunoprecipitate these proteins. With this knowledge, the substrates of YopH in HeLa cells were identified as two focal adhesion located proteins, p130Cas and FAK (figure 8; Black and Bliska, 1997; Persson et al., 1997).

In accordance with YopHs specificity to focal adhesion proteins, a sequence between amino acids 223-226 is important for the virulence in vivo and the phagocytic blocking in vitro. This sequence might be responsible for bringing the protein to focal adhesions well inside the host cell (Persson et al., 1999). Recently, it has been shown that YopH interacts with tyrosine phosphorylated p130Cas and paxillin via four amino acids in the N-terminal region (Gln11, Val321, Ala33 and Asn34; Black et al., 1998; Montagna et al., 2001). The p130Cas and YopH interaction is also important for the localization of YopH to cell-matrix adhesion and for virulence (Deleuil et al., 2003).

**YopE**

Upon translocation into host cells, YopE causes fragmentation of F-actin leading to rounding up of the cell, while still leaving tail-like cytoplasmic membrane remnants that disappear upon prolonged incubation, leading to the detachment of host cells from the substratum (Rosqvist et al., 1990; Rosqvist et al., 1991; Rosqvist et al., 1994; Rosqvist and Wolf-Watz, 1986). YopE has an N-terminal region that is needed for secretion (amino acid 1-11), translocation (amino acid 15-50) and for stable complex formation and efficient secretion by the chaperone YerA/YscE (79 first amino acids; Schesser et al., 1996; Sory et al., 1995; Woestyn et al., 1996). In addition, YopE is a GAP towards the Rho family of small G-proteins that has been shown to inactivate Rho, Rac and Cdc42 in vitro (figure 5 and 8; Black and Bliska,
Common to all GAPs is an arginine-finger that is essential for the GAP activity (Scheffzek et al., 1998) and in YopE the region surrounding Arg144 makes up an arginine-finger (Black and Bliska, 2000; Von Pawel-Rammingen et al., 2000). Mutation of this arginine completely abolished both cytotoxicity and in vitro GAP activity of YopE showing that the GAP activity is essential for the function of YopE (Aili et al., 2002; Black and Bliska, 2000; Von Pawel-Rammingen et al., 2000). In addition, mutations surrounding the YopE arginine-finger are also important for cytotoxicity and in vitro GAP activity towards Rho, Rac and Cdc42 (Aili et al., 2003). However, YopE mutants that are defective in GAP activity towards Rho, Rac and Cdc42 are still cytotoxic, indicating that YopE may well have other small G-protein targets apart from Rho, Rac or Cdc42 in eukaryotic cells (Aili et al., 2003).

Figure 8: The cellular targets and hypothetical mechanisms for the antiphagocytic effectors of Yersinia. The Yops effectors are secreted and translocated into the host cell following bacterial attachment to β1-integrins. YopH destroys cell-matrix adhesions and associated signaling by dephosphorylating FAK and p130Cas. YopH also inhibits TcR communication to integrins in T cells by dephosphorylation of ADAP and SKAP55, which also are YopH targets in macrophages. YopE inactivates Rho family GTPases to affect actin structures. YopT releases RhoA from the cell membrane by cleaving off the prenyl group of the GTPase. YpkA interacts with Rac and Rho and interferes with regulation of the actin cytoskeleton.

YopT

YopT is a non-essential virulence effector found in Y. enterocolitica and some Y. pseudotuberculosis strains, but not in YPIII/pIB103 of Y. pseudotuberculosis (Iriarte and Cornelis, 1998, Forsberg, Å FOI NBC-defence, Umeå, Sweden, unpublished). Translocated YopT induces cytotoxic effects on the host cell due to its cysteine protease domain. YopT localizes to cellular membranes and irreversibly cleaves posttranslationally modified RhoA near the C-terminus to release it from the membrane by removing the prenyl group in the CAAX motif (Aepfelbacher et al., 2003; Iriarte and Cornelis, 1998; Shao et al., 2002), which inactivates RhoA. This leads to a disruption of actin stress fibers and focal adhesions and thus to a rounding up of the cell. In addition, YopT traps RhoA in the cytosol by releasing it from GDI (guanine dissociating factors; Aepfelbacher et al., 2003). The function of YopT is redundant since YopE also inactivates RhoA.

YpkA

Yersinia protein kinase A (YopO in Y. enterocolitica) is essential for virulence in mice and was identified as a Yop that is targeted to the inner surface of HeLa cell membranes causing the host cell to round up while still maintaining focal adhesion contacts (Galyov et al., 1993; Galyov et al., 1994; Håkansson et al., 1996a). The N-terminus of YpkA is homologous to eukaryotic Ser/Thr protein kinases (Galyov et al., 1993). Upon translocation into the host cell, YpkA binds to actin through the last C-
terminal 20 residues. This binding leads to an intrinsic autophosphorylating activity of YpkA, while the protein is inactive in the bacterium (Galyov et al., 1993; Juris et al., 2000). Active YpkA potentially phosphorylates proteins that play key roles in the actin cytoskeleton leading to the depolymerization of F-actin and to the rounding up of the cells (Juris et al., 2000). YpkA has also been shown to bind to the small GTPases RhoA and Rac-1 independent of YpkA phosphorylation and nucleotide loading of RhoA and Rac-1 (Barz et al., 2000; Dukuzumuremyi et al., 2000), but the function of this remains elusive.

**YopM**

YopM is essential for full virulence of *Yersinia* in the mouse model (Cornelis et al., 1998). It is an acidic protein composed almost entirely of leucine-rich repeats, which are thought to be protein-protein interactive motifs (Kobe and Deisenhofer, 1994; Leung and Straley, 1989). YopM binds to thrombin but not prothrombin and it competitively inhibits thrombin-elicited aggregation of platelets, but not aggregation by other agonists (Leung et al., 1990; Reisner and Straley, 1992). However, this is not the reason for its essentiality in virulence, since a YopM mutant lacking thrombin-binding features is still completely virulent (Hines et al., 2001). Once translocated into the host cell, YopM associates with vesicles and traffics to the nucleus (Skrzypek and Straley, 1996). Recently McDonald et al. (2003) showed that YopM interacts and activates two intracellular proteins, protein kinase C-like 2 (PRK2) and ribosomal S6 protein kinase 1 (RSK1). However, despite these studies, the function of YopM is still unknown.

**YopJ**

YopJ (YopP in *Y. enterocolitica*) is an ubiquitin-like protein protease. These proteases disrupt highly conserved ubiquitin-like molecules, which are covalently added to numerous regulatory proteins (Orth, 2002; Orth et al., 2000). However, a role for YopJ in virulence in the mouse model is not clear at this stage (Monack et al., 1998). YopJ inhibits several signaling responses when translocated into the host cell, including induction of apoptosis in macrophages possibly by blocking the activation of NFκB (Mills et al., 1997; Monack et al., 1997; Schesser et al., 1998). In addition, YopJ downregulates the expression of JNK, MAP kinase p38 and ERK (extracellular regulated kinase) in macrophages (Palmer et al., 1998; Palmer et al., 1999). The inactivation of NFκB and the MAPKs occurs by inhibition of the upstream superfamily of mitogen activated protein kinase kinases, IKKβ and MKKs respectively (Orth et al., 1999). YopJ also suppresses the secretion of two cytokines, IL-8 and TNFα, in *Yersinia*-infected HeLa cells (Schesser et al., 1998).
Aim

The aim of this thesis is to get a deeper understanding in how the actin cytoskeleton is regulated, in particular in the mediation of bacterial uptake (phagocytosis) and during cell spreading.

The aim of the first paper was to elucidate the protein targets for YopH in macrophages, since these cells most likely are important targets for the antiphagocytic activity of *Yersinia*.

In paper II, we aimed at elucidating signaling mechanisms of importance for \(\beta_1\)-integrin-mediated uptake of bacteria.

In paper III, the aim was to understand the spreading morphology that the GD25\(\beta_1\)B cells form on invasin (which turned out to be filopodia) and to elucidate which signaling mechanisms that are involved in this spreading.

In paper IV, the aim was to elucidate the role of the actin motor myosin X in the formation of the GD25\(\beta_1\)B filopodia.
Results and discussion

What are the targets for YopH in macrophages (paper I)

In previous studies in our group, YopH was shown to dephosphorylate FAK and p130Cas in HeLa cells (see YopH section this thesis; Persson et al., 1997). In this study, the targets of YopH in monocytes/macrophages were elucidated. Macrophages, that distinct from HeLa cells, are professional phagocytes and thus supposedly the target cells for YopH activity. To identify proteins that interact with YopH, cell lysates from cells infected with the YopHC403A (PTPase-inactive substrate trapping; see YopH section of this thesis) mutant were immunoprecipitated with anti-YopH antibodies, and tyrosine-phosphorylated proteins interacting with YopH were analyzed by Western blot. Two distinct bands, one around 120 kDa and one around 76 kDa, were found. The interaction between YopHC403A and the 120-kDa phosphotyrosine proteins occurred immediately upon infection while the 76-kDa phosphotyrosine protein(s) only could be seen after 20 minutes of infection, indicating that the former are more likely to be involved in antiphagocytosis while the later are not.

To identify the 120-kDa phosphotyrosine proteins targeted by YopH, Western blotting with antibodies directed against various proteins in the size of 120 kDa were performed. As seen in HeLa cells, YopHC403A did interact with p130Cas, however FAK or its close relative Pyk2 could not be found in the YopHC403A immunoprecipitates. Interestingly, we could identify ADAP as a target for YopH in macrophages. This protein interacted with YopHC403A and became phosphorylated upon infection with a multiple yop mutant strain (MYM that among other lack YopH expression) or MYMYopHC403A. In addition, ADAP and p130Cas were both dephosphorylated when infected with a Yersinia strain expressing wild type YopH. Thus, strongly indicating that they are targets of YopH and the constituents of the 120-kDa band.

The finding of ADAP as a target of YopH is intriguing since ADAP only is expressed in hematopoietic cells. The role in macrophages is still elusive, but ADAP has been shown to upregulate FcεR-mediated degranulation release in mast cells (Geng et al., 2001) and to be involved in TCR-stimulation and FcγR-mediated phagocytosis by linking SLP-76 to VASP. The ADAP-VASP-SLP76 complex is involved in regulation of integrin activity, and VASP also interacts with actin (Coppolino et al., 2001; Peterson, 2003). Thus, ADAP could be an important target for YopH to block phagocytosis, but further work is needed to fully understand the role of ADAP in macrophages.

In the previous study in HeLa cells, it was shown that the YopHC403A mutant localized to peripheral cell-matrix adhesions and that FAK and p130Cas were recruited to these sites following infection (Persson et al., 1997). To determine if the situation was similar in macrophages, J774A.1 cells were infected with the MYMYopHC403A strain and were labeled by immunofluorescent methods with the cell-matrix adhesion marker vinculin and YopH, which were shown to co-localize at focal complexes. Following infection, p130Cas localized at these structures while ADAP rather was enriched in and around the focal complexes. In addition, infection with wild type YopH disrupted these sites. Thus, as in HeLa cells, YopH targets peripheral cell-matrix adhesions and dephosphorylate proteins associated with these sites causing disruption of the adhesions.

Later studies have also identified SKAP-HOM as a target of YopH in monocytes/macrophages (Black et al., 2000). The function of this protein is still largely unknown, but it is activated following integrin ligation, localizes to membrane ruffles and associates with ADAP, indicating that it, as ADAP, could be involved in phagocytosis. The targets for the PTPase activity of YopH (p130Cas, FAK, ADAP, SKAP55HOM) are thus all involved in β1-integrin mediated signaling events and suggests that the role of YopH is to inactivate signaling from the receptor to which Yersinia binds.
Which parts of the cytoplasmic tail of β1-integrins are important for uptake of Yersinia and spreading on invasin (paper II)

β1-integrins bind with high affinity to invasin of Yersinia and this interaction promotes uptake of the bacterium (Isberg et al., 2000). To elucidate signaling mechanisms of importance for β1-integrin-mediated uptake of bacteria, a β1-integrin-knockout mouse cell line of fibroblastic origin, GD25, was used (Fässler et al., 1995). This cell line was transfected with wild type β1B, β1A (Armulik et al., 2000; Wennerberg et al., 1996) or various mutants of β1A (Sakai et al., 1998; Wennerberg et al., 2000; Wennerberg et al., 1998; paper II). GD25 and the stable transfectants were then studied with regard to bacterial binding and uptake when exposed to a multiple Yersinia mutant (MYM) or to morphology when spread on invasin-coated surfaces. This cell model system is very handy for these studies since these cells lack endogenous β1-integrin expression, the cells are adherent and the interference of other ligands as fibronectin can be excluded by doing the analysis in serum-free conditions. However, since these cells are fibroblasts and thus synthesize matrix proteins, the protein synthesis inhibitor, cycloheximide, was added to the cells during the experiments. In addition, these cells express β3-integrins, but these were blocked by the integrin-binding blocking peptide GRGDS, which at the concentration used specifically block β3-integrins (Wennerberg et al., 1998). During these conditions the GD25 cell line was unable to bind bacteria, while all the β1-integrin transfectants could, which shows that the binding and subsequent uptake is strictly dependent on the interaction between β1-integrin and invasin. β1A mediated uptake of MYM while the β1B splice variant was negative for uptake, showing that the cytoplasmic tail of β1A is important for uptake.

The cytoplasmic tail of β1A contains two NPXY motifs (NPIY and NPKY) with a double threonine site between them, and these three sites can potentially be phosphorylated. To investigate their importance for uptake of Yersinia, GD25 cells stably transfected with the β1A splice variant carrying point mutations of the tyrosines or threonines were infected with MYM. The single or double mutations of the NPXY motifs to NPXF did not have any affect on the uptake of MYM and the cells showed a similar morphological spreading pattern as β1A. This implies that the tyrosines of the β1-integrin not have to be phosphorylated for uptake to occur. The phosphorylation of the NPXY motif is important for the interaction with some proteins, including IRS-1 (insulin receptor substrate 1) and Shc, but other proteins including X11, Fe65 and Disabled have been shown to bind to non-phosphorylated NPXY motives (Schlessinger and Lemmon, 2003; Yan et al., 2002). The phosphorylation of the first tyrosine of β1A-integrins has been shown to inhibit binding of talin (Tapley et al., 1989) indicating that phosphorylation of this site acts to negatively regulate integrin-actin interactions.

However, in contrast to the tyrosine to phenylalanine mutations, the expression of β1A-integrins with a mutation of the first tyrosine to alanine (Y783A) in GD25 cells resulted in very low uptake of MYM, without affecting binding, and these cells were also affected in spreading. These differences most likely depend on the nature of the substituting amino acids. Phenylalanine is very similar to tyrosine and can thus maintain the tight turn conformation of the NPXY motif and allow protein-protein interactions, but cannot be phosphorylated. In contrast, the substitution with the smaller amino acid, alanine, causes a conformational change, and alanine cannot be phosphorylated. These results thus imply that the NPIY motif is important for bacterial uptake and cell spreading but does not need to be phosphorylated.

The mutation of one threonine to alanine in the double threonine site did not have any effect on uptake or spreading of the cells, while GD25 cells expressing the β1-integrin double threonine to alanine mutation only phagocytosed slightly better than the β1B-expressing cell line. When looking at the morphology of the double threonine mutated cell line, it was similar to wild type β1A, but the cells were less spread when plated on invasin. The double threonine site has been shown to be crucial for the activation state of β1-integrins and the TT788-9AA mutation abolishes binding to fibronectin and the 9EG7 antibody, which only recognizes ligand-binding forms of integrin (Wennerberg et al., 1998). In addition, this site has been shown to bind to ICAP-1α, which however is involved in disruption of cell-
matrix adhesions and does not localize to these structures (Bouvard et al., 2003). Thus, this result implies that invasin has the ability to interact with integrins also when integrin is in a low-affinity state for ligand binding. However, the GD25β1B cells were markedly affected in cell spreading. Thus, the cytoplasmic tail of β1A is important both for uptake and cell spreading where at least the first (unphosphorylated) NPIY motif and the double threonines play important roles possibly by allowing the correct conformation of the cytoplasmic interaction sites of integrin.

Which proteins are important for filopodia formation (paper II and III)

We made an intriguing observation during the investigation of spreading of the β1-integrin-expressing GD25 cells on invasin. The initial observations indicated that the GD25β1B cells were unable to spread. In these experiments, the cells were stained with an antibody to the focal adhesion marker vinculin, which only showed a weak staining of the nucleus. However, when staining the cells with an antibody that recognizes tyrosine phosphorylated proteins, clear dots of phosphotyrosine in a ring 30-40 µm away from the nucleus were detected, indicating that these cells exhibited some kind of spreading. These dots also stained weakly with an anti-β1-integrin antibody indicating that β1B binds to invasin and mediates the formation of these dots.

To further characterize these dots, the cells were stained with the F-actin marker phalloidin, which showed that thin bundles of F-actin reached out to the dots indicating that they represented initial adhesions/tip complexes of filopodia. In addition, this showed that the GD25β1B cells could form filopodia but were deficient in lamellipodia upon spreading on invasin. This intriguing observation that β1B only supported spreading with filopodia made us curious on which proteins that were involved in this process since this could clarify how cells form filopodia and distinguish the proteins involved in this process from those involved in lamellipodia formation. The GD25β1B cells adhered to invasin-coated plates for up to 6 h but started to detach already after 4 h, which might indicate that the adhesions only are transient and not stabilized in the same manner as classical cell-matrix adhesions, which are flexible structures that allow adhesion for long times. In addition, these initial adhesions, seen when GD25β1B spread on invasin, formed independent of microtubules but required actin and Src kinase activity since they were unable to form in cells pretreated with cytochalasin D (F-actin disruption, data not shown) or PP1 (Src inhibitor) but not with nocodazole (cause microtubule depolymerization, data not shown).

To identify proteins that localize to the tip complexes of the β1B-induced filopodia, a screen for proteins that are known to localize to these structures and/or cell-matrix adhesions was made. As previously shown in other tip complexes, VASP and talin were found at these sites (DePasquale and Izzard, 1991; Lanier et al., 1999; Rottner et al., 1999a). In addition and shown for the first time, the p130Cas-Crk-DOCK180 scaffold was found at these sites. We could also show that both p130Cas and Crk were required for the formation of these filopodia, since the overexpression of either a mutant of p130Cas that lacks the substrate domain, the CrkIIISH2 or the CrkIIISH3 domains disrupted the formation of filopodia in GD25β1B cells.

The p130Cas-Crk-DOCK180 scaffold has the ability to activate Rac1 (Brugnera et al., 2002; Cote and Vuori, 2002), which would be expected to promote the formation of focal complexes and lamellipodia (Hall and Nobes, 2000). In accordance, GD25β1B cells were able to activate Rac1 in response to infection with a plasmid-cured Yersinia strain (expresses invasin). Thus, the binding between invasin and β1B leads to Rac1 activation, but the cells were still unable to form focal complexes or lamellipodia on invasin, indicating that active Rac1 by itself is unable to induce these structures. The general belief to date is that tip complexes (or initial adhesions) either disappear or mature into focal complexes a short distance away from the leading edge of the cell. This maturation is marked by the recruitment of vinculin, a decrease in the distance between the adhesion and the ECM coated surface and the formation of cytoskeletal force since the inhibition of actinomyosin contractile forces also inhibits the formation of focal complexes (DePasquale and Izzard, 1987; Galbraith et al., 2002; Izzard, 1988). In
addition, focal complexes contain paxillin, FAK and many more proteins as well as all proteins found at the initial adhesions (Nobes and Hall, 1995). Thus, the reason why GD25β1B cells are unable to form focal complexes and mediate further spreading probably depends on their inability to recruit and activate vinculin, paxillin, FAK, α-actinin or some other protein. These players are activated in an integrin-dependent manner and are recruited to cell-matrix adhesions in normal fibroblast cells (Zamir and Geiger, 2001a). In addition, it is not known whether β1B has the ability to cluster and if this is important in the formation of focal complexes.

**The role of myosin X (paper IV)**

The overexpression of dominant negative Cdc42 or Rac1 did not block filopodia formation in GD25β1B cells spreading on invasin (paper III), indicating that these not are involved and thus some other mechanism must be present. One factor known to induce filopodia formation is the actin motor myosin X, which was shown to localize to tip complexes of filopodia in a variety of cell types (Berg and Cheney, 2002), including GD25β1A and GD25β1B. However, the expression of myosin X tail did not block filopodia formation in GD25β1B cells spreading on invasin, indicating that this protein not is responsible for these filopodia structures. While performing these studies, the cells transfected with myosin X were stained for FAK, paxillin, vinculin and p130Cas to elucidate if these proteins localized in a different manner in this situation. Indeed, in contrast to non-transfected GD25β1B cells, cells transfected with myosin X had vinculin and FAK localized to the tip complexes together with p130Cas suggesting that myosin X recruits vinculin and FAK to the tip complexes. In contrast, paxillin could not be found at the tip complexes in GD25β1B cells. The myosin X HNCC construct was not able to bring FAK and only weakly vinculin to the tip complex indicating that at least FAK bind to the tail domain of myosin X, but the exact motif to which FAK bind is not clear at the moment. The recruitment of FAK and vinculin to tip complexes of filopodia did not stimulate further spreading of the GD25β1B cells, which either is due to that FAK and vinculin are dispensable or unable to induce lamellipodia, but more likely that the β1B-integrins are unable to activate FAK and vinculin even if these proteins are brought to them. This finding still provide further clues to the role of myosin X in filopodia and suggests that myosin X has the function to provide the tip complexes of filopodia with proteins needed for later spreading to progress into lamellipodia, but that this function most likely is deficient in cells expressing β1B-integrin due to that this integrin is unable to activate these proteins (at least FAK).

**Is VASP involved in the formation of filopodia**

Interestingly, VASP was found at the initial adhesions, which suggests that it could be involved in the formation of filopodia in the GD25β1B cells. The recruitment of VASP to a localized site in a cell leads to an increase in F-actin length and a decrease in degree of filament branching (Bear et al., 2002; Samarin et al., 2003), which are conditions needed for filopodia formation. The VASP protein has three domains EVH1, PRR and EVH2. The EVH1 domain plays an essential role in targeting VASP to focal adhesions by interacting with proteins that has the EVH1 binding motif, FPPPP, which is found in vinculin, zyxin, paladin and ADAP (Kwiatkowski et al., 2003). The PRR domain binds to profilin and to proteins containing SH3 and WW domains, but the exact role of this domain remains unclear; it is dispensable for random motility of cells, but is essential for intracellular motility of *Listeria* (Kwiatkowski et al., 2003). The EVH2 domain contains a G-actin binding region, a F-actin binding region and a region that mediates oligomerization of VASP. The EVH2 domain is sufficient to complement loss of Ena/VASP function in random motility of cells (Loureiro et al., 2002) and has the ability to bundle and induce actin polymerization *in vitro* (Harbeck et al., 2000; Walders-Harbeck et al.,
In addition, the F-actin binding region of EVH2 and the EVH1 domain are required for targeting VASP to the leading edge (Bear et al., 2002).

**Figure 9: VASP is involved in the filopodia spreading of GD25β1B cells on invasin.**

A) GD25β1A and GD25β1B cells transfected with FLAG-tagged VASP or indicated VASP mutants were allowed to spread on GST-invasin-coated (10 µg/ml) cover slips for 3 h, fixed and triple-stained for detection of FLAG-tagged protein (mouse anti-FLAG; Sigma), F-actin and phosphotyrosine as described in paper III. Arrows indicate filopodial tips where an apparent localization of the indicated protein can be seen. The size bar represents 10 µm. B) Attached and transfected GD25β1B cells were quantified for spreading and characterized within three groups, not spread, cells with only filopodia and cells having any kind of lamellipodia-like spreading. The values represent percentage of cells in each category ± s.e.m. of three separate experiments.

To elucidate if VASP is involved in the formation of filopodia in GD25β1B cells, these cells were transfected with VASP or the VASP domains EVH1, EVH2, EVH1-PRR or PRR-EVH2, allowed to spread on invasin for 3 h and assessed for cell spreading morphology (figure 9). The transfected VASP localized to cell-matrix adhesions in GD25β1A cells (figure 9A). In GD25β1B cells, VASP was observed in the perinuclear area but also along the filopodia (figure 9A). Expression of VASP did not affect the
spreading morphology of the cells (figure 9B). On the other hand, expression of EVH1 or EVH1-PRR reduced the formation of filopodia in GD25β1B cells with 54% and 48%, respectively (figure 9B), which indicates that VASP is involved in the formation of filopodia. These constructs were localized in patches throughout the cytoplasm in GD25β1A cells (figure 9A) and did not seem to specifically localize to cell-matrix adhesions as would have been expected based on previous results (Kwiatkowski et al., 2003). In contrast, PRR-EVH2 could be found at the tips of filopodia and at the rim of lamellipodia in GD25β1B cells and was found in patches along actin filaments in GD25β1A cells (figure 9A). EVH2 was markedly enriched in the nuclear or perinuclear area of the cells with some enrichment at the rim of lamellipodia and at cell-matrix adhesion-like structures in GD25β1A (figure 9A). These two constructs induced actin polymerization leading to the formation of small lamellipodia-like structures in 24% (EVH2) or 11% (PRR-EVH2) of the GD25β1B cells (figure 9B), which probably is due to that EVH2 is the functional domain of VASP that can exert action in a non-controlled manner since it is overexpressed without the EVH1 and PRR domains.

Thus, these data show that VASP is involved in the formation of filopodia in GD25β1B cells binding to invasin, but it remains to elucidate the mechanism behind how VASP is recruited and activated in these cells. One intriguing question is how the β1B-integrin signals to VASP. The mechanism for how VASP is activated is largely unknown. Previous work has shown that VASP is phosphorylated by PKA, PKG and PKC (Chitaley et al., 2004; Kwiatkowski et al., 2003) and that this phosphorylation is important for the function of Ena/VASP in cell movement (Kwiatkowski et al., 2003; Loureiro et al., 2002). Thus, one plausible way could be that β1B-integrins activate VASP through PKC. Another possibility is that the NRPTK Abl, which is activated upon β1-integrin activation, connects to and regulates VASP through the Abl-interacting protein (Abi; Hernández et al., 2004). However, at this stage it is not clear whether this interaction inhibits or activates VASP (Hernández et al., 2004).

Mechanism behind invasin-induced filopodia formation in GD25β1B cells

At this time, it is unclear which proteins those directly are responsible for the formation of the β1B-integrin-mediated filopodia, i.e. which proteins that nucleates and stimulate unbranched F-actin formation. One mechanism could be through the activation of N-WASP and Arp2/3 at the very early binding between integrins and invasin, as was shown in the in vitro studies performed in the group of Borisy (Vignjevic et al., 2003). In addition, N-WASP has been found in initial adhesions in brain (Ho et al., 2001). Activation of N-WASP is usually attributed to Cdc42, however, overexpression of dominant negative Cdc42 did not block filopodia formation in GD25β1B cells, showing that these filopodia are formed by a Cdc42-independent mechanism (paper III). Since the p130Cas-CrkII complex is needed for formation of filopodia, it could be that proteins working downstream of these induce the filopodia formation. The links between p130Cas-CrkII and N-WASP identified so far is through the SH2-SH3-SH3 adapter Nck2 and the small GTPase TC10.

Nck has been shown to bind to both p130Cas and DOCK180 and has the ability to activate N-WASP in conjunction with PI(4,5)P₂ (Rohatgi et al., 2001; Tu et al., 2001; Vuori et al., 1996). TC10 and its close relative RhoT belong to the Cdc42-subgroup of Rho family of small GTPases. Both TC10 and RhoT were shown to bind to and activate N-WASP to induce Arp2/3-complex-mediated actin polymerization leading to formation of long filopodia-like structures in fibroblasts or neurite outgrowth in PC12 and N1E-115 nerve cells (Abe et al., 2003). In addition, TC10 and its target N-WASP are essential for the translocation of the insulin-stimulated glucose transporter 4 (GLUT4) from intracellular compartments to the cell surface. Intriguingly, the upstream activation of TC10 in the relocalization of GLUT4 has been elucidated both in response to insulin and osmotic shock, and in both responses CrkII in complex with C3G, which is a GEF for TC10, is essential (Chiang et al., 2001; Gaul et al., 2002). Thus, a hypothetical pathway for induction of filopodia-formation in GD25β1B cells spread on invasin could be
through the pathway p130Cas-CrkII-C3G-TC10 that activates N-WASP, which induces Arp2/3-complex-mediated actin polymerization to induce filopodia.

Apart of Nck, TC10 and Cdc42, WASP-interacting SH3 protein (WISH) and Grb-2 have been reported to activate N-WASP (Carlier et al., 2000; Fukuoka et al., 2001) and could potentially be involved in activating N-WASP to form filopodia in GD25β1B cells in a Cdc42-independent manner. Other possibilities are that WAVEs are involved in the filopodia formation in GD25β1B cells or that filopodia are formed in response to some other small GTPase, where RhoD, Wrch-1 and Rif could be potential candidates since they have been shown to induce filopodia in other situations (Aspenström et al., 2004; Ellis and Mellor, 2000; Tao et al., 2001). So far, we have investigated whether Nck acts as a link between p130Cas-CrkII and filopodia formation. However, overexpression of either an SH2 point mutated or a triple SH3 point mutated construct of Nck2 in the GD25β1B cells did not block filopodia formation on invasin (data not shown), thus showing that Nck2 is dispensable for filopodia formation.

Another interesting point in filopodia formation is the role of RhoA. This protein has recently been shown to be downregulated in the leading edge of cells, which is an effect attributed to smurf-1 and/or an indirect association of PKCε with p190RhoGAP (Troller et al., 2004; Wang et al., 2003a). In addition, overexpression of the regulatory domain of PKCε or p190RhoGAP in NIH3T3 cells induced long extensions (Troller et al., 2004), indicating that the downregulation of RhoA is an important factor to allow cell spreading and migration associated to filopodia formation. Thus, we hypothesize that RhoA also would be downregulated in the GD25β1B cells spreading on invasin, and it will be interesting to investigate whether this is the case, to provide further clues to the role of RhoA in early spreading.

**Upstream of p130Cas**

One intriguing question is how β1B activates p130Cas following binding to invasin. This is since p130Cas is a crucial protein for the invasin-mediated induction of filopodia in GD25β1B cells. In addition, YopH rapidly dephosphorylates p130Cas leading to a block in β1-integrin-mediated phagocytosis, which must occur more rapidly than the time it takes for the cell to phagocytose the bacterium (that also is a rapid process). Thus, we have found roles for p130Cas in the early spreading as well as in the blockage of bacterial uptake, indicating that p130Cas is an important protein for early integrin-mediated events and a very appropriate target for YopH.

Paxillin and FAK were not phosphorylated, whereas a weak phosphorylation of p130Cas could be observed upon adhesion of GD25β1B cells to invasin (figure 2, paper II), thus showing that p130Cas can be partially phosphorylated independent of FAK. Previous data indicates that FAK interacts with the membrane-proximal cyto-1 domain of β3-integrins but that the membrane-distal region is required for FAK phosphorylation, thus activation (LaFlamme et al., 1997; Liu et al., 2000). These observations support our finding that FAK not is phosphorylated upon invasin binding to GD25β1B cells. Thus, p130Cas is important for uptake of *Yersinia* and for the partial spreading seen in GD25β1B cells on invasin and both these effects can be mediated in the absence of FAK. How is then p130Cas activated (phosphorylated) in the GD25β1B cells? The clearly elucidated way for p130Cas phosphorylation downstream of integrin is through FAK and Src (as described in the introduction). In addition, BMX/Etk in cooperation with FAK can phosphorylate p130Cas (Abassi et al., 2003). However, in the GD25β1B cells, FAK is not recruited to initial adhesions nor is it phosphorylated (thereby kinase inactive) and thus not involved in phosphorylating p130Cas in this situation. It is therefore possible that Src or BMX/Etk are involved in p130Cas phosphorylation in pathways that are independent of FAK. Our finding that the Src kinase inhibitor PP1 blocks phosphorylation in GD25β1B cells supports this. However, a total inhibition was seen only when rather high concentrations were used, and PP1 is supposed to be Src-specific at low concentrations but more promiscuous at higher concentrations. Hence, the interpretation of this result can
be that Src or maybe another kinase phosphorylates p130Cas. BMX/Etk would then be a plausible candidate for an alternative kinase associated to integrin-mediated signaling.

Interestingly, studies by Boutons group have shown that FAK and p130Cas both are involved in Rac1-dependent uptake of *Yersinia* and both FAK and p130Cas/Crk can activate Rac1 independently (Bruce-Staskal et al., 2002; Weidow et al., 2000). The activation of p130Cas, in this situation, required activity of Src and Pyk2, where the former is a close relative to FAK (Bruce-Staskal et al., 2002; Weidow et al., 2000). Thus, Pyk2 could be a candidate for activation of p130Cas downstream of β1B-integrin. However, due to the close similarity to FAK, Pyk2 is also likely to be activated in the same manner as FAK and thus remain inactive in the GD25β1B cells upon binding to invasin, but it cannot be completely excluded as a candidate acting as the link between β1B-integrin and p130Cas at this stage.

The NRPTK Abl could also be the protein that mediates the signal from β1B-integrin to p130Cas in GD25β1B cells spreading on invasin. Abl has been shown to be activated by adhesion to fibronectin or by antibody crosslinking of α5β1-integrin (Lewis et al., 1996), to induce actin microspikes in fibroblasts spreading on fibronectin in a Cdc42-, Rac1-, RhoA- and Src-independent manner (Woodring et al., 2002) and it can phosphorylate p130Cas and Crk at least *in vitro* (Woodring et al., 2003). However, the phosphorylation of CrkII occurs at the tyrosine that is implicated in CrkII autoinactivation (Feller, 2001; Woodring et al., 2003), thus implicating Abl in inactivation rather than in activation of the p130Cas-Crk scaffold. Since this phosphorylation only has been shown *in vitro* at this stage, it is possible that Abl first activate p130Cas to allow formation of the p130Cas-CrkII complex and at later stages inhibits this interaction to balance the activation-inactivation cycle of downstream proteins as Rac and TC10.

**Figure 10:** The effect of PKC inhibitors on GD25β1A adhesion to invasin (A) and uptake of *Yersinia* (B).

A) Cells were detached and pretreated with the indicated PKC inhibitor or left untreated for 30 minutes in serum-free medium followed by adhesion to indicated concentration of invasin for 1 h. Cells that adhered were detected using crystal violet as described in the material and method of paper II. B) Adherent cells were pretreated with indicated PKC inhibitor or left untreated followed by infection with the *Yersinia* strain MVM for 30 minutes. Extracellular and intracellular bacteria were distinguished by immunofluorescence methods as described in material and methods in paper II.

Another interesting finding is that PKCε or another novel PKC (PKCe, PKCδ, PKCη and PKCθ) activates Src and FAK in TPA- or growth factor-induced differentiation of neuroblastoma cells (SH-SY5Y; Bruce-Staskal and Bouton, 2001; Fagerström et al., 1998). The activation of FAK and Src further induced phosphorylation of p130Cas and the interaction of p130Cas with Crk (Bruce-Staskal and Bouton, 2001; Fagerström et al., 1998). The finding of PKC as an upstream activator of the p130Cas-Crk complex is interesting and indicates that a PKC could be an upstream activator of p130Cas also in other cells/situations, possibly independent of FAK (Fagerström et al., 1998). In addition, several PKCs have been implicated to either regulate integrin activity or to be involved in signaling downstream of integrins, including PKCα, PKCε, PKCδ and PKCγ (Berrier et al., 2000; Chun et al., 1997; Ivaska et al., 2003; Ivaska et al., 2002; Kiley et al., 1999; Lam et al., 2001; Lee et al., 2002; Ng et al., 1999; Palmantier et al., 2001; Platet et al., 1998). Thus, PKCs could be involved in filopodia formation by GD25β1B cells.
spreading on invasin. To investigate this, we have done experiments to elucidate the potential role of PKC in the β1-integrin-mediated events following binding to invasin. In these studies, pre-treatment of GD25β1A cells with 10 µM of Ro31-8220, which inhibits PKCα, PKCβI, PKCβII, PKCε and PKCγ, was shown to inhibit adhesion to invasin and bacterial uptake (figure 10). In contrast, cell adhesion or bacterial uptake were unaffected by the pre-treatment with two other PKC-inhibitors, Gö 6976 (inhibitor for PKCα and PKCβ) and GF109203x (inhibitor for PKCα, PKCβ, PKC δ and PKCε). Hence, these results from studies using inhibitors suggests that there is a need for activation of PKCγ to allow adhesion of GD25β1-expressing cells to invasin and this PKC could thus also be involved in the integrin-mediated signals leading to tyrosine phosphorylation of p130Cas. Taken together, we speculate that PKC, the NRPTKs Src, BMX/Etk, Pyk2 or Abl are possible candidates for signaling proteins acting upstream of p130Cas in GD25β1B cells adhering to invasin and further experiments needs to be performed in order to elucidate whether any of these are involved.
Conclusions

The *Yersinia* protein tyrosine phosphatase YopH rapidly dephosphorylates p130Cas and ADAP in macrophages causing a disruption of cell-matrix adhesions and a block in phagocytosis.

Expression of β1-integrins, either β1A or β1B, is required for invasin-mediated binding of *Yersinia pseudotuberculosis* to host cells. In addition, the membrane-distal part of β1A, including the first NPXY motif (phosphorylation is not important) and the double threonine sites are important in the ability of β1-integrins to mediate uptake of *Yersinia*.

β1B-integrin-mediated spreading on invasin only induces formation of filopodia (no lamellipodia). The tip complex of these filopodia contains VASP, talin and the p130Cas-Crk-DOCK180 scaffold, but lacks vinculin, paxillin and FAK. VASP, p130Cas and Crk are important for the formation of the filopodia, while Cdc42, Rac1 and myosin X are not.

Myosin X recruits FAK and vinculin to the tip complexes of filopodia.
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