Antiphagocytosis by *Yersinia pseudotuberculosis*  
–Role of the YopH Target Proteins

Ming Yuan
# TABLE OF CONTENTS

ABSTRACT ......................................................................................................................... 5  
PAPERS INCLUDED IN THIS THESIS .................................................................................. 6  
ABBREVIATIONS ................................................................................................................ 7  
INTRODUCTION .................................................................................................................. 8  
1. Pathogenic *Yersinia* species .......................................................................................... 8  
   1.1 Overview ..................................................................................................................... 8  
   1.2 Virulence mechanisms .............................................................................................. 8  
      1.2.1 The virulence plasmid ......................................................................................... 8  
      1.2.2 Bacterial adhesion and invasion ......................................................................... 9  
      Invasin.......................................................................................................................... 9  
      YadA............................................................................................................................ 10  
   1.2.3 Yop effectors ......................................................................................................... 11  
      1.2.3.1 Antiphagocytic Yop effectors ......................................................................... 11  
      YopH.......................................................................................................................... 11  
      YopE.......................................................................................................................... 13  
      YopT........................................................................................................................... 13  
      YpkA.......................................................................................................................... 13  
      1.2.3.2 Other intracellular Yop effectors ..................................................................... 15  
      YopJ........................................................................................................................... 15  
      YopM.......................................................................................................................... 15  
2. Targets of YopH in host cells ......................................................................................... 16  
   2.1 Epithelial cells ............................................................................................................ 16  
      Cas............................................................................................................................. 16  
      FAK............................................................................................................................ 17  
   2.2 Professional phagocytes ......................................................................................... 18  
      Fyb............................................................................................................................. 19  
      SKAP-HOM.............................................................................................................. 21  
3. Phagocytosis ................................................................................................................ 22  
   3.1 Background ............................................................................................................. 22  
      3.1.1 FcγR-mediated phagocytosis ............................................................................. 22
3.1.2 CR3-mediated phagocytosis ................................................................. 23
3.1.3 Bacterial uptake mediated by integrin-invasin interaction ...................... 23
3.2 Actin dynamics during phagocytosis ....................................................... 24
  3.2.1 Actin-based complex ........................................................................ 25
  3.2.2 Regulators of the actin cytoskeleton .................................................. 25
3.3 Endocytic fusion and recycling during phagocytosis ............................... 27

AIM ............................................................................................................. 30

RESULTS and DISCUSSION ..................................................................... 31

Interaction between YopH and Cas is required for YopH-mediated disruption of focal
adhesion ..................................................................................................... 31

YopH N terminus binds Fyb and it is important for YopH-mediated effects in
macrophages .............................................................................................. 34

mAbp1 is a novel interactor of Fyb ............................................................... 35

mAbp1 influences spreading of macrophages and antiphagocytosis mediated by
pathogenic Yersinia ..................................................................................... 38

Role of the FYB/SKAP-HOM complex in macrophages ............................... 40

CONCLUSIONS ........................................................................................... 46

ACKNOWLEDGEMENTS .......................................................................... 47

REFERENCES ............................................................................................... 49

PAPERS I–IV
ABSTRACT

The enteropathogenic bacterium *Yersinia pseudotuberculosis* binds to β1 integrins on a host cell via its surface protein invasin. This event stimulates signal transduction to the actin cytoskeleton of the eukaryotic cell, which allows the cell to engulf the bacterium that is attached to its surface. However, the pathogen *Y. pseudotuberculosis* can evade such phagocytosis by injecting virulence effectors that interfere with the antipathogenic machinery of the host cells. One of these virulence effectors is the tyrosine phosphatase YopH. Through its enzymatic activity, YopH blocks phagocytosis by affecting the signalling that is associated with cytoskeletal rearrangements.

Cas is a substrate of YopH in both professional and non-professional phagocytes. We showed that YopH binds to the central substrate domain of Cas and that this interaction is required for YopH to target focal adhesion structures in host cells. We also demonstrated that YopH binds another substrate, FAK, through Cas. Moreover, we suggested that targeting of Cas is necessary for the cytotoxic effects mediated by YopH.

The protein Fyb is specific to immune cells, and it has been identified as a substrate of YopH in macrophages. We discovered that both the N-terminal substrate-binding domain and the C-terminal catalytic region of YopH bind Fyb in a phosphotyrosine-dependent manner. Moreover, we observed that both the substrate-binding domain and the phosphatase activity of YopH are essential for the effects of this protein on macrophages, which include dephosphorylation of Fyb, blocking of phagocytosis, and cytotoxicity.

The role of Fyb in macrophages is largely unknown, although there is evidence that this protein is involved in integrin-linked actin organization. We identified a novel interaction partner of Fyb, mAbp1, which is a protein that binds to F-actin. Studies in vitro indicated that mAbp1 binds to the N terminus of Fyb via a C-terminal SH3 domain. We also found that both Fyb and mAbp1 co-localize with F-actin at the leading edges of macrophages. Further studies suggested that mAbp1 influences the spreading of macrophages and the antiphagocytosis mediated by pathogenic *Yersinia*. These results support a role for Fyb in signalling that affects F-actin dynamics, and they also provide additional insight into the mechanisms involved. Fyb has been shown to form a complex with SKAP-HOM, another substrate of YopH in macrophages. Our data implied that the level of SKAP-HOM protein depends on the presence of Fyb, but the function of the Fyb/SKAP-HOM complex in macrophages has not been determined. However, since Fyb is the only known haematopoietic-specific substrate of YopH, it is possible that Fyb is involved in other antimicrobial functions.

**Key words**: Cas, Fyb, mAbp1, SKAP-HOM, *Yersinia pseudotuberculosis*, YopH.
This thesis is based on the following publications, which are referred to in the text by their roman numerals (I–IV).


The following paper also concerns part of the present research, but it is not discussed extensively in this thesis.

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADAP</td>
<td>adhesion- and degranulation-promoting adapter protein</td>
</tr>
<tr>
<td>ADF</td>
<td>actin-depolymerizing factor</td>
</tr>
<tr>
<td>Arp2/3</td>
<td>actin-related protein 2/3</td>
</tr>
<tr>
<td>Cas</td>
<td>Crk-associated substrate</td>
</tr>
<tr>
<td>CR3</td>
<td>complement receptor 3</td>
</tr>
<tr>
<td>ENa/VASP</td>
<td>enabled/vasodilator stimulated phosphoprotein</td>
</tr>
<tr>
<td>FAK</td>
<td>Focal adhesion kinase</td>
</tr>
<tr>
<td>F-actin</td>
<td>filamentous actin</td>
</tr>
<tr>
<td>FcγR</td>
<td>Fcγ receptor</td>
</tr>
<tr>
<td>Fyb</td>
<td>Fyn-T-binding protein</td>
</tr>
<tr>
<td>G-actin</td>
<td>globular actin</td>
</tr>
<tr>
<td>GST</td>
<td>glutathione S-transferase</td>
</tr>
<tr>
<td>HIP-55</td>
<td>haematopoietic progenitor kinase 1 interacting protein of 55 kDa</td>
</tr>
<tr>
<td>HPK1</td>
<td>haematopoietic progenitor kinase 1</td>
</tr>
<tr>
<td>mAbp1</td>
<td>mammalian actin-binding protein 1</td>
</tr>
<tr>
<td>MEF</td>
<td>mouse embryonic fibroblast</td>
</tr>
<tr>
<td>MYM</td>
<td>multiple Yop mutant</td>
</tr>
<tr>
<td>PRAM-1</td>
<td>promyelocytic leukaemia RARα target gene encoding an adapter molecule-1</td>
</tr>
<tr>
<td>PTPase</td>
<td>protein tyrosine phosphatase</td>
</tr>
<tr>
<td>SH2</td>
<td>Src homology 2</td>
</tr>
<tr>
<td>SH3</td>
<td>Src homology 3</td>
</tr>
<tr>
<td>SKAP55</td>
<td>Src kinase-associated protein of 55kDa</td>
</tr>
<tr>
<td>SKAP-HOM</td>
<td>Src kinase-associated protein of 55kDa homologue</td>
</tr>
<tr>
<td>SHP-1</td>
<td>SH2 domain-containing protein-tyrosine phosphatase 1</td>
</tr>
<tr>
<td>SHPS-1</td>
<td>SH2 domain-containing protein tyrosine phosphatase substrate 1</td>
</tr>
<tr>
<td>SLP-76</td>
<td>SH2 domain-containing leukocyte phosphorylase of 76 kDa</td>
</tr>
<tr>
<td>SLAP-130</td>
<td>SLP-76-associated protein of 130 kDa</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>WASP</td>
<td>Wiskott-Aldrich syndrome protein</td>
</tr>
<tr>
<td>Yop</td>
<td><em>Yersinia</em> outer protein</td>
</tr>
</tbody>
</table>
INTRODUCTION

1. Pathogenic *Yersinia* species

1.1. Overview

The *Yersinia* species belong to the family Enterobacteriaceae, and they are gram-negative, catalase-positive, oxidase-negative facultative anaerobic rods that can grow at temperatures of 4 to 40 ºC (Nilehn, 1969; Smego et al., 1999). Eleven *Yersinia* species are known, three of which are human pathogens: *Y. pestis*, *Y. enterocolitica*, and *Y. pseudotuberculosis* (Sulakvelidze, 2000). *Y. pestis* is non-motile, whereas *Y. enterocolitica* and *Y. pseudotuberculosis* are flagellated and motile at 25 ºC but no longer motile at 37 ºC in a mammalian host (Boyce, 1985; Hanna et al., 1979).

*Y. pestis* is the causative agent of bubonic and pneumonic plague and is thereby one of the most pathogenic bacterial species in human history. *Y. pestis* is normally maintained in infected rodents, and it can be transmitted by infected fleas to humans, where it invades lymphatic tissue and proliferates in the lymph nodes. It can also be transmitted by air and blood (Perry and Fetherston, 1997). *Y. enterocolitica* and *Y. pseudotuberculosis* cause a self-limited enteric disease called yersiniosis. These pathogens are orally transmitted to humans in contaminated food or water (Bottone, 1999; Smego et al., 1999), and, similar to *Y. pestis*, they proliferate in lymphatic tissue, which they enter though the M cells overlying the surface of Peyer’s patches in the small intestine (Autenrieth and Firsching, 1996; Clark et al., 1998; Fallman et al., 1997).

1.2. Virulence mechanisms

1.2.1 The virulence plasmid
Common to all three human pathogenic *Yersinia* species is that they can invade and persist in lymphatic tissue and thereby evade the primary immune defence, and this feature is due to a 70-kb plasmid that is essential for virulence (Gemski et al., 1980a; Gemski et al., 1980b; Portnoy and Falkow, 1981; Portnoy et al., 1981; Zink et al., 1980). This plasmid encodes the following: (i) a type three secretion system (TTSS); (ii) the virulence effectors called *Yersinia* outer proteins (Yops); (iii) a translocation system for delivery of Yops; (iv) regulators of secretion and translocation (Cornelis, 1998; Cornelis et al., 1998). *Y. pestis* has two additional plasmids of 100- and 9.5-kb, respectively, which contribute to the virulence effect of this pathogen (Ferber and Brubaker, 1981).

### 1.2.2 Bacterial adhesion and invasion

Enteropathogenic *Yersinia* species must adhere to host cells in order to cause infection, and the two main adhesins expressed by these bacteria are invasin and *Yersinia* adhesin A (YadA) (Isberg and Barnes, 2001; Isberg et al., 2000; Isberg et al., 1987).

**Invasin**

Invasin is a 103-kDa outer membrane protein that is chromosomally encoded by the gene designated *inv* (Isberg et al., 1987). Maximal expression of invasin in *Y. enterocolitica* and *Y. pseudotuberculosis* occurs at the low temperature of 26 °C, but not at 37 °C (Isberg et al., 1987; Pepe and Miller, 1993), whereas invasin is not expressed in *Y. pestis* (Rosqvist et al., 1988b; Simonet et al., 1996). Recently, two regulators of invasin expression were described: RovA, which is required for expression of invasin (Nagel et al., 2001; Revell and Miller, 2000); and YmoA, which...
is involved in the negative regulation of *inv* (Ellison et al., 2003).

Invasin binds to β1 integrins (α3β1, α5β1, and α6β1) in host cells through the 192 amino acids of its C terminus and thereby triggers bacterial uptake (Isberg and Leong, 1990; Leong et al., 1990). A central region in the invasin of *Y. pseudotuberculosis* causes self-association and enhances uptake of the bacteria (Dersch and Isberg, 1999). However, invasin is not required for the virulence of *Yersinia* (Mecsas et al., 2001; Rosqvist et al., 1988b). The vital effects of invasin in *Yersinia* infection are exerted at an early stage of the process, and they include mediating both the attachment and entry of the bacteria into host cells, such as the M cells in mouse Peyer’s patches (Clark et al., 1998; Isberg et al., 2000; Isberg and Tran Van Nhieu, 1994; Rankin et al., 1992; Wiedemann et al., 2001; Young et al., 1990). In agreement with this, a *Y. pseudotuberculosis* strain that expresses a mutated variant of invasin has been reported to have a slower rate of oral infection in mice (Rosqvist et al., 1988b). The invasion-integrin interaction has also been shown to stimulate T cell activity (Brett et al., 1993), activate human peripheral B cells (Lundgren et al., 1996), trigger platelet aggregation (Simonet et al., 1992), and mediate production of the proinflammatory chemokines interleukin (IL)-8 in intestinal epithelial cells (Grassl et al., 2003).

**YadA**

YadA is a 44–47-kDa protein, which, along with Yops, is encoded by the virulence plasmid (El Tahir and Skurnik, 2001). Unlike invasin, YadA expression is induced at 37 °C (Kapperud et al., 1987). YadA is essential for the virulence of *Y. enterocolitica*, but not *Y. pseudotuberculosis*, and it is not present in *Y. pestis* (El Tahir and Skurnik, 2001).

YadA binds indirectly to β1 integrin in host cells through extracellular matrix (ECM) components such as collagen, fibronectin, and laminin (Bliska et al., 1993; El Tahir and Skurnik, 2001). YadA has been implicated in the formation of fibrillae (Kapperud and Namork, 1987) and in bacterial adhesion and invasion during *Yersinia* infection (Eitel et al., 2005; Heesemann et al., 1987; Kapperud et al., 1987). The YadA of *Y. pseudotuberculosis* has a unique N-terminal amino acid sequence that mediates tight anchoring of fibronectin to α5β1 integrins (Heise and Dersch, 2006), and, in the absence of invasin, it can promote adherence of the bacteria to, and to some degree
uptake of the bacteria by, cultured cells (Eitel and Dersch, 2002; Grosdent et al., 2002; Roggenkamp et al., 1995; Roggenkamp et al., 1996).

Two other adhesins are pH6 antigen and attachment invasion locus (Ail), both of which are expressed at 37 °C (Bliska and Falkow, 1992; Iriarte et al., 1993; Lindler and Tall, 1993). Studies of murine models have indicated that Ail is not required for establishing systemic infection (Miller et al., 1989; Wachtel and Miller, 1995). It is also known that pH6 antigen contributes to the virulence of Y. pestis (Lindler et al., 1990), although the roles of this protein in Y. enterocolitica and Y. pseudotuberculosis are still unclear.

1.2.3 Yop effectors

When Yersinia comes in contact with a target cell, the Yop effectors are delivered from the extracellularly located bacterium into the eukaryotic cell via the type III secretion/translocation mechanism (Cornelis, 1997; Cornelis et al., 1998; Francis et al., 2002). The six Yop effectors that have been identified are YopH, YopE, YopJ/P, YpkA/YopO, YopT, and YopM, and four of those (YopH, YopE, YopJ/P, and YpkA/YopO) are responsible for the disruption of actin cytoskeleton rearrangement (Barbieri et al., 2002; Viboud and Bliska, 2005).

1.2.3.1 Antiphagocytic Yop effectors

YopH: a protein tyrosine phosphatase

YopH is 51-kDa protein tyrosine phosphatase (Guan and Dixon, 1990) that is essential for Yersinia virulence (Andersson et al., 1996; Bliska et al., 1991; Rosqvist et al., 1988a). This protein contains 468 amino acids arranged in an N-terminal non-catalytic domain (residues 1–130), followed by a proline-rich region and a C-terminal phosphatase domain (residues 201–468). Previous studies have shown that the 130 N-terminal amino acids of YopH form a multifunctional domain that is essential for secretion (residues 1–17) and translocation into host cells (residues 18–71), and also binds to the chaperone SycH in the bacteria to deliver the YopH to
tyrosine-phosphorylated target proteins in the eukaryotic cells (Cornelis, 2002; Montagna et al., 2001; Wattiau et al., 1994; Woestyn et al., 1996). A region comprising residues 223–226 has been shown to be important for immediate targeting of YopH to host cell peripheral focal adhesion complexes (Persson et al., 1999). The C-terminal phosphatase domain of YopH contains a phosphate-binding loop (P-loop; residues 403–410), and substitution of the Cys at position 403 with either Ser or Ala inactivates the phosphatase (Guan and Dixon, 1990). This catalytically inactive variant of YopH can form stable complexes with its substrates in vivo, and it has been used as a substrate trap to identify the host cell proteins targeted by this effector. In HeLa cells, YopH dephosphorylates focal adhesion kinase (FAK) and Crk-associated substrate (Cas) (Black and Bliska, 1997; Bliska et al., 1992; Persson et al., 1997), which disrupts the peripheral focal adhesion complexes. In macrophages, besides Cas, YopH targets Fyn-T-binding protein (Fyb) and Src-kinase-associated protein of 55kDa homology (SKAP-HOM) (Black et al., 2000; Hamid et al., 1999).

![Figure 2. Schematic representation of YopH.](image)

S, secretion; T, translocation; Pro, proline-rich region; PTPase, protein tyrosine phosphatase domain; FCT, focal complex targeting; P-loop, phosphatase-binding loop.

It is assumed that the primary function of YopH is to block phagocytosis in macrophages and neutrophils, and, through its PTPase activity, also to inhibit the oxidative burst in these cells (Bliska and Black, 1995; Ruckdeschel et al., 1996). The results of infection studies in a murine model have suggested that YopH counteracts innate immunity, as indicated by the observation that a yopH mutant failed to pass the stage of Peyer’s patches (Logsdon and Mecsas, 2003; Trulzsch et al., 2004; Viboud and Bliska, 2005). YopH has also been implicated in the signalling cascades associated with antigen-induced activation of T or B cells (Yao et al., 1999).
**YopE: a GTPase-activating protein**

YopE is a 23-kDa protein that is essential for *Yersinia* virulence (Forsberg and Wolf-Watz, 1988; Viboud et al., 2006). It contains N-terminal secretion and translocation sequences, and a C-terminal Rho GTPase-activating protein (GAP) domain (Schesser et al., 1996; Sory et al., 1995) that is necessary for the function of the protein (Aili et al., 2002; Black and Bliska, 2000). YopE can deactivate the Rho family GTPases Rac1 and RhoA, but not Cdc42 (Aepfelbacher, 2004; Aili et al., 2006; Black and Bliska, 2000), and it thereby disrupts the actin cytoskeleton, which causes rounding and detachment of infected cells, a phenomenon known as cytotoxicity. Besides blocking phagocytosis, YopE inhibits activation of caspase-1 by deactivating Rac1, and it thereby prevents the maturation and secretion of IL-1β and IL-18 as a means of regulating the inflammatory response to the *Yersinia* infection (Schotte et al., 2004).

**YopT: a cysteine protease**

YopT is a 35.5-kDa protein, which, similar to YopE, affects the cytoskeleton of eukaryotic cells (Iriarte and Cornelis, 1998). YopT acts as a cysteine protease to cleave the prenyl groups of Rho GTPases and in that way inactivate those proteins, and subsequently remove them from membranes (Aepfelbacher et al., 2005; Shao et al., 2002; Shao et al., 2003; Sorg et al., 2001; Zumbihl et al., 1999). *In vivo*, YopT targets RhoA but not Rac or Cdc 42 (Aepfelbacher et al., 2003; Aepfelbacher et al., 2005; Zumbihl et al., 1999). The polybasic sequence in the C terminus of RhoA is essential for substrate recognition and cleavage by YopT (Shao et al., 2003). The role of YopT in *Yersinia* pathogenesis is still unclear, since this protein is not essential for the virulence of *Y. enterocolitica*, and it is not expressed in *Y. pestis* (Iriarte and Cornelis, 1998). Recently, in a study of mouse infection and cell culture models (Viboud et al., 2006), it was found that YopE has stronger antiphagocytic activity than YopT, and the latter protein can only partially compensate for loss of the former.

**YpkA: a serine/threonine kinase**

YpkA (YopO in *Y. enterocolitica*) is an 81.7-kDa autophosphorylating protein with a serine/threonine kinase catalytic domain in the N terminus (Galyov et al., 1993;
Galyov et al., 1994; Hakansson et al., 1996). The C-terminal region of YpkA has been shown to bind to RhoA and Rac1 (Barz et al., 2000), however, the function of such attachment is still unknown. YpkA can disrupt the actin cytoskeleton of cultured cells through both kinase-dependent and kinase-independent mechanisms when it is overexpressed and in the absence of other Yop effectors (Hakansson et al., 1996; Juris et al., 2002; Wiley et al., 2006). YpkA-mediated morphological phenotypes differ from the YopE-mediated counterparts, as demonstrated by an investigation showing that infected HeLa cells do not detach from the substrate (Hakansson et al., 1996). YpkA is also essential for *Yersinia* virulence (Galyov et al., 1993; Wiley et al., 2006), as suggested by a previous study indicating that this protein is involved in the antiphagocytic effect of *Y. enterocolitica* on macrophages and polymorphonuclear neutrophils (Grosdent et al., 2002).

**Figure 3. The *Yersinia* effectors target multiple signalling pathways.** The Yops are delivered into the host cells via a type III secretion system. YopT modifies the Rho family GTPases; YopE inactivates the Rho family of GTPases; YpkA binds to Rac and Rho (function unknown); YopH dephosphorylates Cas and FAK in epithelial cells, and Cas, Fyb and SKAP-HOM in macrophages. These four Yops alter or disrupt the actin cytoskeleton and thereby block phagocytosis. YopJ impairs activation of MAPKKs and NF-κB, which induces apoptosis and inhibits cytokine production. YopM is translocated into the nucleus (function unknown).
1.2.3.2. Other intracellular Yop effectors

**YopJ: a cysteine protease**

YopJ (YopP in *Y. enterocolitica*) is a 33-kDa ubiquitin-like cysteine protease. It has been reported to act downstream of the small GTPase Rac1 to inhibit the signalling of mitogen-activated protein kinases (MAPKs), such as c-Jun N-terminal kinase (JNK), p38, and extracellular signal-regulated kinases (ERK) 1 and 2, and that effect is achieved by preventing activation of the family of MAPK kinases (MAPKKs) (Mukherjee et al., 2006; Orth, 2002; Palmer et al., 1999). YopJ also inhibits the nuclear factor κB (NFκB) signalling pathway by targeting IκB kinase β (IKKβ) in the host cells (Aepfelbacher et al., 1999; Orth, 2002; Ruckdeschel, 2002). YopJ is the only Yop effector that is required to induce *Yersinia*-mediated apoptosis in macrophages (Mills et al., 1997; Monack et al., 1997). An early study indicated that YopJ is dispensable for the virulence of *Y. pseudotuberculosis* in a murine model (Galyov et al., 1994), whereas later experiments demonstrated that this protein participates in the establishment of systemic infection (Monack et al., 1998). A recent study suggested that YopJ inhibits MAPK-mediated uptake of *Y. enterocolitica* in dendritic cells (Autenrieth et al., 2006).

**YopM: a leucine-rich protein**

YopM is a 41-kDa acidic protein that contains leucine-rich repeats (Leung and Straley, 1989) and C-terminal nuclear localization sequences (NLSs) (Benabdillah et al., 2004). During *Yersinia* infection of HeLa cells, YopM can translocate to the nucleus via a vesicle-associated pathway (Skrzypek et al., 1998). This Yop forms a complex with two cytoplasmic kinases called ribosomal protein S6 kinase 1 (RSK1) and protein kinase C-like2 (PRK2) (McDonald et al., 2003). The function of YopM has not yet been fully elucidated, although it is known that this molecule is an important virulence factor in *Yersinia*. YopM mutants of both *Y. pestis* and *Y. enterocolitica* have been found to exhibit altered virulence in a murine model (Kerschen et al., 2004; Trulzsch et al., 2004), and it has also been demonstrated that YopM can interfere with innate immunity by causing depletion of NK cells (Kerschen et al., 2004).
2. Targets of YopH in host cells

2.1. Epithelial cells

YopH is an important virulence effector of *Yersinia* that can block host cell signalling through its PTPase activity (Andersson et al., 1996; Bliska et al., 1991; Guan and Dixon, 1990; Rosqvist et al., 1988a). In many types of cultured cells, such as HeLa cells, the targets of YopH are Cas and FAK (Black and Bliska, 1997; Bliska et al., 1992; Persson et al., 1997). YopH has also been found to bind and dephosphorylate paxillin, but that effect has only been seen *in vitro* (Black and Bliska, 1997).

**Cas**

Cas was originally identified as a 130-kDa highly tyrosine-phosphorylated protein in v-Src- and v-Crk-transformed cells, and it forms a complex with the mentioned molecules (Sakai et al., 1994). As an adaptor, Cas promotes protein-protein interactions. An N-terminal SH3 domain in Cas interacts with FAK and protein tyrosine kinase 2 (Pyk2), and the C terminus of Cas contains Src-binding sequences. Moreover, a central substrate-binding domain containing 15 potential tyrosine phosphorylation sites mediates association of Cas with the SH2 domain of Crk (Kanner et al., 1991; Nakamoto et al., 1996; Sakai et al., 1994). The phosphorylation of Cas can be regulated via interaction with tyrosine-phosphatases such as PTP-1B and PTP-PEST, and serine-phosphorylated Cas has been found to bind 14-3-3 proteins (O’Neill et al., 2000).

Cas is ubiquitously expressed, and deletion of this protein is lethal in mouse embryos (Honda et al., 1998). Fibroblasts from Cas<sup>−/−</sup> mice exhibit short actin filaments and display delayed cell spreading as well as reduced migration phenotype (Honda et al., 1998). Studies have indicated that Cas is involved in cell migration (Panetti, 2002), cell transformation and the progression of cancer (Behrens et al., 2003; Burnham et al., 1999; Chodniewicz and Klemke, 2004), and cell survival and apoptosis (Defilippi et al., 2006; Dolfi et al., 1998; Oktay et al., 1999).
FAK

FAK is a 125-kDa protein tyrosine kinase (Hildebrand et al., 1993). It contains an N terminus with an autophosphorylation site that may interact with the cytoplasmic domain of β1 integrin (Schaller et al., 1995), a central kinase domain, and also a C terminus with a focal adhesion targeting sequence (FAT) that interacts with several proteins, including paxillin (Hildebrand et al., 1995), Cas (Harte et al., 1996; Polte and Hanks, 1995), and some other proteins that comprise an SH2 domain. The cell-surface integrin receptor binds ECM, which induces integrin clustering and recruitment of FAK. FAK is subsequently phosphorylated and becomes associated with Src (Schaller, 1996; Schaller et al., 1994), and this activation of FAK has been implicated in cell cycle progression, adhesion, and migration (Brown et al., 2005; Cary and Guan, 1999; Choma et al., 2006; Ilic et al., 1997; Klemke et al., 1998). Like Cas, FAK deficiency is also lethal in mouse embryos (Ilic et al., 1995).

The Cas-FAK interaction has been shown to increase the tyrosine phosphorylation of Cas, which in turn causes recruitment of Crk to stimulate cell migration (Cary et al., 1998; Klemke et al., 1998). The possible downstream effector of Crk is DOCK180 (Gu et al., 2001), which may act as an activator of Rho GTPase Rac1 (Brugnera et al., 2002), and the Caenorhabditis elegans counterpart of DOCK180 has been implicated in the regulation of cell migration and phagocytosis (Wu and Horvitz, 1998). In addition to cell migration, Cas and FAK have also been found to participate in host-pathogen interactions, including those involving the bacteria species Yersinia pseudotuberculosis (Bruce-Staskal et al., 2002; Weidow et al., 2000), Pseudomonas aeruginosa (Deng et al., 2005), Salmonella typhimurium (Shi and Casanova, 2006), and Streptococcus pyogene (Humtsoe et al., 2005).
2.2. Professional phagocytes

It is assumed that the first immune cells to encounter infecting *Yersinia* are the professional phagocytes and antigen-presenting cells (APCs), which include the macrophages, neutrophils, and dendritic cells. YopH is essential for the pathogenic *Yersinia*, because it impairs the functions of the mentioned immune cells, which is demonstrated by the finding that *yopH* mutants are rapidly eliminated upon infection (Viboud and Bliska, 2005). In mouse macrophage-like J774 cells, besides Cas, Fyb and SKAP-HOM also serve as substrates for YopH (Black et al., 2000; Hamid et al., 1999).
Fyb

Fyb is a haematopoietic cell-specific protein that is also called SLAP-130 (SLP-76-associated protein of 130 kDa) (Musci et al., 1997) and ADAP (adhesion- and degranulation-promoting adaptor protein) (Geng and Rudd, 2001). Expression of Fyb occurs in T cells, thymocytes, and myeloid cells, but not in B cells (da Silva et al., 1997; Geng and Rudd, 2001; Musci et al., 1997). Fyb contains a number of regions that have the potential to mediate protein-protein interactions; these include several proline-rich motifs in the N-terminal half of the protein and a C-terminal SH3-like domain. In addition, Fyb has two putative NLSs and multiple tyrosine-containing motifs (da Silva et al., 1997). Two YDDV motifs have been shown to be tyrosine-phosphorylated by Fyn kinase, which is required for binding to the 76-kDa SH2-domain-containing leukocyte phosphoprotein (SLP-76) (Geng et al., 1999; Raab et al., 1999). Fyb also harbours a FPPPP motif that has been reported to bind the Ena/VASP homology 1 (EVH1) domain of enabled/vasodilator-stimulated phosphoproteins (Ena/VASP), which constitute a family of proteins that bind and regulate actin (Krause et al., 2000; Renfranz and Beckerle, 2002; Scott et al., 2006). Two isoforms of Fyb have been identified, Fyb-120 and Fyb-130, which differ with regard to a 46-amino-acid insert that is found in the C terminus between the two tyrosine motifs YDGI and YDDV in the 130-kDa form (Veale et al., 1999). Both Fyb-120 and Fyb-130 bind to the SH2 domains of Fyn and SLP-76 (da Silva et al., 1997; Musci et al., 1997; Veale et al., 1999). Other dissimilarities between these two isoforms include more efficient binding of Fyb-120 to SLP-76 and preferential expression of Fyb-130 in mature T cells. It has been demonstrated that Fyb-130 plays a role in up-regulation of TCR-induced IL-2 production in mature T cells, and Fyb-120 has an important function in thymic differentiation (Veale et al., 1999).

Fyb-120 is the most well characterized isoform. Studies of Fyb have been focused on T cells in which Fyb was initially found, and several reports have suggested that Fyb is included in the TCR signalling pathway (Boerth et al., 2000; da Silva et al., 1997; Fang et al., 1996; Musci et al., 1997; Raab et al., 1999; Wardenburg et al., 1996; Wu et al., 1996). Fyb undergoes tyrosine phosphorylation upon TCR activation, and it forms a complex with Wiskott-Aldrich syndrome protein (WASP), Ena/VASP, Nck,
and SLP-76 (Krause et al., 2000). In T cells, Fyb binds SKAP55 (Schraven et al., 1997), and that association is mediated by the SH3 domain of SKAP55 and the proline-rich sequence of Fyb (Marie-Cardine et al., 1998a). In macrophages, Fyb forms a similar complex with SKAP55 homology protein SKAP-HOM (Black et al., 2000; Bliska et al., 1992; Timms et al., 1999).

Previous studies have provided evidence that Fyb is involved in integrin-regulated cellular responses. Fyb enhances integrin-mediated adhesion and migration in T cells and mast cells (Geng et al., 2001; Hunter et al., 2000; Wang et al., 2003), which agrees with the finding that Fyb^{−/−} T cells failed to enhance integrin-dependent adhesion (Peterson et al., 2001). This TCR-induced adhesion may be regulated by the interaction between Fyb and SLP-76 that is mediated by receptor-stimulated generation of intracellular reactive oxygen species (ROS) (Kwon et al., 2005). Recently, Kliche et al. (2006) reported that Fyb and SKAP55 participate in TCR-promoted inside-out signalling through recruitment of the activated small GTPase Rap1 to the plasma membrane. Fyb has been found to co-localize with F-actin in membrane ruffles, adhesion plaques/podosomes, and phagocytic cups (Coppolino et al., 2001; Geng et al., 2001; Koga et al., 2005). The observation that Fyb is a ligand for the EVH1 domain of the actin-binding Ena/VASP proteins (Krause et al., 2000) might explain the observation that Fyb co-localizes with cellular structures that exhibit extensive actin dynamics (Coppolino et al., 2001; Krause et al., 2000). The cited results indicate that Fyb is involved in actin organization, although it is not necessary for TCR-induced actin polymerization (Peterson et al., 2001). It is also plausible that Fyb is required for T cell proliferation (Griffiths et al., 2001; Peterson et al., 2001; Wu et al., 2006), and it may take part in cell migration and progression to the multinucleated cell stage in osteoclastogenesis (Koga et al., 2005). Notwithstanding, it is known that Fyb is dispensable for the development and function of NK cells (Fostel et al., 2006). The role of Fyb in macrophages is largely unknown, but the finding that this adaptor protein is rapidly dephosphorylated by the \textit{Yersinia} antiphagocytic effector YopH suggests that it may participate in macrophage antibacterial function, perhaps phagocytosis (Black et al., 2000; Hamid et al., 1999).
SKAP-HOM

SKAP-HOM is a cytoplasmic adaptor protein with homology to SKAP55 (Curtis et al., 2000; Liu et al., 1998), and it is also designated SKAP55R (SKAP55-related protein) (Marie-Cardine et al., 1998b) and RA70 (retinoic acid-inducible protein) (Kouroku et al., 1998). Both SKAP-HOM and SKAP55 contain a pleckstrin homology (PH) and a SH3 domain. However, in contrast to SKAP55, SKAP-HOM includes an additional N-terminal coiled-coil region, which is assumed to mediate non-covalent protein-protein interactions by homo- or hetero-oligomeric associations effected via charged residues (Lupas, 1996; Marie-Cardine et al., 1998b). SKAP55 is expressed exclusively in T lymphocytes (Marie-Cardine et al., 1997), whereas expression of SKAP-HOM seems to be essentially ubiquitous (Marie-Cardine et al., 1998b). Both these proteins can be tyrosine phosphorylated by Fyn kinase, and they have been implicated in TCR signalling (Marie-Cardine et al., 1997; Marie-Cardine et al., 1998b). Unlike SKAP55, SKAP-HOM is not tyrosine phosphorylated in resting T cells, but it is phosphorylated after activation of the cells (Marie-Cardine et al., 1998b; Schraven et al., 1997).

Timms et al. (1999) detected tyrosine phosphorylation of SKAP-HOM and Fyb in macrophages adhering to fibronectin, and Black et al. (2000) subsequently confirmed that SKAP-HOM forms a complex with Fyb and is tyrosine-phosphorylated in response to macrophage adhesion. Considering those results, along with the knowledge that both Fyb and SKAP-HOM are targets of YopH in macrophages (Black et al., 2000), it appears that SKAP-HOM affects actin dynamics under the control of integrin-mediated signals.

In a study of SKAP-HOM−/− mice (Togni et al., 2005), it was noted that SKAP-HOM was not required for growth of the animals or development of the haematopoietic system, nor was it needed for T cell and platelet functions, or for the Fc or complement receptor-mediated phagocytosis in bone marrow derived macrophages (BMMs). In contrast, the proliferative responses of anti-IgM- and LPS-stimulated B cells in knockout mice were significantly decreased. Further experiments demonstrated that SKAP-HOM is involved in the coupling between B cell receptors (BCRs) and integrin activation, whereas it is dispensable for BCR-mediated reorganization of the cytoskeleton.
3. Phagocytosis

3.1. Background

Phagocytosis (“cell eating”) entails the engulfment of solid particles (0.5–5 μM in diameter), such as microbial pathogens, apoptotic cells, large immune complexes, and cell debris. It is a major defence mechanism of the innate immune system of the hosts, and it is accomplished by professional phagocytes, including macrophages (the “classic” phagocytes) and neutrophils. However, cells referred to as non-professional phagocytes are also capable of phagocytosis.

The phagocytic process can be divided into several steps: (i) binding of particles to the cell surface; (ii) formation of a phagocytic vesicle termed a phagosome; (iii) maturation of the phagosome to a phagolysosome and digestion of engulfed particles in the phagolysosome. The initial step of phagocytosis involves recognition of the particles by special receptors on the plasma membrane of the phagocytes. These receptors can be either opsonic dependent or opsonic independent. Opsonins in serum consist of the Fc portion of immunoglobulins (Igs) bound to the particles, and the complement components C3b and iC3b deposited on the particle surface. Opsonic-dependent receptors, such as Fc and complement receptors, are recognized by opsonins supplied by the host. Structural determinants present on the surface of target particles bind directly to opsonic-independent receptors, which include complement receptor 3 (CR3), endotoxin receptors, β1 and β3 integrins, mannose receptor, the β-glucan receptor, the galactose receptor, and scavenger receptors (Tjelle et al., 2000). The best characterized of these receptors are FcγR (Fcγ receptor for IgG) and CR3 (receptor for complement protein iC3b). In response to *Yersinia* infection, β1 integrin expressed on the host cells can bind the bacterial surface protein invasin, and that mediates uptake of the bacteria in the absence of antiphagocytic Yop effectors (Isberg and Leong, 1990).

3.1.1 FcγR-mediated phagocytosis

Three classes of Fcγ receptors have been identified, which are designated FcγRI, FcγRII, and FcγRIII. These receptors differ with regard to their expression patterns,
but they share a highly homologous extracellular portion that contains the IgG-binding domain. Upon receptor clustering induced by binding of IgG-coated particles, the immunoreceptor tyrosine-based activation motifs (ITAMs) on the intracellular part are phosphorylated by Src family kinases, which leads to recruitment and activation of the protein tyrosine kinase Syk. This results in formation of signalling complexes close to the membrane, in which Syk-mediated phosphorylation of several adaptor proteins causes activation of downstream pathways that give rise to the phagocytic effect (Berton et al., 2005). The signalling molecules of those pathways include intracellular calcium, protein kinase C (PKC), phospholipase D (PLD), phosphatidylinositol 3-kinase (PI3-K), ERK, and GTPases of the Rho family (Garcia-Garcia and Rosales, 2002; May and Machesky, 2001; Swanson and Hoppe, 2004).

3.1.2 CR3-mediated phagocytosis

CR3 is a β2 integrin heterodimer that can bind to several ligands. One of those is iC3b, which is produced as follows: C3b is produced upon complement activation, and it binds to microbes, and acts as an opsonin; C3b is subsequently cleaved by plasma factors to yield iC3b. Ligation of CR3 by iC3b leads to opsonic-dependent phagocytosis. CR3 can also bind molecules on target particles such as β-glucan to mediate opsonic-independent phagocytosis (May and Machesky, 2001; Tohyama and Yamamura, 2006). Unlike FcγR-mediated phagocytosis, it seems that C3-opsonized particles simply sink into the host cells, with little formation of pseudopodia (Allen and Aderem, 1996). A plausible explanation for this difference is that CR3-mediated phagocytosis requires activation of GTPase RhoA, but it does not need the Rac and Cdc42, which are involved in the extension of pseudopodia that occurs to engulf the bacteria (Caron and Hall, 1998).

3.1.3 Bacterial uptake mediated by integrin-invasin interaction

Integrins are large heterodimeric transmembrane proteins that are involved in the spreading, migration, and survival of eukaryotic cells (van der Flier and Sonnenberg, 2001). Integrins can transmit signals to the cytoskeleton when cells adhere to substrates, and they bind the *Yersinia* surface protein invasin with high affinity (Isberg
Integrin-invasin binding is necessary for uptake of non-opsonized *Yersinia* (Fallman et al., 1995; Grosdent et al., 2002; Hudson et al., 2005). In addition to professional phagocytes, non-professional phagocytes such as epithelial cells and intestinal M cells also can internalize *Yersinia* through that binding (Clark et al., 1998; Isberg and Leong, 1990.

The integrin-mediated uptake of *Yersinia* has not yet been thoroughly elucidated, although a model has been proposed to explain internalization in non-phagocytic cells (Fallman et al., 1997). According to that model, *Yersinia* infection causes invasin on the bacterium to interact with β1 integrins on the host cell, which provokes clustering of the integrins and subsequent assembly of focal complex structures. These signalling complexes transduce signals to the cytoskeleton, resulting in actin reorganization that allows engulfment of the surface-attached bacterium. It has been shown that the β1 chain of the integrin is critical for downstream signalling during the uptake (Gustavsson et al., 2002; Van Nhieu et al., 1996). The proteins involved in downstream signalling include FAK, Cas, Paxillin, c-Src, Syk, WASP, and the small GTPase Rac1 (Alrutz and Isberg, 1998; Alrutz et al., 2001; Andersson et al., 1996; Black and Bliska, 1997; Black et al., 2000; Hudson et al., 2005; McGee et al., 2001; Persson et al., 1997; Wiedemann et al., 2001). FAK, Cas, and Paxillin are involved in formation of the focal adhesion complexes that connect integrins with the actin cytoskeleton (Brugge, 1998). Scibelli et al. (2005) have recently observed that flavoridin (a member of the disintegrin family) binds β1 integrin with high affinity and thereby prevents the disruption of focal adhesion complexes and inhibits the uptake of *Y. enterocolitica* by HeLa cells.

### 3.2. Actin dynamics during phagocytosis

Phagocytosis is an actin-dependent process that is mediated by several different receptors. Internalization of the target particle requires actin polymerization, pseudopod extension or membrane invagination, membrane recruitment, and closure of the phagocytic cup forming an intracellular phagosome (Aderem and Underhill, 1999).
3.2.1 Actin-based complex

Actin is an ATP-binding protein that exists in a monomeric form (G-actin) and as polymerized filaments (F-actin). Actin monomers have distinct ends and are assembled into filaments in a “head-to-tail” manner, which gives the filaments two different ends that are respectively called barbed (plus) and pointed (minus). Free barbed ends can be created by uncapping of pre-existing filaments or by de novo nucleation of monomeric actin, and that serves as a template for actin polymerization. Nucleation of free monomers is catalyzed by cellular actin nucleation factors, such as the actin-related protein (Arp) 2/3 complex (Welch et al., 1997). Nucleation of the actin can be controlled by proteins of the WASP family (Blanchoin et al., 2000; Machesky and Insall, 1998; Mullins et al., 1998; Zigmond, 2000).

The linkage of the actin cytoskeleton to the signalling pathway is generally mediated by the interaction between an active Rho family GTPase and a “connector” on the plasma membrane. Activation of cell surface receptors generates signals that induce the “connector” to recruit the Arp2/3 complex and thereby initiate growth at the barbed-end of actin filaments (Higgs and Pollard, 1999). Filaments grow when actin monomers are added at a fixed angle of 70° from the side of pre-existing actin filaments, and thus push the plasma membrane forward. Actin filament disassembly can be executed by cofilin, which is a member of the actin-depolymerizing factor (ADF)/cofilin family (Carlier et al., 1997; Lappalainen et al., 1998). This ADP-bound G-actin is exchanged for the ATP-bound form under the influence of profilin and thereby recycled back to achieve rapid elongation of new barbed ends. Elongation can be blocked upon depletion of ATP-bound G-actin or by binding of capping proteins to the barbed end (Pollard et al., 2000).

3.2.2 Regulators of the actin cytoskeleton

Regulation of actin rearrangement during phagocytosis involves various proteins, including GTPases, WASP, Ena/VASP, and some other actin-binding molecules.

Rho GTPases are small GTP-binding proteins, and most studies have focused on three members of this family, namely, Rac1, Cdc42, and RhoA. These three Rho
GTPases cycle between a GDP-bound (inactive) and a GTP-bound (active) state, and this process is catalyzed by the following molecules: guanine nucleotide-exchange factors (GEFs) such as Vav (Garcia-Garcia and Rosales, 2002), which enhance the switch from bound GDP to GTP; the GTPase-activating proteins (GAPs), which increase the intrinsic GTP hydrolysis; and the guanine nucleotide dissociation inhibitors (GDIs), which inhibit both the exchange of GDP for GTP and the hydrolysis of bound GTP (Van Aelst and D'Souza-Schorey, 1997). In the cytosol, a Rho GTPase forms a complex with a GDI and is in that way maintained in the GDP-bound inactive state, which can be released from GDI to be able to convert to the GTP-bound active form under the action of a GEF. The GTP-bound form interacts with a downstream effector and is transformed into the GDP-bound state by the action of a GAP. The GDP-bound molecule then forms a complex with the GDI and returns to the cytosol. In fibroblasts, Cdc42 generates cell polarity and elicits formation of filopodia; RhoA activity generates contractile actin bundles (stress fibres) and focal adhesion structures; and Rac1 activity induces actin polymerization to drive lamellipodial protrusion (Wittmann and Waterman-Storer, 2001). Studies have indicated that Rho GTPases are also involved in phagocytosis: Cdc42 controls pseudopod extension during FcR-mediated phagocytosis (Castellano et al., 2001); RhoA is necessary for CR3-mediated phagocytosis (Caron and Hall, 1998); and Rac1 controls phagosome closure during FcR-mediated phagocytosis (Castellano et al., 2001), and it is also required for β1 integrin-mediated uptake of Y. pseudotuberculosis (Alrutz et al., 2001; McGee et al., 2001).

The dynamin family of proteins comprises three large GTPases called dynamins 1, 2, and 3. Dynamin 1 is expressed solely in neurons, dynamin 2 is produced ubiquitously, and dynamin 3 is generated in testis (Urrutia et al., 1997). These proteins contain an N-terminal GTPase domain, a PH domain that binds phosphatidylinositol lipids to associate with membranes, and a C-terminal proline-rich region that binds to SH3 domains of actin-linked proteins such as profilin, mAbp1, and cortactin (Orth and McNiven, 2003). It has been reported that dynamin 2 is essential for the formation of clathrin-coated endocytic vesicles (Takei et al., 1995), and it is also involved in membrane trafficking in the trans-Golgi network (TGN) (Cao et al., 2000; Maier et al.,
1996). Moreover, this dynamin variant is required for FcγR-mediated phagocytosis in macrophages (Gold et al., 1999).

**Actin-binding proteins** constitute a large family that can be divided into several groups, only a few of which are listed below. Members of this family include the **WASP proteins**, which are located downstream of Rac1 and Cdc42 during cytoskeletal rearrangement (Blanchoin et al., 2000; Mullins, 2000). The WASP C terminus initiates the growth of actin filaments by recruiting actin monomers and the **Arp2/3 complex**. The Arp2/3 complex contains seven subunits, two of which (Arp2 and Arp3) are actin-related proteins. During phagocytosis the Arp2/3 complex is co-localized with F-actin in the phagosome (Alrutz et al., 2001; May et al., 2000). **Ena/VASP** contains a central proline-rich domain that binds the G-actin-binding protein **profilin** and a C-terminal Ena-VASP-homology domain 2 (EVH2) that binds F-actin (Krause et al., 2002). It has been shown that VASP, WASP, Fyb, Nck, and SLP-76 form a complex that represents a link between the actin cytoskeleton and FcγR-mediated phagocytosis (Coppolino et al., 2001).

### 3.3. Endocytic fusion and recycling during phagocytosis

Internalization of a particle results a nascent phagosome that fuses with endocytic compartments to reach maturation (Fig. 5). Finally, the matured phagosome merges with late endocytic organelles to form a phagolysosome, which is responsible for the killing and degradation of intracellular pathogens. The phagosome-lysosome fusion is probably a “kiss-and-run” interaction that involves brief exchange of solutes from the two compartments without complete intermixing of their fluid membranes (Tjelle et al., 2000). In addition to transient fusion, phagosome maturation can occur through complete fusion (Tjelle et al., 2000), but the mechanisms that control phagosome-endosome merging are still largely unknown. Phagosomes can also fuse with endoplasmic reticulum (ER) (Desjardins, 2003; Gagnon et al., 2002) and Golgi-derived vesicles (Fratti et al., 2003).
Figure 5. The phagocytic pathway. Microorganisms bind to receptors (X) on the surface of a host cell, which induces actin polymerization to internalize the microorganisms and form a nascent phagosome. The phagosome undergoes a series of fusions with endocytic compartments (early endosome, late endosome, and lysosome) to reach maturation, and microorganisms are completely degraded in the phagolysosome. Peptides from that process can be presented by major histocompatibility complex (MHC) class I molecules (active CD8⁺ T lymphocytes (Lehner and Cresswell, 2004)) or MHC class II molecules (active CD4⁺ T lymphocytes (Ramachandra et al., 1999)) to adaptive immune system (Niedergang and Chavrier, 2004).

ADP-ribosylation factor (ARF) and the Rab family members are small GTPases that are associated with distinct organelle membranes and regulate vesicular transport (Chavrier and Goud, 1999; Kawasaki et al., 2005). Arf6 has been found to be activated during FcγR-mediated phagocytosis, and it is necessary for membrane extension (Niedergang et al., 2003; Zhang et al., 1998). Rab5 and Rab7 are required for phagosome maturation (Deretic et al., 1997; Via et al., 1997), and, in macrophages, Rab5 is necessary for recruitment of Rab7 (Vieira et al., 2003). Rab5 is also a key molecule regulating the earlier events in phagosome-endosome fusion. In a study by Duclos et al. (2000) it was observed that expression of Rab5 mutants in macrophages
caused uncontrolled fusion, which led to the appearance of giant phagosomes that could begin to mature and acquire lysosome marker LAMP1, but could not kill intracellular parasites.

Another process in phagolysosome formation occurs through recycling of the phagosomal membrane to the plasma membrane or other endocytic/phagocytic vesicles. Rab11 is found in endosomes and nascent phagosomes, and it has been implicated in this recycling process based on the observation that expression of a GTP-binding-deficient mutant of Rab11 decreased the rate of transferrin efflux and impaired FcγR-mediated phagocytosis, whereas expression of a GTPase-deficient Rab11 mutant had the opposite effect (Cox et al., 2000). Arf6 has also been shown to target recycling vesicles to the plasma membrane (Radhakrishna and Donaldson, 1997). Other proteins involved in recycling include PI3-K, soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) and the ATPase NSF (Braun and Niedergang, 2006; Greenberg and Grinstein, 2002).

Macrophages lack a morphologically distinct pericentriolar recycling compartment and instead have an extensive network made up of transferrin receptor-positive tubules and vesicles that participate in plasma membrane recycling. As professional phagocytes, macrophages possess a mechanism for rapid renewal of the plasma membrane. This is confirmed by the observation that the rate of transferrin recycling in thioglycollate-elicited murine peritoneal macrophages is about two- to threefold higher than seen in most other types of cells (Cox et al., 2000).
AIM

The general aim of the research underlying this thesis was to investigate the mechanisms of YopH-mediated antiphagocytosis, particularly the roles of the YopH targets Cas and Fyb. More specific objectives were as follows:

- To analyse the effects of Cas during *Y. pseudotuberculosis* infection.

- To explore the functions of Fyb in macrophages and to investigate the effects of the YopH-induced dephosphorylation of Fyb.
RESULTS and DISCUSSION

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>YPIII</td>
<td>virulence plasmid cured</td>
<td>(Bolin et al., 1982)</td>
</tr>
<tr>
<td>MYM(YPIIIpIB29MEKA)</td>
<td>yadA::Tn5, yopHMEKypkA</td>
<td>(Hakansson et al., 1996)</td>
</tr>
<tr>
<td>MYMYopH</td>
<td>pyopH</td>
<td>(Persson et al., 1997)</td>
</tr>
<tr>
<td>MYMYopHC/A</td>
<td>pyopHC/A</td>
<td>(Persson et al., 1997)</td>
</tr>
<tr>
<td>MYMYopHQ11N34</td>
<td>pyopHQ11N34</td>
<td>(Deleuil et al., 2003)</td>
</tr>
<tr>
<td>YPIIIpIB102</td>
<td>yadA::Tn5, wild type</td>
<td>(Bolin and Wolf-Watz, 1984)</td>
</tr>
</tbody>
</table>

Table I. Y. pseudotuberculosis strains used in the research. MYM is a mutant strain of Y. pseudotuberculosis that lacks the effectors YopH, YopE, YopK, YopM, and YpkA, but still has intact genes encoding the proteins that are necessary to regulate, secrete, and translocate Yops (Hakansson et al., 1996). The MYM strain was used to express YopH and mutants thereof in order to investigate the role of that effector protein in host cells.

Interaction between YopH and Cas is required for YopH-mediated disruption of focal adhesion (Paper I)

Previous studies had shown that a catalytically inactive variant of YopH called YopHC/A forms stable complexes with its substrates in vivo (Guan and Dixon, 1990), and thus that variant was used to identify the protein targets of YopH in host cells. YopH targets p130Cas in HeLa cells, fibroblasts, and macrophages (Black and Bliska, 1997; Hamid et al., 1999; Persson et al., 1997), and YopHC/A co-localizes with p130Cas in adhesion structures of infected cells (Black and Bliska, 1997; Deleuil et al., 2003; Hamid et al., 1999; Persson et al., 1997). We used p130Cas−/− mouse embryonic fibroblasts (MEFs) to explore the effects of Cas on host cell responses during Yersinia infection. The results revealed that YopHC/A does not localize to focal adhesions in these cells, even though those structures are similar to the ones seen in wild-type MEFs (Fig. 1A in Paper I). The localization of YopHC/A to focal adhesion structures
could be restored by re-expression of full-length Cas in the p130Cas−/− cells (Fig. 1B in Paper I), which implies that Cas is responsible for the subcellular localization of YopH. Interestingly, in our study, when Cas lacking the central substrate-binding domain (ΔSDCas) was expressed in p130Cas−/− cells, it was unable to recruit YopHC/A to focal adhesions, whereas the variant itself was localized to these structures (Fig. 1B in Paper I). Further immunoprecipitation experiments demonstrated that the central substrate-binding domain of p130Cas is required to allow association with YopH (Fig. 1C in Paper I). There are 15 tyrosine motifs in the central substrate-binding domain of Cas that may serve as potential phosphorylation sites for cellular protein tyrosine kinases (Songyang and Cantley, 1998), and thereby as the target sites of bacterial phosphatase YopH. Those motifs have been implicated as binding molecules that transduce signals to control cell motility (Klemke et al., 1998).

It has been observed that host cells infected with Yersinia strains that express active YopH become round and detached (considered to be the effects of cytotoxicity) due to the disruption of focal adhesion structures containing p130Cas (Persson et al., 1997). We found that P130Cas−/− MEFs were less sensitive to this effect, and the sensitivity could be restored by re-expression of full-length Cas but not by expression of the substrate-domain-depleted Cas mutant (Fig. 4 and Table 1 in Paper I). These results suggested that the interaction between YopH and Cas is important for the cytotoxic effect mediated by YopH. The tyrosine kinase FAK binds to the SH3 domain of p130Cas (Polte and Hanks, 1995). Our research group has previously shown that FAK and p130Cas form a complex with YopHC/A in HeLa cells (Persson et al., 1997), and we obtained similar results when using wild-type fibroblasts (Fig. 2A in Paper I). However, in macrophages, FAK is not a substrate of YopH but Cas is (Hamid et al., 1999), and that finding suggested that FAK binds YopH via Cas. In agreement with that assumption, we noted that FAK did not associate with YopHC/A in p130Cas−/− MEFs, whereas p130Cas coupled with YopHC/A in FAK−/− MEFs (Fig. 2A in Paper I). Furthermore, immunofluorescence staining of those cells indicated that Cas, but not FAK, is required to recruit YopH to focal adhesion structures (Fig. 2B in Paper I).

Both Cas and FAK have been shown to participate in β1 integrin-mediated internalization of Yersinia in epithelial cells (Alrutz and Isberg, 1998; Bruce-Staskal et al., 2002; Weidow et al., 2000). Surprisingly, we did not observe any reduced uptake of
Yersinia in p130Cas"/− MEFs, as compared to wild-type cells from same littermate (Fig. 3 in Paper I). This suggested that p130Cas is not essential for the uptake of Yersinia, which was confirmed by the fact that re-expression of full-length Cas in Cas"/− cells did not have a significant impact on the internalization of the bacteria (Fig. 3 in Paper I). In the absence of p130Cas, it is possible that other members of the Cas family such as HEF-1/Cas-L and Sin/Efs can function in uptake, since they are similar to p130Cas with regard to structure and localization (O’Neill et al., 2000). Presumably, Cas that lacks the substrate-binding domain acts as a dominant-negative for other members of the Cas family, which is supported by the observation that expression of ΔSDCas inhibited uptake of Yersinia by p130Cas"/− MEFs (Fig. 3 in Paper I). FAK has been implicated to play a role in integrin-mediated uptake (Alrutz and Isberg, 1998), in consistence with that, internalization of the bacteria was greatly impaired in FAK"/− MEFs as compared to littermate wild-type cells (Fig. 3 in Paper I). Except for that FAK-dependent uptake of Yersinia, Bruce et al. (2002) have reported a FAK-independent pathway that requires Pyk2, as well as p130Cas, Crk, and Src. Pyk2 is a FAK-related proline-rich protein tyrosine kinase (Avraham et al., 1995) that has been found to associate with Cas in FAK"/− MEFs (Ueki et al., 1998). Expression of Pyk2 is elevated in both FAK"/− MEFs (Sieg et al., 1998) and normal macrophages (Bruce-Staskal et al., 2002). Recently, Owen et al. (2006) have suggested that FAK is required for invasin-integrin mediated uptake of Y. pseudotuberculosis by macrophages, while Pyk2 is essential for YadA/ECM-integrin mediated uptake. The mechanism of Cas/Pyk2-mediated uptake is still unclear, since Pyk2 does not contain the sequences in FAK that have been shown to bind to integrins (Klingbeil et al., 2001; Schaller et al., 1995). It is possible that Cas/Pyk2 functions the same as Cas/FAK to bring Cas to Src kinase and thereafter to induce phosphorylation of Cas. That phosphorylation results in the recruitment of Crk and activation of Rho GTPase Rac1, which causes the cytoskeletal rearrangement (Ridley, 2000; Weidow et al., 2000) that is a prerequisite of bacteria internalization. YopH dephosphorylates Cas in infected cells and thereby disrupts the Cas-mediated signalling process, which in turn leads to impaired uptake of Yersinia.
Macrophages are professional phagocytes that are believed to be targets for pathogenic *Yersinia* to inject virulence effectors, Yops, via the type III secretion system (Marketon et al., 2005). YopH is one of the Yops that is delivered into macrophages and blocks their capacity for phagocytosis (Fallman et al., 2002), and it also appears to be highly critical for the ability of pathogenic *Yersinia* to remain in the lymphoid system and establish an infection (Logsdon and Mecsas, 2003; Trulzsch et al., 2004; Viboud and Bliska, 2005). In the mouse macrophage-like J774 cells, YopH selectively dephosphorylates Cas, Fyb, and SKAP-HOM (Black et al., 2000; Hamid et al., 1999). Of all the substrates of YopH, Fyb is the only one that is a haematopoietic-specific protein. We initiated our studies of Fyb by investigating the way it interacts with YopH (Paper II). GST-YopHC/A was used to pull down lysates of J774 cells that were infected with MYM or with MYM expressing active YopH or catalytically inactive YopHC/A. The results showed that YopH interacted with tyrosine-phosphorylated Fyb (Fig. 1 in Paper II). In addition, proteins associated with YopHC/A exhibited enhanced phosphorylation, which agrees with earlier findings and probably indicates that YopHC/A protects Fyb from endogenous phosphatases (Hamid et al., 1999). To ascertain which region in YopH is involved in the interaction with Fyb, we performed *in vitro* binding studies using different variants of His-Fyb and GST-YopH protein fragments (Fig. 2, a and b in Paper II). This showed that the 1–130 amino acids in the N-terminal region of YopH were linked to tyrosine phosphorylated Fyb (Fig. 2d in Paper II), which is similar to what has been reported regarding the interaction of YopH with Cas (Black et al., 1998).

Research has revealed that the C-terminal PTPase domain of YopH harbours an additional substrate-binding domain, which interacts with Cas in a phosphotyrosine-dependent manner. It has been suggested that this domain co-operates with the N-terminal substrate-binding domain of YopH to promote efficient recognition of Cas by YopH in epithelial cells (Montagna et al., 2001). However, the substrate-binding ability of the YopH N terminus is about 12-fold higher than that of
the phosphatase domain (Montagna et al., 2001). The three-dimensional structure of the YopH N-terminal region has been determined (Evdokimov et al., 2001), and it is known that residues Q^{11}, V^{31}, A^{33}, and N^{34} are involved in substrate binding (Deleuil et al., 2003; Montagna et al., 2001). Our immunoprecipitation analyses using YopHQ^{11}N^{34} confirmed that these residues are also necessary for the interaction of YopH with Fyb (Fig. 2e in Paper II).

Our next intention was to reveal the biological importance of the YopH N-terminal substrate-binding region with regard to the effects that this protein has on macrophages. An important task of YopH is to block phagocytosis during *Yersinia* infection (Fallman et al., 2002), and the present immunoprecipitation experiments showed that YopHQ^{11}N^{34} has a reduced capacity to dephosphorylate Fyb in macrophages (Fig. 3a in Paper II). That finding is in analogy with a previous study conducted by our research team, showing that MYMYopHQ^{11}N^{34} was less efficient than MYMYopH at blocking bacterial uptake by HeLa cells (Deleuil et al., 2003). Similar results were obtained using the more phagocytosis-efficient J774 cells (Fig. 3b in Paper II). YopH also has toxic effects on cultured cells, and we found that J774 cells infected with MYMYopHQ^{11}N^{34} were less sensitive to the YopH-mediated cytotoxicity compared to cells exposed to MYM expressing wild-type YopH (Fig. 3c in Paper II). Those observations suggested that the N-terminal substrate-binding domain of YopH is necessary for both a full antiphagocytic effect and cytotoxicity towards macrophages. In addition, the substrate-binding ability of YopH N terminus is also important for the virulence of *Y. pseudotuberculosis* (Deleuil et al., 2003).

The role of Fyb in macrophages is not yet fully understood. Nonetheless, the fact that YopH targets Fyb implies that this immune-cell-specific adaptor protein plays an important role in the antimicrobial function of macrophages.

**mAbp1 is a novel interactor of Fyb (Paper III and unpublished data)**

Previous studies have suggested that Fyb is involved in integrin-mediated cell adhesion (Geng et al., 2001), which indicates that this protein is associated with signalling to the actin cytoskeleton. Furthermore, Krause et al. (2000) have reported
that Fyb is localized to actin-rich structures in cells, potentially via interaction between the FPPPP motif of Fyb and the EVH1 domain of Ena/VASP proteins.

To study the function of Fyb in macrophages, and especially in phagocytosis, we first investigated the effects of the Fyb-Ena/VASP interaction on localization of Fyb to actin-rich cellular structures. Surprisingly, our results showed that the FPPPP motif is not required for the association of Fyb with such structures in J774 cells (Fig. 1 in Paper III). Also, a pull-down assay using GST-Fyb truncated variants and J774 cell lysates revealed that only a minor portion of VASP binds Fyb (supplementary data Fig. 1 in Paper III). Taken together, these findings indicated that an additional part of Fyb directly or indirectly mediated the linking between Fyb and the actin cytoskeleton.

To identify new Fyb-interacting proteins in immune cells, we performed a yeast two-hybrid screen using a mouse lymphoma cDNA library. This approach detected a novel interactor of Fyb called mammalian actin-binding protein 1 (mAbp1), which is also known as SH3P7 (Sparks et al., 1996) and HIP-55 (haematopoietic progenitor kinase 1 (HKP1) interacting protein of 55kDa) (Ensenat et al., 1999). The protein mAbp1 is ubiquitously expressed, and it contains a single actin-depolymerizing factor homology (ADF-H) domain in its N terminus, which is followed by an α-helical structure region, a stretch of PXXP and YXXP motifs, and a C-terminal SH3 domain (Fig. 6) (Ensenat et al., 1999; Kessels et al., 2001; Larbolette et al., 1999).

Our in vitro interaction studies showed that the N-terminal part (residues 1–339) of Fyb, which contains several proline-rich regions, binds to the mAbp1 SH3 domain (Fig. 4 E, F, and G in Paper III). We also used immunofluorescence staining of J774 cells to determine the subcellular localization of mAbp1. These experiments revealed mAbp1 in the perinuclear region, as well as in the leading edge of cells where it co-localized with F-actin (Fig. 3B in Paper III). Those locations correspond to what has previously

Figure 6. Schematic representation of mAbp1 and the proteins that interact with mAbp1.
been shown for Fyb in other types of cells (Geng et al., 2001). In accordance with that, co-staining of Fyb and mAbp1 demonstrated that these proteins co-localized in areas displaying high actin dynamics, namely, in cell edges and ruffles (Fig. 3C in Paper III). The co-localization of Fyb and mAbp1 was observed solely at the cell edges and neither of these proteins was found in punctuated structures resembling focal adhesions. The latter observation agrees with published data indicating that Fyb is not localized to adhesion structures (Krause et al., 2000). Overexpression of the Fyb N terminus in HeLa cells led to distinct accumulation of the protein in the edges of the cells (Fig. 7), which further supports the notion that the Fyb-mAbp1 interaction is responsible for the localization of Fyb in actin-rich structures.

Additional infection experiments using *Yersinia* and J774 cells showed that both Fyb and mAbp1 were tyrosine dephosphorylated by YopH, but this did not affect the Fyb-mAbp1 interaction (Fig. 5 in Paper III). In accordance with that observation, co-staining of Fyb and mAbp1 revealed both these proteins at the cell edges even in the presence of active YopH (supplementary data Fig. 2 in Paper III). Hence, the Fyb-mAbp1 interaction is not interrupted by YopH, which is consistent with studies *in vitro* in which it was found that the association between Fyb and mAbp1 did not depend on the tyrosine phosphorylation of those two proteins. It has previously been reported that mAbp1 is localized to areas that harbour newly formed actin structures, and such assignment of the protein does not require tyrosine phosphorylation (Kessels et al., 2000). Moreover, in our experiments, the tyrosine phosphorylation of Fyb, but not mAbp1, was stimulated by the β1 integrin engagement caused by the *Yersinia* infection (Fig. 5, A and B in Paper III). This supports a model in which tyrosine phosphorylation of Fyb is involved in the receptor-mediated clustering of β1 integrin, whereas such phosphorylation of mAbp1 is less important.
Fyb has been reported to form a complex with Nck, VASP, and WASP that is translocated to phagocytic cups during FcγR-mediated phagocytosis (Coppolino et al., 2001). However, Fyb does not bind directly to Nck or WASP. Fyb is probably tyrosine phosphorylated upon stimulation and can therefore operate via SLP-76 to associate with Nck and Vav. The latter protein is a GEF of Rho GTPases and it has WASP as a downstream effector. Thus WASP activates the Arp 2/3 complex, which results in local actin rearrangements at the contact site. However, our observation that tyrosine-dephosphorylated Fyb co-localized with mAbp1 in the cell edges implicated an additional way for Fyb to connect actin cytoskeleton. Indeed, the yeast orthologue of mAbp1 has been found to be an activator of the Arp 2/3 complex, and it is critical for regulating the actin cytoskeleton (Goode et al., 2001; Qualmann and Kessels, 2002). mAbp1 is also co-localized with Arp 2/3 in the leading edges of newly formed lamellipodia, though it lacks the acid regions that are required for the activating capacity of Arp 2/3 (Kessels et al., 2000). Accordingly, our finding of mAbp1 as an interactor of Fyb suggests a novel linkage occurs between the Fyb-containing complex and the F-actin network.

mAbp1 influences spreading of macrophages and antiphagocytosis mediated by pathogenic Yersinia (Paper IV and unpublished data)

Considering mAbp1, the ability to bind F-actin and the observed localization to F-actin-rich structures suggested that this protein participates in the regulation of actin dynamics. However, we found that neither RNA interference (RNAi) of mAbp1 nor overexpression of the different variants of mAbp1 (Fig. 1A in Paper IV) in HeLa cells affected β1 integrin-mediated cell spreading (Fig. 4A in Paper IV and unpublished data), which also concurs with findings in human embryonic kidney cells (Mise-Omata et al., 2003). This does not rule out the possibility that mAbp1 is involved in regulating F-actin dynamics, probably not for forces leading to that kind of membrane extensions in these cell types. In contrast to what has been observed regarding mAbp1 in HeLa cells, ectopic expression of full-length mAbp1 in J774 cells increased both the
spreading areas and the number of spreading cells, whereas expression of the mAbp1<sup>W/A</sup> mutant and the two SH3 variants resulted in impaired spreading (Fig. 4 B and C in Paper IV). These results indicated that both the actin-binding region and the SH3 domain of mAbp1 are required to induce an increase in spreading. The actin-binding region is likely important for localization to the cell periphery where the dynamic rearrangement and growth of the F-actin network occurs, whereas the SH3 domain likely contributes by binding proteins important for this event, such as Fgd1, which is the Cdc42 GEF that stimulates F-actin reorganization via the Rho GTPase Cdc42 (Hou et al., 2003; Olson et al., 1996), or other proteins binding the mAbp1 C terminus. The finding that mAbp1 greatly promoted cell spreading specifically in macrophages also suggested that mAbp1 might have different functions in haematopoietic and non-haematopoietic cells. The mAbp1 protein interacts with at least two haematopoietic-specific proteins, Fyb (Paper III) and HPK1 (Ensenat et al., 1999), the latter of which is a protein serine/threonine kinase and a member of the MAPK kinase kinase kinases family (Han et al., 2003). In T cells, mAbp1 is mainly associated with HPK1 (Ensenat et al., 1999) and that complex has been found to regulate TCR signalling, such as T cell proliferation, cytokine production, and immune responses (Le Bras et al., 2004; Chen and Tan, 2000; Han et al., 2005). HPK1 can activate JNK, which has been implicated in cell migration (Huang et al., 2004). However, it is still unclear whether mAbp1 and HKP1 form a complex in macrophages, and the role of HPK1 in that cell type is largely unknown. Interestingly, Fyb, the other haematopoietic-specific protein that can interact with mAbp1, has been implicated in integrin signalling in T cells (Peterson et al., 2001). In macrophages, Fyb is a target of YopH (Black et al., 2000; Hamid et al., 1999), which is responsible for the impairment of β1 integrin-mediated cytoskeletal rearrangements (Fallman et al., 2002). Hence, mAbp1 might influence the β1 integrin-mediated spreading via the association with Fyb.

The mAbp1 protein has also been implicated in the coordination of endocytic and cytoskeletal activities with the evidence that this protein binds directly to the following molecules: dynamin, which is the GTPase that controls fusion of endocytic vesicles (Kessels et al., 2001); and piccolo, which is the presynaptic zinc finger protein that can
interact with both profilin and actin to participate in synaptic vesicle endo- and exocytosis (Fenster et al., 2003). Thus, via the mentioned proteins, mAbp1 might influence F-actin-dependent events that are essential for membrane invagination and vesicle trafficking. Inasmuch as that mAbp1 is involved in endocytosis (Connert et al., 2006; Kessels et al., 2000; Kessels et al., 2001; Mise-Omata et al., 2003; Sauvonnet et al., 2005 and Fig. 2B in Paper IV), we conducted experiments to determine whether this protein also participates in the phagocytic process, which in many ways resemble endocytosis. Expression of the different mAbp1 variants in HeLa and J774 cells indicated that mAbp1 is not required for internalization of the non-virulent Y. pseudotuberculosis strain YPIII (Fig. 3, A and B in Paper IV). In contrast, HeLa cells overexpressing mAbp1 showed a slight increase in uptake of the wild-type Y. pseudotuberculosis strain YPIIIpIB102 (Fig. 3A in Paper IV). Furthermore, enforced expression of mAbp1W/A (in which residue 411 is mutated to Ala, a change that blocks interaction with the proline-rich region of associated proteins such as Fyb (Paper III)) in both HeLa and J774 cells led to impaired uptake of YPIIIpIB102 (Fig. 3, A and B in Paper IV). Hence, mAbp1FLW/A might acts as a dominant-negative protein by occupying sites for endogenous mAbp1 and thereby reducing the phagocytic capacity of the cells even further. These results suggested that mAbp1 can participate in, but is not essential for, β1 integrin-mediated uptake of Yersinia, and support for that assumption was provided by assays of the uptake of such bacteria by HeLa cells with mAbp1 RNAi (Fig. 3D in Paper IV). The exact role of mAbp1 in this process awaits further analysis.

Role of the Fyb/SKAP-HOM complex in macrophages (unpublished data)

To investigate the role of Fyb in macrophages, we used both overexpression of Fyb and RNAi to knock down Fyb in J774 cells. However, we failed to maintain overexpression of Fyb, whereas Fyb RNAi reduced the expression with about 70% (Fig. 8). In addition, the level of SKAP-HOM, but not mAbp1, was also impaired in Fyb RNAi cells (about 60% reduction, Fig. 8). SKAP-HOM is also a substrate of YopH in macrophages, and it binds Fyb via its SH3 domain (Liu et al., 1998). These
results suggested that the level of SKAP-HOM protein depends on the presence of Fyb. Similar findings regarding Fyb and SKAP55 have been obtained in T cells (Huang et al., 2005), in which Fyb binds SKAP55 to protect against proteolysis of SKAP55. In addition, deficiency of SKAP-HOM does not alter expression level of Fyb in T cells (Togni et al., 2005). Hence, a possible explanation for the unsuccessful overexpression of Fyb in our experiments is that the enforced expression of Fyb may have increased the level of SKAP-HOM, which has been reported to inhibit the growth of myeloid cells (Curtis et al., 2000).

![Relative expression of proteins in J774 and Fyb RNAi cells.](image)

**Figure 8. Relative expression of proteins in J774 and Fyb RNAi cells.** Results are the mean ± SD of three separate experiments.

Fyb and SKAP-HOM have been identified in complex with SHPS-1 in macrophages (Timms et al., 1999). SHPS-1 is also known as SIRPα (signal regulatory protein α) (Kharitonenkov et al., 1997), BIT (brain Ig-like molecule with tyrosine-based activation motifs) (Sano et al., 1997), or p84 (Chuang and Lagenaur, 1990), which is an Ig-like transmembrane receptor that contains cytoplasmic immunoreceptor tyrosine-based inhibitory motifs (ITIMs) (Fujioka et al., 1996). Tyrosine phosphorylation of those motifs mediates association between SHPS-1 and tyrosine phosphatase SHP-1, and in that way SHPS-1 recruits SHP-1 to the macrophage cell membrane. SHP-1 is generally viewed as a negative regulator of cell signalling. Moreover, SHPS-1 is extremely abundant in macrophages (Veillette et al., 1998), and hence it has been suggested that protein participates in the negative control of macrophage functions (Oshima et al., 2002; Yamao et al., 2002). Recruitment of Fyb to SHPS-1 occurs through SKAP-HOM (Timms et al., 1999). Both Fyb and SKAP-HOM are the specific targets of the antiphagocytic effector YopH in
macrophages, thus they might be involved in the transmission and/or regulation of signals from integrins. In addition, SHPS-1 also has been implicated in integrin-mediated signalling (Inagaki et al., 2000; Maile et al., 2003; Tsuda et al., 1998). To ascertain whether Fyb/SKAP-HOM acts via SHPS-1 to influence uptake of Yersinia, we treated J774 cells with SHPS-1 antibodies for 30 min and then subjected them to a Yersinia uptake assay. Those antibodies bind to the extracellular Ig-like part of SHPS-1 and thereby inhibit the activation through this region. There was no obvious difference between antibody-treated and untreated cells with regard to their ability to internalize YPIII or YPIIIpIB102 (Fig. 9, A and B). This is an unexpected finding, since it has been shown that SHPS-1 negatively regulates macrophage phagocytosis of antibody-coated red blood cells (Ikeda et al., 2006; Oshima et al., 2002). Perhaps the amounts of SHPS-1 antibodies we used were insufficient to result in a significant difference between the treated and untreated cells. However, it is also possible that different signalling pathways or molecules participate in the phagocytosis of different types of particles. In other words, previous studies have used antibody-coated red blood cells in uptake assays, which represent FcγR-mediated phagocytosis, whereas we focused on β1 integrin-mediated internalization of pathogens.

Figure 9. Uptake of YPIII (A) and wild-type YPIIIpIB102 (B) by J774 cells. The J774 cells were left untreated or were treated with 1 or 10 μg/ml anti-SHPS-1 antibodies before performing the uptake assays. Results are the mean ± SD of three separate experiments.

It is still not clear whether formation of a Fyb/SKAP-HOM/SHPS-1 complex is induced by Yersinia infection or if it is mediated by tyrosine phosphorylation. To address that issue, we used J774 cells infected with MYM, MYM expressing wild-type YopH, or catalytically inactive YopHC/A; uninfected cells were used as controls. Cell
lysates were immunoprecipitated with SKAP-HOM antibodies, and immunocomplexes were detected with Fyb, SHPS-1, or SKAP-HOM antibodies. The results showed that a major portion of cellular Fyb was bound to SKAP-HOM (Fig. 10, A and B), and, even after an infection time of one hour, this interaction was not abolished in the presence of active YopH, and that Yop induces tyrosine dephosphorylation of Fyb and SKAP-HOM (Fig. 10B). This observation further confirmed the previous finding that Fyb binds SKAP-HOM in a phosphotyrosine-independent manner (Black et al., 2000). In contrast, an interaction between SHPS-1 and SKAP-HOM was detected only in the lysates of cells infected with MYMYopHC/A (Fig. 10A). The role of SHPS-1 during Yersinia infection has not been fully elucidated, although it is apparently not required for internalization of the bacteria. It has been suggested that MyD-1, which is the bovine homologue of SHPS-1, can promote the attachment of APCs to CD4+ T cells (Brooke et al., 1998), and hence Fyb/SKAP-HOM may act through SHPS-1 to activate the adaptive immune system.

Figure 10. Immunoprecipitation of J774 cell lysates with anti-SKAP-HOM antibodies. A: J774 cells were left uninfected or infected with MYM, or MYMYopH, or MYMYopHC/A for 45 min. B: J774 cells were left uninfected or infected with MYMYopH for various times.

Another transmembrane receptor, PIR-B (paired immunoglobulin-like receptor B), has been reported to form a complex with Fyb/SKAP-HOM in macrophages during Yersinia infection (Black et al., 2000). PIR-B is a negative regulator of β2 integrin-mediated spreading of BMMs (Pereira et al., 2004), but its role in Yersinia
uptake has not yet been explained. Similar to SHPS-1, PIR-B also contains cytoplasmic ITIM motifs that can be tyrosine phosphorylated and thereby mediate association of PIR-B with SHP-1 (Pereira and Lowell, 2003). It is not known whether the extracellular domains of these receptors bind directly to invasin, or if they must associate with an integrin receptor to induce activation of the Fyb/SKAP-HOM signalling complex. However, we cannot rule out the possibility that Fyb/SKAP-HOM is primarily cytosolic or that it interacts with some other type of transmembrane receptor in J774 cells.

The functions of Fyb in macrophages have been obscure, although the discovery that this adaptor is a substrate for the bacterial antiphagocytic factor YopH suggested that it is involved in phagocytosis. However, in our experiments, Fyb RNAi did not change J774 cells with regard to the internalization of Yersinia (data not shown). It has been shown that the retinoic-acid-induced protein designated PML-RARalpha target gene encoding an adaptor molecule-1 (PRAM-1) shares structural homologies with Fyb (two tyrosine motifs and an SH3-like domain), and it also binds the adaptors SLP-76 and SKAP-HOM (Moog-Lutz et al., 2001). Recently, it was also noted that, like Fyb, PRAM-1 can bind mAbp1 (Denis et al., 2005). Experiments on PRAM-1−/− mice have demonstrated that PRAM-1 is necessary for integrin-mediated adhesion-dependent production of reactive oxygen intermediates and cellular degranulation, but not for other integrin-dependent responses, such as cell spreading and activation of several signalling pathways in neutrophils (Clemens et al., 2004). PRAM-1 is expressed and regulated during normal human myelopoiesis (in haematopoietic tissue, such as bone marrow and peripheral blood leukocytes), whereas there is only negligible expression of this protein in BMMs and mast cells, even though they contain PRAM-1 mRNA (Clemens et al., 2004). It is still unclear whether knock down of Fyb can affect the expression of PRAM-1 or if expression of PRAM-1 in Fyb−/− cells can restore the function of Fyb.

Macrophages constitute the first line of defence in lymphoid tissues, and they are considered to be the major targets for the antiphagocytic effectors of Y. pseudotuberculosis. Besides phagocytosing pathogens, macrophages perform the
important functions of killing the invaders and presenting antigens to the adaptive immune system. It is plausible that Fyb is involved in the killing of bacteria or other immune responses.
CONCLUSIONS

During infection, pathogenic *Y. pseudotuberculosis* can inject virulence effectors (Yops) into host cells to interfere with the antipathogenic machinery. YopH is a PTPase, and as such it blocks phagocytosis by targeting proteins that mediate the signalling associated with cytoskeletal rearrangements.

The present research has shown the following:

1. In non-professional phagocytes, YopH binds the substrate domain in Cas, and this interaction is required for YopH-mediated disruption of focal adhesions and cytotoxicity, which are associated with antiphagocytosis.

2. In macrophages, the N terminus of YopH binds tyrosine-phosphorylated Fyb, and this domain is important for YopH-mediated antiphagocytosis.

3. The protein mAbp1 is a novel interactor of Fyb in macrophages. The binding between these two proteins occurs via the SH3 domain in mAbp1 and the N terminus in Fyb. mAbp1 is implicated to influence β1 integrin-mediated spreading and the antiphagocytic effects of pathogenic strains of *Y. pseudotuberculosis*.

4. Fyb binds another YopH target, SKAP-HOM, in a tyrosine-independent manner, and the endogenous level of SKAP-HOM is regulated by Fyb in macrophages.

The functions of Fyb and mAbp1 in macrophages are the subjects of our ongoing research.
ACKNOWLEDGEMENTS

First, I would like to express my gratitude to my supervisor, Maria Fällman, for giving me the chance to be her graduate student. I had almost no background in molecular biology experiments, could you believe it? Thank you, Maria, for your great guidance and strong support throughout my studies.

I want to thank the following former members of “Maria’s girls”: Nivia Hamid, Karen McGee, Fabienne Deleuil, Anna Gustavsson, Lena Mogemark, Ritu Andersson, and Nina Ericsson for their assistance in the lab. Karen, Anna, and Fabienne were very supportive at the beginning of my studies. Thank you Lena for your excellent collaboration, and Ritu for your help with my son’s daycare. Also thanks to all for the tasty contributions to Friday’s group meeting. Karen and Lena, I miss your soft chocolate cakes; Anna, you made such a beautiful birthday cake, I could not believe you made it yourself! I also want to thank the present members of our group, Anna Fahlgren, Sara Carlsson, and Anders Fagerström, for sharing good times in the lab. Special thanks to Sara for help with the manuscript. I am also grateful to the former and present subjects and summer students for helping with my experiments.

Thanks to the all my teaching colleagues. It’s almost Christmas time again, and I still remember when I got a card and chocolate from students during my first year of teaching. Edmund, I will never forget that funny lab report you shared with us: “Bacteria look like the needles that fall off the Christmas tree.”

I am also grateful to all the past and present members of the “Journal Club” and the “Lab meeting” for your constructive discussions and suggestions. Thank you everybody at the department for help with the equipment and other things…..!

Special thanks to Patricia Ödman for correcting the English in this thesis.

Thanks to my Chinese friends: Jufang Wu, Bo Huang, Guangwei Ou, Changchun Chen, Professor Jin and his wife Gu. Shall we go fishing somewhere again next summer? Suyan Wang and her family, we had a good time together in Greece during that EMBO course!

I am very grateful to my parents, Yongqiang Ji and Suyun Yuan. I miss you very much! Mama, you always understand me. Dad and mama, I think you are the best
parents! I love you! 感谢父母多年来的支持! Thanks to my brother Tao Ji and his wife Min Zhang for their support and understanding during these years. Min, good luck with your own thesis!

Last, but not at all least, I want to thank my husband Jian. You helped me enormously at the beginning of my studies, you are my “second supervisor” of molecular biology, and without you I would never have got this far! My little boy, Yuanyang, you bring me so much joy everyday! I love you!

This work was supported by the Swedish Medical Research Council, the Swedish Cancer Foundation, the King Gustaf Vth 80 Year Foundation, the Medical Research Foundation at Umeå University, and the J.C. Kempes Memorial Foundation.
REFERENCES


protein tyrosine phosphatase binding proteins in cattle that are expressed on monocytes and a subpopulation of dendritic cells and which mediate binding to CD4 T cells. *Eur J Immunol*, **28**, 1-11.


Gagnon, E., Duclos, S., Rondeau, C., Chevet, E., Cameron, P.H., Steele-Mortimer, O., Paiement, J.,


Lindler, L.E., Klemmper, M.S. and Straley, S.C. (1990) Yersinia pestis pH 6 antigen: genetic, biochemical,


Olson, M.F., Pasteris, N.G., Gorski, J.L. and Hall, A. (1996) Faciogenital dysplasia protein (FGD1) and Vav,


Radhakrishna, H. and Donaldson, J.G. (1997) ADP-ribosylation factor 6 regulates a novel plasma membrane...


