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Cell invasion of *Yersinia pseudotuberculosis* by invasin and YadA requires protein kinase C, PLC- γ 1 and Akt kinase

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Summary

The outer membrane proteins YadA and invasin of *Y. pseudotuberculosis* promote invasion into mammalian cells through β_1 -integrins and trigger the production of interleukin-8. FAK, c-Src, and the PI3 kinase were previously found to be important for both YadA- and invasin-promoted uptake. Here, we demonstrate that two different downstream effectors of PI3 kinase, Akt and phospholipase C γ 1 are required for efficient cell invasion. Inhibition of Akt or PLC- γ 1 by pharmaceutical agents as well as reduced expression of the isoforms Akt1 and Akt2, and of PLC- γ 1 by RNA interference decreased entry of YadA- and Inv-expressing bacteria significantly. In addition, we report that the conventional protein kinases C α and - β , positioned downstream of PLC- γ 1, are activated upon Inv- or YadA-promoted cell entry. They colocalize with intracellular bacteria and their depletion by siRNA treatment also resulted in a strong reduction of cell entry. In contrast, neither Akt, nor PLC- γ 1 and the PKCs are essential for YadA- and Inv-mediated IL-8 synthesis and release. We conclude that YadA and invasin of *Y. pseudotuberculosis* both trigger similar signal transduction pathways during integrin-mediated phagocytosis into epithelial cells, which lead to the activation of Akt, PLC- γ 1, PKC α and - β downstream of PI3 kinase, separate from the MAPK-dependent pathway that triggers IL-8 production.

Introduction

An efficient infection process of a bacterial pathogen is generally accompanied by a precise subversion of the cellular machinery of the host. This includes the activation of certain host cell signal transduction pathways by bacterial adhesins, which are crucial for triggering cytoskeletal rearrangements to promote binding and entry of the microbe into host cells. The enteric pathogen *Y. pseudotuberculosis* is an excellent model organism for studying signalling events that occur during host pathogen interactions, as it evolved multiple strategies to invade the intestinal epithelial layer to colonize underlying lymphoid follicles (Peyer's patches) after oral ingestion (Schulte *et al.*, 2000; Marra and Isberg, 1996; Grutzkau *et al.*, 1990). *Y. pseudotuberculosis* encodes two outer membrane adhesins, invasin and YadA. Both molecules promote intimate host cell contact by providing high-affinity interactions with the host cell β_1 -integrin receptors. This initiates rapid internalization of the pathogen by a zipper-like mechanism and triggers the synthesis of pro-inflammatory cytokines, such as interleukin-8 (IL-8). In the case of invasin, binding to β_1 -integrins is direct, whereas interaction by YadA is indirect and occurs through an extracellular matrix (ECM) protein bridge of fibronectin (Heise and Dersch, 2006; Eitel and Dersch, 2002; Isberg *et al.*, 2000). How these differences in the binding mechanism affect the quality, strength and persistence of the bacterially induced intracellular signalling cascades in epithelial cells is largely unknown, but clustering of the receptors by both adhesins seems crucial for promoting an appropriate signal (Hudson *et al.*, 2005; Eitel and Dersch, 2002; Dersch and Isberg, 1999b). Several studies have addressed the intracellular signalling factors that are recruited and activated in response to *Yersinia* Inv and YadA. Evidence has been presented that the related non-receptor protein tyrosine kinases FAK and Pyk2, the Src kinase, and the adaptor molecules Cas and Crk form a signalling network that is crucial for the initiation of the uptake process

(Owen *et al.*, 2007; Bruce-Staskal *et al.*, 2002; Weidow *et al.*, 2000; Alrutz and Isberg, 1998). Activation and interplay between these signalling factors upon β_1 -integrin engagement appear to feed into a common Rac1 dependent signalling cascade. Rac1 is a member of the Rho GTPase family that regulates a variety of actin-dependent cellular events, including membrane ruffling, protrusion formation and phagocytosis (Iden and Collard, 2008; Etienne-Manneville and Hall, 2002); it is activated upon substrate binding to β_1 -integrins and implicated in the remodeling of actin filaments associated with many bacterial uptake processes (Ridley, 2006; Rottner *et al.*, 2005). Rac1 was found to be recruited and immediately activated by GTP loading upon polyvalent β_1 -integrin binding of the *Y. pseudotuberculosis* invasin protein at the site of bound bacteria (Wong *et al.*, 2008; Wong and Isberg, 2005; Alrutz *et al.*, 2001; McGee *et al.*, 2001). Moreover, inhibition of FAK or Cas function caused morphological defects in *Y. pseudotuberculosis* uptake that were manifested in incomplete membrane protrusive activity, consistent with an inhibition of Rac1 activity (Bruce-Staskal *et al.*, 2002). This indicated a crucial role for Rac1 signalling from nascent bacterial phagosomes to downstream effectors. A recent study further demonstrated that the Rac1 effector protein, the phosphoinositol-4-phosphate-5-kinase (PIP5K), forming phosphoinositol-4,5-bisphosphate (PIP₂) at the phagocytic cup, as well as the small GTPase Arf6, a regulatory factor of this process, are required for invasin-mediated cell uptake (Wong and Isberg, 2003). It has been speculated that increase of PIP₂ at the site of bacterial uptake could recruit and activate PIP₂-binding proteins involved in the regulation of actin dynamics or it could result in an increase of diacylglycerol production due to phospholipase C- γ activity, leading to products that might provide the crucial signal for bacterial uptake (Wong and Isberg, 2003).

Activation of the mammalian phosphoinositide (PI) 3 kinase p85 α -p110 is required for both invasin and YadA-promoted bacterial uptake. It was shown to occur via a FAK- and Src-depending process, involving the GTPase Ras (Eitel *et al.*, 2005; Eitel and Dersch, 2002). Understanding the mechanisms how activation of PI3 kinase induces uptake of *Y. pseudotuberculosis* requires the identification of signal factors that act downstream of this lipid kinase during the bacterial entry process. The PI3 kinase catalyses the addition of a phosphate to phosphoinositides, and results in the formation of membrane-bound phosphatidylinositol 3,4,5-triphosphate (PIP₃) from phosphatidylinositol 4,5-bisphosphate (PIP₂) at the inner leaflet of the cell membrane (Takenawa and Itoh, 2001). This was shown to mediate membrane recruitment and activation of other host cell protein kinases. One of which is the protein kinase B/Akt, which is associated with the membrane through interactions with PIP₃. Akt membrane recruitment is followed by sequential phosphorylation of residues Thr³⁰⁸ and Ser⁴⁷³ by 3-phosphoinositide-dependent kinases 1 and 2 (PDK1, PDK2), leading to full activation of Akt kinase activity (Manning and Cantley, 2007; Brazil *et al.*, 2004). Several studies have also established, that phospholipase C- γ (PLC- γ) isoenzymes can be directly activated by the PI3 kinase (Bae *et al.*, 1998; Falasca *et al.*, 1998; Gratacap *et al.*, 1998; Rameh *et al.*, 1998).

In this study, we investigated and compared the Inv- and YadA-induced signal transduction cascades in human epithelial cells, and we found that both adhesins require the presence of the PI3 kinase downstream effectors protein kinase B/Akt and PLC- γ 1 for efficient uptake. In addition, we showed that the conventional protein kinases C α (PKC α) and - β are activated upon Inv- or YadA-promoted engagement of β ₁-integrins. Depletion or inhibition of these isoforms resulted in a significant reduction of the triggered cell entry process, although Inv- and YadA-induced

production of IL-8 through the Ras-induced MEK1/2-ERK1/2 MAP-kinase pathway remained unaffected.

Results

Akt is required for invasin and YadA-mediated invasion of Y. pseudotuberculosis

The serine threonine kinase Akt becomes activated in response to many integrin-initiated signalling processes (Parsons, 2003). Activation of Akt involves phosphorylated lipid products such as PIP₃, generated by the PI3 kinase, which is required for the YadA- and invasin-mediated uptake of *Y. pseudotuberculosis* (Eitel and Dersch, 2002). This prompted us to examine whether both *Yersinia* adhesins are able to induce Akt activation. First, Akt phosphorylation upon adhesin-triggered uptake was assessed. Serum starved HEP-2 cells were either infected with YadA- and invasin-expressing *E. coli* K-12 or left uninfected. The recombinant bacteria allowed us to analyze YadA- or invasin-mediated bacterial entry into epithelial cells without interference of other *Yersinia* adhesion factors (e.g. Ail, PsaA). As a positive control mammalian cells were also treated with epithelial growth factor (EGF), which was previously shown to increase Ser⁴⁷³-specific Akt phosphorylation. After different times of infection, cell lysates were collected and analyzed by Western blotting with an antibody specific for Ser⁴⁷³-phosphorylated Akt. In parallel, cell extracts were probed with an antibody directed against Akt. Akt phosphorylation in HEP-2 cells after addition of Inv- and YadA-expressing bacteria was induced at an early time point and remained above basal levels for at least 60 min post infection (Fig. **1A**). The total amount of Akt did not change, demonstrating that the observed increase in Akt phosphorylation is not caused by an increase of protein synthesis.

Akt recruitment in *E. coli* strains that heterologously express *Yersinia* Inv or YadA was also confirmed via infection of HEP-2 cells expressing a green fluorescent

protein chimera linked to the first 167 amino acids of Akt. This portion of Akt includes the pleckstrin homology (PH) domain (Akt-PH-GFP), which binds PIP₂. As shown in Fig. **1B**, internalization of Inv⁺ bacteria led to the formation of prominent phagosomes surrounded by Akt-PH-GFP, whereas cell-bound bacteria were not associated with detectable fluorescence. Akt-PH-GFP was recruited by about 9% of Inv-expressing *E. coli* 60 min after infection. Colocalization of Akt-PH-GFP was also observed during phagocytosis with 5% of the YadA⁺ bacteria, but not with *E. coli* K-12 harboring the empty vector (data not shown). We conclude that *Yersinia* recruits and activates Akt at the contact site between bacteria and host cell by Invasin- and YadA-triggered β_1 -integrin signalling.

Three major isoforms of Akt are present in mammalian cells, namely Akt-1 (PKB α), Akt-2 (PKB β), and Akt-3 (PKB γ), which are encoded by three separate genes with >85% sequence identity (Brazil and Hemmings, 2001; Datta *et al.*, 1999). The Akt inhibitor VIII is a cell-permeable quinoxaline compound that potently and selectively inhibits the main isoforms Akt1 and Akt2. The inhibitor appears to affect the pleckstrin homology (PH) domain involved in PIP₃ binding and does not exhibit any inhibitory effect against PH domain-lacking Akts, or other closely related AGC family kinases, PKA, PKC, and serum and glucocorticoid-inducible kinase, even at concentrations as high as 50 μ M (Logie *et al.*, 2007; Barnett *et al.*, 2005). Akt inhibitor VIII treatment resulted in a significant dose-dependent inhibition of Inv- and YadA-mediated internalization at 5 μ M. At concentration $\geq$ 25 μ M less than 5% of the bacteria were internalized, whereas cell adhesion via YadA and invasin remained unaffected at all tested inhibitor concentrations (Fig. **1C**).

To further investigate the role of Akt in bacterial adhesion and internalization, Akt expression was inhibited in HEp-2 cells via RNA interference prior to infection with Inv- and YadA-expressing *E. coli*. Despite a high degree of sequence homology, the

main isoforms Akt-1 and Akt-2 seem to serve distinct cellular functions with regard to cell survival and metabolism. Therefore, HEP-2 cells were transfected with small interfering RNA (siRNA) duplexes directed towards isoforms Akt-1 or/and Akt-2. Selective inhibition of Akt-1 resulted in the most efficient down-regulation of total Akt, and this reduced cell entry of YadA⁺ and Inv⁺-bacteria down to 20-30% (Fig. 2). Also inhibition of Akt-2 reduced the overall amount of Akt within the cells, and this led to a decrease of uptake levels of the Inv- and YadA-expressing bacteria to 40-60%. Application of both AKT specific siRNAs reduced uptake by both *Yersinia* ligands to less than 20%. In contrast, cells transfected with the non-silencing control siRNA did not show any defect in cell binding and entry compared with the Mock control (Fig. 2). Taken together, these results strongly suggest a role for Akt in the β_1 -integrin mediated entry of *Y. pseudotuberculosis*.

PLC- γ is essential for invasin and YadA-mediated invasion of Y. pseudotuberculosis

It has been previously demonstrated that phospholipase C (PLC)- γ isozymes PLC- γ 1 and PLC- γ 2 can also be activated downstream of the PI3 kinase through the interaction of their SH2 and/or PH domains with PIP₃ (Falasca et al. 1998; Rameh et al. 1998; Scharenberg & Kinet 1998). PLC- γ 1 is widely expressed and can be activated by a variety of receptors, including integrins and other non-receptor tyrosine kinases through phosphorylation of the Tyr⁷⁸³ residue (Choi et al., 2007; Tvorogov et al., 2005). In the present study, the role of PLC- γ 1 was tested in the context of YadA and Inv-mediated invasion. To demonstrate PLC- γ 1 activation during the invasion process, HEP-2 cells were infected with Inv- or YadA-expressing bacteria and lysed after different time periods of infection. Total cell lysates were immunoprecipitated with an anti-PLC- γ 1 antibody. The immuno complexes were analyzed by Western blotting with a phospho-PLC- γ 1 antibody that specifically recognizes the phosphory-

lated form of Tyr⁷⁸³. To confirm that equal amounts of the protein were used, the cell extracts were also probed with anti-PLC γ 1 antibodies. No higher levels of phospho-PLC- γ 1 were observed in cells infected with *E. coli* K-12 without YadA or invasin (Fig. **3A**). In contrast, a significant increase of Tyr⁷⁸³ phosphorylation in PLC- γ 1 was observed after infection with YadA- and Inv-expressing *E. coli* K-12 and remained elevated above basal levels for at least 60 min. To test whether PLC- γ 1 activation occurs through PI3 kinase, we preincubated the HEp-2 cells with two different inhibitory concentrations of the PI3 kinase inhibitors LY294002 and wortmannin. As shown in Fig. **3A**, no activation of PLC- γ 1 can be obtained upon infection with YadA-expressing bacteria after LY294002 treatment, indicating that PI3 kinase is responsible for PLC- γ 1 activation. A similar effect of the PI3 kinase inhibitors was detected with Inv-expressing bacteria (data not shown).

Having demonstrated the activation of PLC- γ 1 upon invasin- and YadA-promoted uptake, we next examined the role of PLC- γ 1 in invasion using a cell-permeable PLC-specific inhibitor U73122. At 15 μ M, U73122 reduced Inv- and YadA-mediated invasion to about 20-25%, whereas adhesion of the bacteria by both adhesins remained the same (Fig. **3B,C**). The inhibitor was neither cytotoxic nor bacteriocidal at the concentrations used in the assay. This suggests that *Yersinia* invasion requires the activity of phospholipase C.

To further confirm that PLC- γ 1 activity is important for Inv- and YadA-mediated invasion, PLC- γ 1 production in HEp-2 cells was inhibited with two PLC- γ 1 specific siRNAs, differing in their target sequences within the PLC- γ 1 gene. Westernblot analysis demonstrated that HEp-2 cells transfected with PLC- γ 1 siRNAs produced significantly less PLC- γ 1 compared with cells treated with the transfection agents only (Mock), or cells transfected with non-silencing control siRNAs (Fig. **4A**). HEp-2 cells

transfected with the control siRNA did not show any decrease in bacterial entry compared with non-transfected control cells (Fig. **4C**), whereas uptake efficiency of Inv- or YadA-expressing bacteria was reduced about two-fold in the PLC- γ 1 knock-down cells. Residual amounts of PLC- γ 1 (Fig. **4A**) after siRNA treatment or complementation by other PLC isoforms may account for partial inhibition of cell uptake. We also tested Inv- and YadA-mediated adhesion to transfected HEp-2 cells and found comparable numbers of cell-bound bacteria (Fig. **4B**), demonstrating that inhibition of invasion by PLC- γ 1 siRNAs was not due to reduced cell binding.

PKC inhibition blocks YadA- and invasin-mediated invasion

The phospholipase PLC- γ 1 plays a critical role in several receptor-mediated signal transduction pathways through the generation of two second messengers: inositol 1,4,5-triphosphate (IP₃) and diacylglycerol (DAG) after hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP₂) (Rebecchi and Pentyala, 2000). Both molecules modulate the activity of downstream proteins of important cellular signalling cascades. IP₃ interacts with a receptor on the endoplasmic reticulum to release Ca²⁺ from internal stores into the cytoplasm. DAG remains bound to the inner leaflet of the plasma membrane, where it recruits a large family of Ca²⁺/phospholipid-dependent protein kinase (PKC) isoenzymes (Dempsey *et al.*, 2000; Mellor and Parker, 1998). To further characterize the signal transduction pathways implicated in *Yersinia* invasion, we analyzed the role of PKCs in the Inv- and YadA-promoted uptake process. First, cell uptake assays were performed following treatment of the cells with increasing concentrations of different PKC inhibitors. The selective PKC inhibitor NPC 15437, interacting at the regulatory domain of the enzyme, was effective in blocking the invasion of YadA⁺ and Inv⁺ bacteria in a dose-dependent manner with a >90% inhibition of the Inv- and YadA-mediated internalization pathway (Fig. **5A**).

Similarly, a significant inhibitory effect on both internalization pathways was also observed when HEp-2 cells were pretreated with increasing concentrations of the PKC inhibitors Calphostin C (0.1-1 μ M) (Fig. **5A**), Bis-BIM I (1-5 μ M) and Rottlerin (1-10 μ M) (data not shown). However, the total number of cell-bound bacteria did not differ between untreated and PKC inhibitor treated cells, indicating that the reduction of internalized bacteria is not due to inefficient binding of Inv- and YadA-expressing *E. coli* to HEp-2 cells (Fig. **5A**, data not shown).

Implication of PKC in the β_1 -integrin mediated bacterial internalization process was further underlined by the application of PKC activators. Quantification of bacterial internalization revealed that pretreatment with the PKC activator PMA (1-5 μ M; Fig. **5B**) and PDD (1-5 μ M) improved the capacity of invasin- and YadA-producing bacteria to invade epithelial cells considerably (2- and 4-fold). Also adhesion of the bacteria was slightly increased (Fig. **5B**). This may be explained through effects on the inside-out signalling of β_1 -integrins, which can improve integrin-ligand interactions (Luo *et al.*, 2007). Taken together these data indicated that activation of PKC and its translocation to the membrane play an important role for the invasin- and YadA-promoted cell entry process.

YadA and invasin induce activation of conventional PKCs during HEp-2 cell invasion.

The importance of PKC in the internalization process made us to examine the activation of different PKCs in HEp-2 cells exposed to Inv- and YadA-expressing *E. coli*. The PKC family consists of various isoforms that differ in primary structure, expression patterns, subcellular localization as well as substrate specificity. To identify which isoforms contribute to YadA- and Inv-driven uptake, the phosphorylation status of the (i) conventional PKCs (PKC α and PKC β II, reported to be the major isoforms implicated in actin rearrangements), (ii) the novel PKC δ , and (Maina

et al.) the atypical PKCs PKC ζ/λ were investigated during internalization into host cells. For this purpose, HEp-2 cells were infected with *E. coli* K-12, which surfacely expressed YadA or invasin. Westernblot analysis of the cell extracts was performed using antibodies directed against different phosphorylated forms of the PKC isoforms, e.g. PKC α and PKC β II phosphorylated at the Thr^{638/641}, conventional and novel PKC isoforms phosphorylated at Ser⁶⁶⁰, PKC δ phosphorylated at residues Thr⁵⁰⁵ or Ser^{643/676}, and PKC ζ/λ phosphorylated at Thr^{410/403}. To exclude that differences in phosphorylation are attributed to an unequal protein quantity, total amount of PKC was examined by using anti-PKC α antibodies. The results showed that YadA and invasin induced phosphorylation of the Thr^{638/641} and Ser⁶⁶⁰ residues found in the conventional PKC α and - β isoforms (Fig. **6A**). Phosphorylation was detectable within 5 min and sustained until 45 min post infection. In contrast, no or a very small increase of the phosphorylated forms of PKC ζ/λ and PKC δ was detected in HEp-2 cells upon treatment with YadA⁺ and Inv⁺ expressing *E. coli* (data not shown). Moreover, no activation of the conventional PKCs was found when *E. coli* K-12 harboring the empty vectors was incubated onto HEp-2 cells over 60 min (data not shown). These results indicate that predominantly phospholipid- and Ca²⁺-dependent PKCs are specifically activated by YadA and invasin, which correlates with the phosphorylation kinetics of the PKC interaction partner PLC- γ 1.

To confirm this result, we also studied recruitment of conventional PKCs to cell contact sites of Inv- and YadA-expressing bacteria by immunofluorescence, using HEp-2 cells transfected with a PKC α -eGFP fusion construct. High PKC α -eGFP expression levels in the cell cytoplasm hampered the analysis. However, enhanced fluorescence due to translocation of PKC α -eGFP to the bacteria cell contact sites was still visible in the proximity of 10% of the bacteria located in the less fluorescent periphery or near the nucleus of the cells (Fig. **6B**). Similarly, PKC α -eGFP was

recruited by 4% of the YadA-expressing bacteria, but no colocalization was found with *E. coli* K-12 carrying the empty vector (data not shown). We also observed colocalization of a PKC β II-eGFP fusion protein with about 15-20% of the Inv⁺ and YadA⁺ bacteria, although the fluorescence intensity was lower compared to PKC α -eGFP (data not shown). We conclude that both PKC isoforms are recruited and activated during Inv- and YadA-induced cell uptake.

Expression of PKC- α siRNA blocks Inv- and YadA-mediated invasion of HEp-2 cells.

In order to confirm the role of conventional PKCs in Inv- and YadA-mediated cell uptake, HEp-2 cells were infected with siRNA directed against PKC α or the PKC β isoforms I and II. Westernblot analysis of cell lysates of transfected HEp-2 cells with PKC α , PKC β I and - β II specific antibodies showed significantly lower levels of the protein kinases when compared with Mock cells or cells transfected with the non-silencing control RNA (Fig. **7A**). The blot was also probed with actin to verify that equal amounts of the protein were loaded. After confirming down-regulation of PKC α and PKC β protein synthesis, transfected HEp-2 cells were used in invasion assays along with Mock and non-silencing siRNA transfected cells as controls. As shown in Fig. **7B** invasion of Inv⁺ and YadA⁺ *E. coli* strains was blocked by 70% to 90% in the PKC α or PKC β knock-down cells, although decrease of both PKC isoforms did not affect the adhesion capability of the bacteria. These data clearly support an important role of PKC α and PKC β in Inv- and YadA-promoted cell entry.

Akt, PLC- γ and PKC- α/β are not required for IL-8 production

It has been demonstrated that binding of YadA and invasins to β_1 -integrin receptors induces the production of the pro-inflammatory cytokine IL-8 (Eitel & Dersch; 2005).

IL-8 induction by both adhesins involves autophosphorylation of FAK, which in turn activates the c-Src kinase and the small GTPase Ras. We further showed that IL-8 synthesis is the result of the Ras-stimulated Raf-MEK1/2-ERK1/2 MAPK signal transduction pathway, which appears to be separate from the PI3K-mediated cellular signalling cascade responsible for bacterial invasion (Eitel & Dersch; 2005). In the present study, we tested whether activation of Akt, PLC- γ 1 as well as the PKC α and - β isoforms plays a role for the generation of IL-8 in the course of the Inv- and the YadA-promoted cell uptake process. HEp-2 cells transfected with siRNA directed against the different signalling molecules were infected with YadA and Inv-producing *E. coli*, and the amount of IL-8 produced and released by HEp-2 cells into the culture supernatant was determined by enzyme-linked immunoabsorbent assay (ELISA). HEp-2 cells transfected with non-silencing siRNA was used as control. Addition of both YadA- and Inv-expressing bacteria resulted in a strong increase of IL-8 compared to the bacteria that harbour the empty vector (Fig. 8), but knock-downs of Akt1, Akt2, PLC- γ 1, PKC α and PKC β synthesis had no negative effect on secreted IL-8 levels. This is in full agreement with our previous study, demonstrating that also PI3 kinase, which is located upstream of Akt, PLC- γ 1 and the PKCs, is also required for Inv- and YadA-mediated cell invasion, but not for Inv- and YadA-promoted IL-8 induction (Eitel *et al.*, 2005).

Akt, PLC- γ 1 and PKC- α/β are required for Listeria monocytogenes, but not for Salmonella typhimurium uptake into HEp-2 cells

Although YadA- and Inv-induced IL-8 secretion was not prevented by inhibitors or siRNAs directed against Akt, PLC- γ 1, PKC α and - β , we wanted to investigate whether these treatments exerted a general effect on bacterial uptake, e.g. through inhibition of actin cytoskeletal rearrangements. Therefore, we tested whether the

inhibition of these signal molecules also exerted an influence on cell adhesion and invasion of *Listeria monocytogenes* and *Salmonella enterica* serovar Typhimurium. *L. monocytogenes* uptake into HEp-2 cells, which occurs through a zipper mechanism, is mainly dependent on the adhesin InlB, and implicates PI3 kinase, similar to YadA and Inv of *Yersinia* (Bierne *et al.* 2000). We found that this internalization mechanism is also affected by the inhibition of Akt, PLC- γ 1, PKC α and - β (Fig. **S1-4,A,B**). However, uptake of *S. typhimurium*, which is internalized by an effector-driven trigger mechanism, was not reduced after inhibition of Akt, PLC- γ 1, PKC α or - β (Fig. **S1-4,C,D**). In contrast, addition of the PKC inhibitor NPC appeared to stimulate *Salmonella* uptake into HEp-2 cells to a certain extent. This demonstrated that inhibition of these signalling molecules did not generally prevent bacterial phagocytosis into host cells.

Discussion

Many pathogens exploit host cell signalling pathways in order to manipulate the actin cytoskeleton for host cell entry, intracellular motility, intracellular survival and replication. In this study we analyzed signalling pathways activated by the *Y. pseudotuberculosis* outer membrane proteins YadA and invasin, which are required for host cell invasion. YadA as well as invasin have been previously shown to activate PI3 kinase upon direct or indirect binding to β_1 -integrins through the protein kinases FAK and Src (Eitel *et al.*, 2005; Alrutz and Isberg, 1998). This leads to the modification of short-lived host phosphoinositides whose production at specific membrane locations controls recruitment and activation of diverse cellular effectors involved in phagocytosis. Here we show that the bacterial adhesins YadA and invasin both utilize Akt kinase, PLC- γ 1 and PKC isoforms PKC α and PKC β as signalling mediators following PI3 kinase activation to promote internalization into host cells (Fig. **9**).

Our evidence that Akt activation is required for *Yersinia* uptake by invasin and YadA, was supported by data demonstrating that Akt inhibition and reduction of the most abundant isoforms Akt-1 and Akt-2 blocked bacterial uptake. The serine threonine kinase Akt is a central regulator of various cellular processes, including regulation of cell-cycle progression, vesicular trafficking, differentiation, apoptosis, gene transcription, and physiological processes such as cell migration and cytoskeletal reorganization (Brazil *et al.*, 2004; Vanhaesebroeck and Alessi, 2000). Activation of Akt generally occurs through PI3 kinase generated 3-phosphoinositides, such as PIP₃, which are virtually absent in quiescent cells. Once activated, Akt propagates stimulation of multiple signal factors in the cytosol and the nucleus. For instance, Akt has been shown to stimulate Rab5-dependent endocytosis and to interact with the host cell actin cytoskeleton in response to the cellular stimulation with platelet derived growth factor (Cenni *et al.*, 2003; Barbieri *et al.*, 1998). Likewise, activated Akt kinase could be recruited to actin filaments upon infection with YadA- or invasin-expressing bacteria, where it participates in the reorganization of the actin cytoskeleton during the invasion process.

Akt activation has also been observed during host cell infections with other bacterial pathogens. For instance, addition of *S. typhimurium* onto human colonic epithelial cells and HeLa cells leads to Akt phosphorylation by SigD (also known as SopB), a bacterial effector protein that is translocated into host cells via a type III secretion system (Huang *et al.*, 2005; Steele-Mortimer *et al.*, 2000). Phosphorylation of Akt was also shown to occur during the uptake process of other pathogens (Pendaries *et al.*, 2006; Sidhu *et al.*, 2005; Coombes and Mahony, 2002; Mansell *et al.*, 2001), but a requirement of Akt for host cell invasion was only demonstrated of *Pseudomonas aeruginosa* (Kierbel *et al.*, 2005) and Streptococci (Bergmann *et al.*, 2009; Burnham *et al.*, 2007).

It is also well documented that an activation of the Akt kinase, e.g. after translocation of the *S. typhimurium* SigD/SopB effector protein, protects mammalian cells from apoptotic events by inhibition of pro-apoptotic mediators such as Bad and procaspase-9 (Knodler *et al.*, 2005; Datta *et al.*, 1999; Cardone *et al.*, 1998). A similar function might be fulfilled by *Yersinia*-induced Akt activation to prolong the intracellular presence of the bacteria for further dissemination to other cells. The bacterial adhesin InlB of *Listeria monocytogenes* was also found to activate the nuclear factor- κ B via Akt, which culminates in the synthesis of pro-inflammatory cytokines, including IL-8 (Mansell *et al.*, 2001). However, depletion of Akt did not affect YadA- and Inv-induced IL-8 expression, which is initiated by the Ras-MEK1/2-ERK1/2 pathway, suggesting that YadA- and Inv-mediated IL-8 induction is different from that of InlB.

This work further exposed that PLC γ 1, which represents another downstream target of PI3 kinase and becomes activated through phosphorylation at position Tyr⁷⁸³, is also required for the YadA- and Inv-mediated uptake process. The PLC family consists of six different groups namely PLC β , - δ , - γ , - ζ , - η , and - ϵ distinguishable by their tissue contribution, substrate specificity, and regulation pathways (Katan, 2005). PLC γ interacts with PIP₃ generated by the PI3 kinase through its SH2 and its PH domains. This high affinity interaction anchors the PLC γ in the plasma membrane and places the enzyme in close proximity to its substrate PIP₂ (Falasca *et al.*, 1998; Singer *et al.*, 1997). PIP₂ is consequently hydrolyzed to DAG and IP₃, which is crucial for the release of Ca²⁺ from intracellular stores by interaction with receptors on the endoplasmatic reticulum (Rhee, 2001). Both PIP₂ and Ca²⁺ have been described as important regulators of actin-binding proteins (Hilpela *et al.*, 2004; Lee and Rhee, 1995) and phosphorylation of PLC γ 1 on Tyr⁷⁸³ by PDGF was shown to induce reorganization of the cytoskeleton (Yu *et al.*, 1998), consistent with

a role in facilitating bacterial invasion. Furthermore, it has been reported that PLC γ 1 plays a key role in integrin-dependent cell adhesion and motility by the activation of Pyk2/paxillin/Rac1 signaling (Choi *et al.*, 2007; Jones and Katan, 2007).

Besides its role in the YadA- and Inv-promoted uptake process of *Yersinia*, presence of PLC γ 1 is also needed for internalization of *E. coli* K1 into human endothelial cells (Sukumaran *et al.*, 2003) and it is activated by *E. coli* EPEC, using intimin for interaction with epithelial cells (Kenny and Finlay, 1997). Bierne and Cossart (2002) also provided evidence for PLC γ 1 activation and transient IP $_3$ formation during InlB-triggered host cell infection by *L. monocytogenes*, although blocking of PLC γ 1 function had no effect on invasion. Therefore, it has been postulated that PLC γ 1 activation by *Listeria* is necessary for post-ingestion events and occurs via a mechanism, which requires association with other tyrosine phosphorylated proteins (Bierne and Cossart, 2002).

The third class of signalling molecules, which were shown to participate in the YadA- and Inv-promoted uptake process of *Yersinia*, includes members of the protein kinase C family. A role of PKCs in *Yersinia* uptake was supported by the observation that the conventional PKC isoforms PKC α and PKC β were activated in the course of host cell infections with YadA- or Inv-producing *E. coli*, whereas a depletion of both isoforms caused a drastic decrease of the uptake process.

The conventional PKCs α and β act in a Ca $^{2+}$ - and phospholipid-dependent manner, and are regulated by two sequential mechanisms: i) phosphorylation induced by PDK1, which is PI3 kinase dependent, and ii) allosteric control mediated by PLC-mediated DAG (Dempsey *et al.*, 2000). Both isotypes participate in the regulation of cell growth, migration, and proliferation. They play a crucial role in cell-cell contacts and are important components of signalling cascades leading to cytoskeletal rearrangements, e.g. stabilization of F-actin (Dempsey *et al.*, 2000;

Mellor and Parker, 1998; Farhadi *et al.*, 2006). This may explain why higher level of both isoforms might be required to promote efficient bacterial uptake. Alternatively, PKC α and - β have individual functions, which might be crucial for integrin-promoted bacterial uptake. For instance, PKC α is implicated in β_1 -integrin-mediated cell migration, and the actin binding components ezrin and fascin were shown to be downstream effectors of PKC α (Anilkumar *et al.*, 2003; Ng *et al.*, 2001), whereas PKC β enhances the architectural integrity of the microtubule cytoskeleton, and normalizes intracellular calcium levels (Farhadi *et al.*, 2006). Strikingly, PKC β was also shown to stabilize I κ B α and prevent NF κ B activation, known to induce IL-8 synthesis (Banan *et al.* 2004). This may explain, why a PKC β knock-down led to a significant stimulation of IL-8 production, even in the absence of YadA- or Inv-expression bacteria (Fig. 8).

The role of PKC-specific phosphorylation in the promotion of bacterial entry has not been widely characterized. Sukumaran & Prasadarao (2002) were able to specify the conventional PKC α as an important molecule involved in the internalization of pathogenic *E. coli* K1 via caveolae into endothelial cells. As also FAK, PI3 kinase and PLC- γ 1 have been shown to be important for *E. coli* K1 uptake into endothelial cells, it is tempting to speculate that a signalling cascade, similar to that induced by Inv- or YadA-bound β_1 -integrins, is activated by *E. coli* K1 upon binding to the human brain microvascular endothelial cell glycoprotein receptor Ecgp (Prasadarao *et al.*, 2003; Sukumaran and Prasadarao, 2002). PKC inhibition studies further support, that certain PKCs may also be important for host cell invasion of *Neisseria gonorrhoea* and phagocytic uptake of *Legionella pneumophila* into monocytes (Freissler *et al.*, 2000; Coxon *et al.*, 1998; Dehio *et al.*, 1998).

Comparative analysis between Inv- and YadA-promoted uptake in this study to-

gether with previous data illustrate that triggered signalling pathways by direct or indirect binding of the *Yersinia* adhesins to β_1 -integrins seem to induce similar, if not identical signalling pathways in epithelial cells (Fig. 9). In both cases, activation of the PI3 kinase through FAK, Src and Ras is crucial to initiate signal transduction cascades, implicating a role for protein kinase B/Akt, PLC- γ 1 and conventional protein kinases PKC α and - β . The activation pattern of the signal molecules in response to YadA or invasin are comparable, indicating that the strength of the triggered host cell processes within human epithelial cells is alike when surface exposed adhesin are produced in concentrations where they mediate equal uptake rates. In this context it should be mentioned that higher levels of YadA are required to promote cell invasion with an efficiency comparable to invasin (Eitel and Dersch, 2002). This might be explained by the fact that YadA interaction with β_1 -integrins is indirect and occurs via an extracellular matrix bridging molecule, i.e. fibronectin, whereas invasin binds directly to β_1 -integrins and has a much higher affinity to the receptors than fibronectin (Heise and Dersch, 2006; Eitel and Dersch, 2002; Tran van Nhieu & Isberg, 1993). In addition to the intracellular factors described in this study, several other signalling molecules have been found to participate in the β_1 -integrin-triggered uptake process by invasin (Fig. 9). They include several signalling components of the focal adhesion complex, and the small GTPase Rac1, shown to induce actin filament nucleation by activating the Arp2/3 complex via WAVE family proteins (Wang *et al.*, 2006; Bruce-Staskal *et al.*, 2002; Alrutz *et al.*, 2001). Rac1 associates and induces phosphoinositol-4-phosphate-5 kinase (PIP5 kinase), involved in PIP₂ formation at the site of bacterial uptake, and PIP5 kinase and its activating small GTPase Arf6, both permit bypass of the Rac1 requirement for invasin-mediated uptake (Wong and Isberg, 2003; Tolia *et al.*, 2000). Thus, it is possible that Rac1-dependent PIP₂ formation also contributes to PLC- γ 1 and PKC α/β

activation and other downstream signals required for uptake. However, there is also evidence that PI3 kinase and PLC- γ 1, both shown to act downstream of PIP₂, are required for Rac1 activation (Choi *et al.*, 2007; Alrutz *et al.*, 2001). This leads to the suggestion that a feedback loop between the different signaling molecules might exist. To solve this issue, we are currently addressing the question of how Rac1 can be selectively activated through invasin-engaged integrins and how it is connected with the PI3 kinase/PLC- γ 1/PKC α/β pathway.

Experimental procedures

Plasmids, bacterial strains, media and growth conditions

The plasmids pBAD18, pPD284 (pBAD18-*yadA*⁺), and pRI203 (pBR322-*inv*⁺) have been described previously (Eitel *et al.*, 2005; Eitel and Dersch, 2002). Plasmid pFU50, encoding the dsRed2 gene, was constructed by insertion of two PCR generated fragments into *Xho*I/*Sac*I sites of pZS24*MCS (Lutz and Bujard, 1997). One fragment with *Xho*I/*Not*I sites were amplified from pDsRed2 (Clontech) with primers 5'-GCA CTG CGG CCG CCT ACA GGA ACA GGT GGT GG-3' and 5'-GCA CGC TCG AGT ACG CAA ACC GCC TCT CC-3'. The second fragment, encoding the low copy ori 29807 with adjacent *Sac*I *Not*I sites was generated by PCR, using primers ACT GTC AGA CCA AGT TTA CG and 5'-GCA CGG CGG CCG CTA TCT AGA GGC ATC AAA TAA AAC G -3' from vector pFU33. Plasmid pFU33 was constructed by insertion of a PCR fragment amplified from pIVmob2 (Strauch *et al.*, 2000), using primers 5'-GCA CGC CTA GGC GGA GAT ATT CCG CTT CCT CG-3' and 5'-GCA CGA CTA GTA AGA TGA ACC TCT TGA GAT CC-3', into the *Spe*I and *Avr*II site. The plasmids were introduced into the *E. coli* K-12 by electroporation. Overnight cultures of *E. coli* were routinely grown at 37°C in LB (Luria-Bertani) broth

and *L. monocytogenes* EGD was cultivated at 37°C in BHI (Brain Heart Infusion) medium to an OD₆₀₀ of 0.9. *S. enterica* serovar Typhimurium strain SL1344 was grown in LB at 37°C to late exponential phase (OD₆₀₀ = 2.0). The antibiotics used for bacterial selection were as follows: ampicillin or carbenicillin 100 µg/ml, and kanamycin 50 µg/ml. The *inv* gene on pRI203 is under the control of the *inv* promoter (Isberg *et al.*, 1987). To induce *yadA* encoded on pPD284 0.2% arabinose was added to the overnight cultures and production of invasin and YadA was proven by Westernblot analysis using a polyclonal antibody directed against invasin and YadA, respectively. The plasmid, encoding the GFP-PH-Akt-construct, was kindly provided by Tamas Balla (NIH, Bethesda). PKC α -eGFP and PKC β 2-HA-eGFP encoding plasmids were purchased from Aequotech (Italy).

Eukaryotic cell lines and culture conditions.

Human HEp-2 cells were cultured in RPMI 1640 media (Biochrom KG, Berlin, Germany) supplemented with 7,5% newborn calf serum (Invitrogen) with 2 mM L-glutamine (Invitrogen, Sigma) at 37°C in the presence of 5% CO₂.

Preparation of cell extracts, gel electrophoresis, and Western blotting

Approximately 8 x 10⁶ HEp-2 cells were seeded into 6-well plates, serum-starved for 20 h and infected with 5 x 10⁷ bacteria. Bacteria were not centrifuged into the cell monolayer to avoid mechanical forces. At various times after infection, the cells were washed with PBS and resuspended in 200 µl lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2,5 mM Na₄P₂O₇, 1 mM β -glycerol phosphate, 1 mM Na₂VO₄, 1 µg/ml leupeptine, 1 mM PMSF) for immune precipitation or 200 µl SDS sample buffer for gel electrophoresis. Immune precipitation was performed for the detection of PLC- γ 1 phosphorylated at position

Tyr⁷⁸³. To do so, antibodies directed against PLC- γ 1 (1:1000, Cell signaling) were added to the lysate and incubated overnight at 4°C. Subsequently, protein A agarose (Roche) was added in (1:10 v/v) and incubated on ice for 2 h. Samples were centrifuged and pellets were washed three times with lysis buffer and finally resuspended in 30 μ l SDS sample buffer. Cell extracts or immune precipitates were boiled for 5 min and 5-20 μ l of the samples were separated on 10-12% SDS-polyacrylamide gels. Subsequently, the proteins were transferred to immobilon membranes (Millipore) and incubated with TBST (Tris pH 7.5, 500 mM NaCl, 0.1% Tween) and 3% BSA for at least 1 h or overnight at 4°C to block non-specific protein binding. Primary antibodies against PKC α (Biomol), PKC β I and PKC β II (Santa Cruz), P^{Thr638/641}-PKC α β II, P ^{β II Ser606}-PKCpan, P^{Thr505}-PKC δ , P^{Ser744/748}-PKC μ , P^{Ser916}-PKC μ , P^{Ser473}-Akt, and Akt (Cell signaling) were diluted 1:1000 in TBST with 3% BSA, and applied to the membrane for at least 1h at room temperature or overnight at 4°C. Following washing, the blots were incubated with HRP-conjugated anti-rabbit IgG (diluted 1:5000) in TBST and 3% BSA. Immunoreactive bands were visualized by enhanced chemiluminescence using the Western Lightning Chemiluminescence Reagent (Perkin Elmer).

Cell infection for IL-8 determination

Before infection, HEp-2 cells were seeded into 24-well plates and serum-starved for 20 h. For cell infection, 500 μ l of an overnight culture of the bacteria were collected by centrifugation and resuspended in 1 ml phosphate-buffered saline (PBS) pH 7.5. The optical density of the culture was determined and appropriate dilutions of the bacteria were taken for cell infection in a bacterium-to-cell ratio of 100:1. HEp-2 cells were washed three times with PBS and incubated in RPMI supplemented with 20 mM HEPES (pH7.0) and 0.4% bovine serum albumin (Rubsamen-Waigmann *et al.*)

before the addition of bacteria. After infection, the cells were incubated at 37°C for 4 h. Subsequently the cultures were washed with PBS and further incubated for at least 4 h or overnight in RPMI in the presence of gentamicin (50 µg/ml) to kill extracellular bacteria. The culture supernatant was removed and centrifuged for 4 min to pellet residual bacteria and cell debris and was then taken for IL-8 detection.

Cell adhesion and invasion assay

In preparation of the cell adhesion and uptake assay, 5×10^4 HEp-2 cells were seeded and grown overnight in individual wells of 24-well culture plates (Biochrom, Berlin, Germany). Cell monolayers were washed three times with PBS and incubated in RPMI 1640 medium supplemented with 20 mM HEPES (pH7.0) and 0.4% BSA before the addition of bacteria. Overnight cultures of the bacteria were washed once in PBS and used for cell infection in a bacterium-to-cell ratio of 100:1. The number of living bacteria in the culture was also determined by plating of 1:10 serial dilutions on LB medium. Infected cell monolayers were incubated at room temperature to test for cell binding or at 37°C to test for invasion. One hour post infection, the cells were washed extensively with PBS. The total number of adherent bacteria was determined by cell lysis using 0.1% Triton in PBS and plating on bacterial media. Bacterial uptake was assessed 60 min after infection as the percentage of bacteria which survived killing by the addition of the antibiotic gentamicin to the external medium, as described previously (Dersch and Isberg, 1999a). For each strain, the level of bacterial adhesion and uptake was determined by calculating the number of colony-forming units relative to the total number of bacteria introduced onto monolayers. Three independent experiments were routinely performed in triplicate. YadA-mediated cell adhesion (2-8%) and invasion (0.5-3%) of the recombinant *E. coli* strain CC118 pPD284 in the presence of 0.2% arabinase were similar to Inv-

promoted cell adhesion (4-5%) and invasion (1-2%) of CC118 pRI203 as described previously (Eitel & Dersch, 2002).

Inhibitors and activators of Akt, PLC- γ , PI3 kinase, and PKC kinases

For inhibitor studies, 0-40 μ l Akt inhibitor VIII (Calbiochem), 0-10 μ M PLC- γ inhibitor U73122 (Sigma Aldrich), 0-10 μ M PKC inhibitor Rottlerin (Sigma Aldrich), 0-1 μ M Calphostin C (Sigma Aldrich), 0-5 μ M Bisindolylmaleimide I hydrochloride (Sigma Aldrich), 0-5 μ M NPC-15437 dihydrochloride (Sigma Aldrich), and 0-5 μ M Gö 6983 (Sigma Aldrich), 0-5 μ M of the PKC activators the phorbol ester PMA or PDD, as well as 0-75 nM of the PI3 kinase inhibitor wortmannin (Sigma Aldrich) or 0-50 μ M LY294002 were diluted in RPMI 1640 medium supplemented with 20 mM HEPES (pH7.0) and 0.4% BSA, added to human cells and incubated for 30-60 min at 37°C before infection with bacteria.

Immunofluorescence and fluorescence microscopy

About 10^4 HEp-2 cells seeded in μ -slide 8 well (Ibidi, Germany) were transfected with plasmid DNA (final concentration 0,4 μ g/ml), encoding Akt-PH-GFP or PKC α -eGFP or PKC β II-eGFP fusions using Lipofectamine LTX reagent in Opti-MEM (10 μ g/ml, Invitrogen) and grown overnight at 37°C and 5% CO₂. Transfected cells were infected with 10^5 bacteria as described above. Recruitment of GFP-fusion constructs to intracellular bacteria was visualized by fluorescence microscopy (Zeiss, Axiovert II, Germany). Acquisition and processing of the images was performed with an AxioCam HRm and the Axiovision Software (Zeiss, Germany).

IL-8 ELISA

The amount of IL-8 produced and released by HEP-2 cells into the culture supernatant was determined by enzyme-linked immunoabsorbent assay (ELISA) with an IL-8 Assay Kit (RayBiotech, US; Trinova, Germany) as described in the manufacturer's instruction. 100 µl of the 1:5 diluted supernatant of the infected cells and a series of two-fold dilutions of the standard were added into the wells of a 96-well plate coated with human IL-8 specific antibodies. Plates were incubated at room temperature for 2,5 h. After washing with PBS, 100 µl of a 1:80 dilution of the biotinylated anti-human IL-8 detection antibody were added and incubated at room temperature for 1 h. Subsequently, plates were washed and 100 µl of a 1:30000 dilution of the horse-radish peroxidase (HRP) conjugated streptavidin was added to each well. Plates were incubated at room temperature for 45 min and washed. The detection reaction was started by addition of tetramethylbenzidine (TMB) and stopped with 2 M H₂SO₄. Optical density at 450 nm (OD₄₅₀) was measured with a Microplate Reader 680 (BioRad). Recombinant IL-8 was used as a standard.

Knock-down of Akt, PLC- γ and PKC kinases by RNA interference

About 2×10^6 HEP-2 cells seeded in 24 well plates were transfected with 1 µg siRNA directed against the different signalling molecules (for details see Tab. **S1**) diluted in 100 µl EC-R buffer (Qiagen, Germany). 6 µl RNAiFect Transfection Reagent (Qiagen, Germany) was added to diluted siRNA samples, mixed and incubated for 10-15 min at room temperature to allow formation of the transfection complexes. Before siRNA was added, HEP-2 cells were washed and 600 µl of RPMI containing 7,5 % NCS was added into each well. The appropriate transfection complex was added drop wise uniformly distributed by gently swirling of the plates. Subsequently, cells were incubated for 48 h at 37°C and 5% CO₂ and infected with bacteria as described above. Knock-down of the equivalent signal molecules was analyzed by

probing extracts of the transfected cells with antibodies directed against the signal factor and actin for loading control.

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Figure Legends

Fig. 1. Role of Akt for Inv- and YadA promoted uptake. **(A)** Activation of protein kinase B/Akt during Inv- or YadA-mediated cell invasion. HEp-2 cells were infected with Inv- or YadA-expressing *E. coli* K-12 (CC118/pRI203; CC118/pPD284). For controls, *E. coli* K-12 or EGF (100 ng/ml) was added. Subsequently, cells were lysed at various times (0 min to 60 min) after infection. Equal amounts of the cell extracts were loaded on 12% SDS-polyacrylamide gels and probed with anti-P^{Ser473}-Akt or anti-Akt antibody to confirm the presence of equal levels of Akt in the cell extracts. All Western blots are representative of three independent experiments. **(B)** HEp-2 cells transiently expressing an AKT-PH-GFP fusion construct were infected with invasin-expressing *E. coli* pFU507/pRI203 with a multiplicity of infection (MOI) of 10. Recruitment of Akt-PH-GFP to invading bacteria was visualized by fluorescence microscopy (Zeiss, Axovert II). Overlay represents merged images of green and red fluorescence channels resulting in yellow-orange colour. Bars, 1 μ m. **(C)** Inv- and YadA-expressing *E. coli* K-12 (CC118/pRI203; CC118/pPD284) were applied to HEp-2 cells preincubated with different concentrations of the Akt inhibitor VIII (5-40 μ M). Adherent and internalized bacteria were enumerated by the gentamicin-protection assay, as described in *Experimental Procedures*. The graphs represent mean values $\pm$ standard deviations of three independent experiments done in duplicate. Data were analysed with a student t test. *, significantly different from non-treated cells (-), p value < 0.05.

Fig. 2. Depletion of Akt reduces Inv- and YadA-mediated uptake to a similar extent. **(A)** Western blotting of total Akt in HEp-2 cells after treatment with siRNA directed against isoforms Akt1 and/or Akt2. HEp-2 cells treated with the transfection agent

(Mock), plus control siRNA, or the Akt1 and/or Akt2 specific siRNA were infected with Inv- or YadA-expressing *E. coli* K-12 (CC118/pRI203; CC118/pPD284). One hour after infection, adherent (**B**) and internalized (**C**) bacteria were determined by the gentamicin-protection assay, as described in Experimental procedures. The graphs represent mean values $\pm$ standard deviations of three independent experiments done in duplicate. Data were analysed with a student t test. *, significantly different from non-treated cells (-), p value < 0.05.

Fig. 3. Role of PLC- γ 1 for Inv- and YadA-mediated invasion. (**A**) Activation of PLC- γ 1 during Inv- or YadA-mediated cell invasion. HEp-2 cells were infected with Inv- or YadA-expressing *E. coli* K-12 (CC118/pRI203; CC118/pPD284). *E. coli* K-12 was added as a negative control, and two different concentrations of the PI3 kinase inhibitors LY294002 (LY) and Wortmannin (Wo) were added to the cells 30 min prior to the infection. Various times (0 min to 90 min) after addition of the bacteria cells were lysed, and PLC- γ 1 was isolated from the cell extracts by immune precipitation as described in *Experimental procedures*. Equal amounts of the extracted samples were loaded on 12% SDS-polyacrylamide gels and probed with anti-P^{Tyr783}-PLC- γ 1 or anti-PLC- γ 1 antibody to confirm the presence of equal levels of the enzyme in the cell extracts. (**B-C**) Inv- and YadA-expressing *E. coli* K-12 (CC118/pRI203, CC118/pPD284) were applied to HEp-2 cells preincubated with different concentrations of the PLC- γ 1 inhibitor U73122 (0.01-1 μ M). Adherent (**B**) and internalized (**C**) bacteria were enumerated by the gentamicin-protection assay, as described in Experimental procedures. The graphs represent mean values $\pm$ standard deviations of three independent experiments done in duplicate. Data were analysed with a student t test. *, significantly different from non-treated cells (-), p value < 0.05.

Fig. 4. Depletion of PLC- γ 1 reduces Inv- and YadA-mediated host cell internalization. **(A)** Western blotting of total PLC- γ 1 in HEp-2 cells after treatment with two different siRNA (siPLC- γ 1-1 and siPLC- γ 1-2) directed against PLC- γ 1. HEp-2 cells treated with the transfection agent (Mock), plus control siRNA, or the PLC- γ 1 specific siRNA were infected with Inv- or YadA-expressing *E. coli* K-12 (CC118/pRI203; CC118/pPD284). One hour after infection, adherent **(B)** and internalized **(C)** bacteria were determined by the gentamicin-protection assay, as described in Experimental procedures. The graphs represent mean values $\pm$ standard deviations of three independent experiments done in duplicate. Data were analysed with a student t test. *, significantly different from non-treated cells (-), p value < 0.05.

Fig. 5. Modulation of the PKC kinase activity by pharmaceutical agents affects Inv- and YadA-mediated cell invasion. Inv- and YadA-producing *E. coli* K-12 (CC118/pRI203; CC118/pPD284) were applied to HEp-2 cells preincubated with two different concentrations of the PKC inhibitors **(A)** NPC (1 and 5 μ M) and Calphostin (0,1 and 1 μ M), and the PKC activators **(B)** PDD (0,5 and 1 μ M) and PMA (1 and 5 μ M). Adherent and internalized bacteria were enumerated by the gentamicin-protection assay, as described in Experimental procedures. The graphs represent mean values $\pm$ standard deviations of three independent experiments done in duplicate. Data were analysed with a student t test. *, significantly different from non-treated cells (-), p value < 0.05.

Fig. 6. Activation and recruitment of PKC isoforms during Inv- or YadA-mediated cell invasion. **(A)** HEp-2 cells were infected with Inv- or YadA-expressing *E. coli* K-12 (CC118/pRI203; CC118/pPD284). Various times (0 min to 60 min) after addition of

the bacteria cells were lysed, and equal amounts of the extracted samples were loaded on 12% SDS-polyacrylamide gels and probed with anti-P-PKC α / β II, anti-P-PKCpan or anti-PKC α antibody to confirm the presence of equal levels of the enzyme in the cell extracts. **(B)** HEp-2 cells transiently expressing a PKC α -eGFP fusion construct were infected with *E. coli* K-12 pFU50/pRI203 (*inv*⁺) or *E. coli* K-12 pFU50/pPD284 (*yadA*⁺) with a MOI of 10. Recruitment of PKC α -eGFP to the cell contact site of the bacteria was visualized by fluorescence microscopy (Zeiss, Axovert II). Overlay represents merged images of green and red fluorescence channels resulting in yellow-orange colour. White boxes indicated areas that were enlarged in the picture shown below. Bars, 1 μ m.

Fig. 7. Depletion of PKC α or PKC β reduces the Inv- and YadA-mediated host cell internalization. **(A)** Western blotting of total PKC α or PKC- β I and - β II in HEp-2 cells after treatment with specific siRNAs directed against PKC α or PKC β I and - β II isoforms. **(B)** HEp-2 cells treated with the transfection agent (Mock), plus control siRNA, or the PKC specific siRNAs were infected with Inv- or YadA-expressing *E. coli* K-12 (CC118/pRI203; CC118/pPD284). One hour after infection, adherent and internalized bacteria were determined by the gentamicin-protection assay, as described in *Experimental procedures*. The graphs represent mean values $\pm$ standard deviations of three independent experiments done in duplicate. Data were analysed with a student t test. *, significantly different from non-treated cells (-), p value < 0.05.

Fig. 8. Akt, PLC- γ 1 and PKC- α and - β knock-downs do not affect YadA and Inv-driven IL-8 secretion. HEp-2 cells were transfected with the indicated siRNAs as described in Experimental Procedures. After 48 h, transfected cells were infected with YadA- or Inv-expressing *E. coli* K-12 (CC118/pRI203; CC118/pPD284).

Supernatants of infected cells were removed 4 h after addition of the bacteria to monitor IL-8 production. siRNA transfected HEp-2 cells, which remained uninfected or were infected with *E. coli* K-12, were used as negative controls. IL-8 production was quantified by an IL-8-specific ELISA kit. Data are presented as means $\pm$ standard deviations of three independent experiments done in duplicate. Data were analysed with a student t test. *, significantly different from non-treated cells (-), p value < 0.05.

Fig. 9. Intracellular signalling induced by YadA and invasin upon direct and indirect binding to β_1 -integrins. The scheme illustrates the effect on intracellular signalling pathways in response to exogenous β_1 -integrins engagement by the *Yersinia* adhesins invasin and YadA. Several kinases (ILK, Cas, FAK and Pyk2) directly associated with β_1 -integrins were shown to be important for Inv- and/or YadA-mediated uptake of *Yersinia*. Subsequent activation of Src and Ras induces the MEK1/2-ERK1/2 MAP kinase pathway leading to IL-8 production and secretion. PI3 kinase downstream of Ras induces activation of Akt and PLC- γ 1, which in turn results in phosphorylation of conventional PKC α and - β . Inv-mediated engagement of β_1 -integrins also activates the small GTPase Rac1, leading to higher PIP₂ levels due to PIP5 kinase activation. Arf6 participates in the activation of the Rac-dependent PIP5 kinase activity and other so far unknown events associated with *Yersinia* uptake.

Fig. 1 Uliczka et al. 2009

A **B**

Fig. 2 Uliczka *et al.* 2009

Fig. 3 Uliczka et al. 2009

A **B**

Fig. 4 Uliczka et al. 2009

Fig. 5 Uliczka et al. 2009

Fig. 6 Uliczka *et al.* 2009

Fig. 7 Uliczka *et al.* 2009

Fig. 8 Uliczka et al. 2009

Fig. 9 Uliczka et al. 2009