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The secretion of the *Shigella flexneri* Ipa invasins is activated by epithelial cells and controlled by IpaB and IpaD

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***Shigella* species are enteropathogens that invade epithelial cells of the human colon. Entry into epithelial cells is triggered by the IpaB, IpaC and IpaD proteins which are translocated into the medium through the specific Mxi–Spa machinery. *In vitro*, *Shigella* cells secrete only a small fraction of the Ipa proteins, the majority of which remains in the cytoplasm. We show here that upon interaction with cultured epithelial cells or in the presence of fetal bovine serum, *S.flexneri* release pre-synthesized Ipa molecules from the cytoplasm into the environment. Evidence is presented that IpaB and IpaD are essential for both blocking secretion through the Mxi–Spa translocon in the absence of a secretion-inducing signal and controlling secretion of the Ipa proteins in the presence of a signal. Subcellular localization and analysis of the molecular interactions of the Ipa proteins indicate that IpaB and IpaD associate transiently in the bacterial envelope. We propose that IpaB and IpaD, by interacting in the secretion apparatus, modulate secretion.**

Key words: Ipa invasins/protein secretion/*Shigella*/type III secretion pathway

Introduction

The virulence processes of pathogenic bacteria are frequently coupled to the secretion of effector molecules into the environment. The secretory pathway of a protein destined to be released from a Gram-negative bacterium starts in the cytoplasm and leads through the cytoplasmic membrane, periplasm and outer membrane. Three distinct secretory pathways have been described in Gram-negative bacteria (reviewed in Lory, 1992; Pugsley, 1993). One of these, the so-called type III secretory pathway, is required for the secretion of proteins which contribute to the virulence of a number of animal and plant pathogens, such as *Yersinia*, *Shigella*, *Salmonella*, *Xanthomonas* and *Pseudomonas* (Van Gijsegem *et al.*, 1993). Recent genetic studies have indicated that enteropathogenic species of *Yersinia*, *Shigella* and *Salmonella* have conserved highly related type III secretion machineries. A number of plasmid-borne genes necessary for the secretion of the Yop anti-phagocytosis proteins of virulent *Yersinia* species have homologues in the plasmid-borne region involved in the secretion of the Ipa entry-associated proteins of *Shigella* species (Michiels *et al.*, 1991; Allaoui *et al.*, 1992, 1993; Venkatesan *et al.*, 1992; Sasakawa *et al.*, 1993; Bergman *et al.*, 1994; Woestyn *et al.*, 1994). Likewise, the chromosomal *spa* gene cluster, which is essential for invasion by *Salmonella typhimurium*, is remarkably similar in order and sequence to the *spa* operon controlling secretion of the Ipa proteins of *Shigella* species (Groisman and Ochman, 1993). Some of these genes are also similar to the flagellar assembly loci of *Bacillus subtilis*, *Escherichia coli* and *S.typhimurium* in both sequence and organization (Groisman and Ochman, 1993). However, the molecular mechanisms involved in secretion by these machineries remain unknown.

Shigella species are enteropathogens that are able to enter human colonic epithelial cells (reviewed in Hale, 1991). Entry of *S.flexneri* into epithelial cells is governed by a 31 kb plasmid-borne locus (Maurelli *et al.*, 1985; Sasakawa *et al.*, 1989), which is composed of two divergently transcribed regions. The first, referred to as the *ipa* region, encodes the IpaB, IpaC, IpaD and IpgC proteins, which have been shown to be essential for the entry process. IpgC, which is not secreted, acts as a molecular chaperone by transiently associating with and impeding the association of IpaB and IpaC in the cytoplasm (Ménard *et al.*, 1994). IpaB, IpaC and IpaD are secretory proteins thought to be the effectors of the entry process (Ménard *et al.*, 1993). The second region of the 31 kb invasion-associated region contains two blocks each of eleven contiguous genes, called *mxi* (*m*embrane e*x*pression of *i*nvasion plasmid antigens) and *spa* (*s*urface *p*resentation of *a*ntigens). Mutations have been created in most of the *mxi* and *spa* genes; they all abolish the ability of *Shigella* to secrete the Ipa proteins and to invade epithelial cells (Andrews *et al.*, 1991; Allaoui *et al.*, 1992, 1993; Venkatesan *et al.*, 1992; Sasakawa *et al.*, 1993). It has recently been shown that the Mxi–Spa system allows the secretion of the Ipa proteins into the medium rather than, or in addition to, their association with the bacterial surface (Andrews *et al.*, 1991; Ménard *et al.*, 1994).

In *in vitro* cultured *Shigella* cells, the majority of the Ipa proteins is retained in the cytoplasm, with only small amounts being secreted. Here we show that *Shigella* respond to interaction with epithelial cells and to the presence of a factor in fetal bovine serum by releasing pre-synthesized Ipa proteins into the medium. Evidence is presented that IpaB and IpaD are both essential to prevent secretion via the Mxi–Spa system in the absence of inducer and to control the level of secretion in the presence of inducer. We propose that IpaB and IpaD, which associate transiently in the

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bacterial envelope, control secretion through the Mxi–Spa translocon.

Results

Lack of IpaB or IpaD enhances secretion through the Mxi–Spa translocon

In *in vitro* cultured *S.flexneri*, ~90% of the IpaB and IpaC proteins accumulate in the cytoplasm (Andrews *et al.*, 1991; Ménard *et al.*, 1994). We have previously constructed strains carrying non-polar mutations in each of the four *ipa* genes and shown that none of the *ipa* mutations significantly affected the total amounts of the other Ipa proteins (Ménard *et al.*, 1993). However, the *ipaB* and *ipaD* mutations led to a marked increase in the extracellular amounts of the other Ipa proteins (Figure 1). To further characterize the phenotypes of these mutants, we first determined whether the increased Ipa secretion by the *ipaD* mutant occured via the Mxi–Spa system. One of the essential components of this secretion system is the *mxiD* gene product (Allaoui *et al.*, 1993). A strain carrying mutations in both *ipaD* and *mxiD* was constructed, and the extracellular proteins of the double mutant were analysed by immunoblotting (Figure 1). Although whole cell extracts of the double mutant contained similar amounts of IpaB and IpaC to the wild-type (data not shown), these proteins could not be detected in the medium of the double mutant. Thus, oversecretion of IpaB and IpaC by the *ipaD* mutant occurred via the Mxi–Spa system. We then investigated whether the *ipaD* mutation resulted in an increased secretion of IpaB and IpaC by enhancing expression of the genes encoding the Mxi–Spa translocon. A *mxiD–lacZ* transcriptional fusion was integrated into the *mxiD* locus of both the wild-type and the *ipaD* mutant, and the β-galactosidase activities expressed by this fusion in each of the strains were compared. Similar levels of β-galactosidase activities were produced by the $ipaD^+$ and $ipaD^-$ strains (815 and 790 units respectively), indicating that IpaD did not alter expression of the genes encoding the components of the secretion machinery. Since neither the secretory nor the translocator proteins were overproduced by the *ipaD* mutant, the increased secretion of the remaining Ipa proteins in the absence of IpaD appeared to be due to increased leakage through the Mxi–Spa apparatus.

To quantify the proportion of the remaining Ipa proteins that was secreted in the absence of either IpaB or IpaD, we compared the amounts of Ipa proteins in the wild-type and in the *ipaB* and *ipaD* mutants that were extracellular, as indicated by their susceptibility to degradation by proteinase K. Proteinase K was added to exponentially growing cultures of these strains and total bacterial extracts were analysed by immunoblotting at indicated times thereafter (Figure 2). In the wild-type, the amounts of IpaB, IpaC and IpaD did not decrease significantly, even after prolonged incubation with proteinase K, which confirmed that the vast majority of these proteins was intracellular. In contrast, in both the *ipaB* and *ipaD* mutants virtually all of each of the remaining Ipa proteins were rapidly degraded by proteinase K, while adenylate kinase (ADK) remained protected from the protease, as expected from its cytoplasmic localization. Control experiments performed with cytoplasmic extracts of the wild-type confirmed that ADK, IpaB, IpaC and IpaD were all sensitive to degradation by proteinase K (data not shown). These results indicate that, in the absence of either IpaB or IpaD, the remaining Ipa proteins are fully secreted. Therefore, IpaB and IpaD are each essential to prevent the complete leakage of Ipa proteins into the medium.

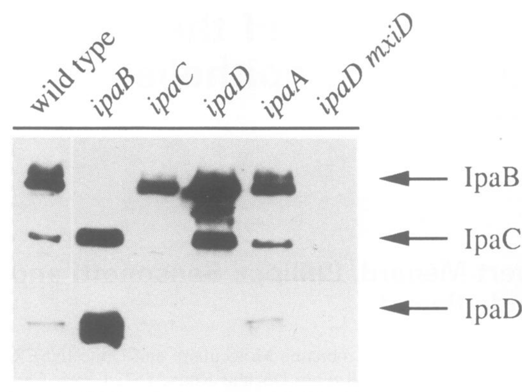

Fig. 1. Secretion of IpaB, IpaC and IpaD by the wild-type strain, the *ipaB*, *ipaC*, *ipaD* and *ipaA* mutants and the *ipaD mxiD* double mutant. The extracellular proteins present in the culture supernatant of these strains were analysed by SDS–PAGE and immunoblotting.

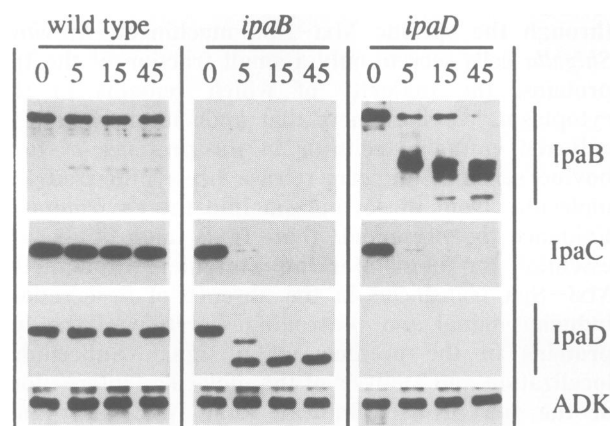

Fig. 2. Proteinase K susceptibility of IpaB, IpaC, IpaD and the cytoplasmic adenylate kinase (ADK) in total cultures of the wild-type strain and the *ipaB* and *ipaD* mutants. Proteinase K was added to exponentially growing cultures of each of these strains and degradation of extracellular bacterial proteins was allowed to proceed at 20°C. Numbers indicate the incubation times (in min) in the presence of proteinase K. At the indicated times, the reaction was stopped by adding PMSF and Laemmli sample buffer and by heating the samples at 100°C for 5 min. The samples were then analysed by SDS–PAGE and immunoblotting.

Subcellular fractionation of IpaB and IpaD

We then attempted to localize IpaB and IpaD in exponentially growing *Shigella* cells. We have previously shown that most of the IpaB protein accumulates in the cytoplasm and that small amounts are secreted into the medium without any detectable periplasmic intermediate (Ménard *et al.*, 1994). When total proteins of *S.flexneri* M90T $PhoA^+$, a derivative of the wild-type that produces a periplasmic alkaline phosphatase, were fractionated into

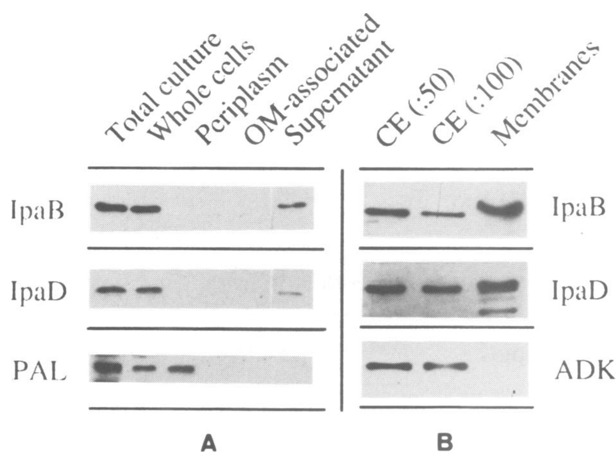

Fig. 3. Subcellular localization of IpaB and IpaD. (**A**) Proteins of a total culture of exponentially growing *S.flexneri* M90T PhoA$^+$ were fractionated into cell-associated (whole cells, containing both cytoplasmic and integral membrane proteins), periplasmic and extracellular proteins (supernatant). Treatment of bacteria with xylene was used to detach proteins weakly associated with the outer membrane (OM-associated). Equivalent amounts of each bacterial fraction were analysed by SDS–PAGE and immunoblotting with antibodies directed against IpaB, IpaD and alkaline phosphatase (PAL). (**B**) Membranes and cytoplasmic extracts (CE) were prepared after disruption of exponentially growing cells of *S.flexneri* M90T in a French pressure cell. Immunoblotting was used to compare the amounts of IpaB, IpaD and the cytoplasmic adenylate kinase (ADK) present in the membranes to those present in the cytoplasmic extracts (CE), diluted 50-fold (CE:50) or diluted 100-fold (CE:100).

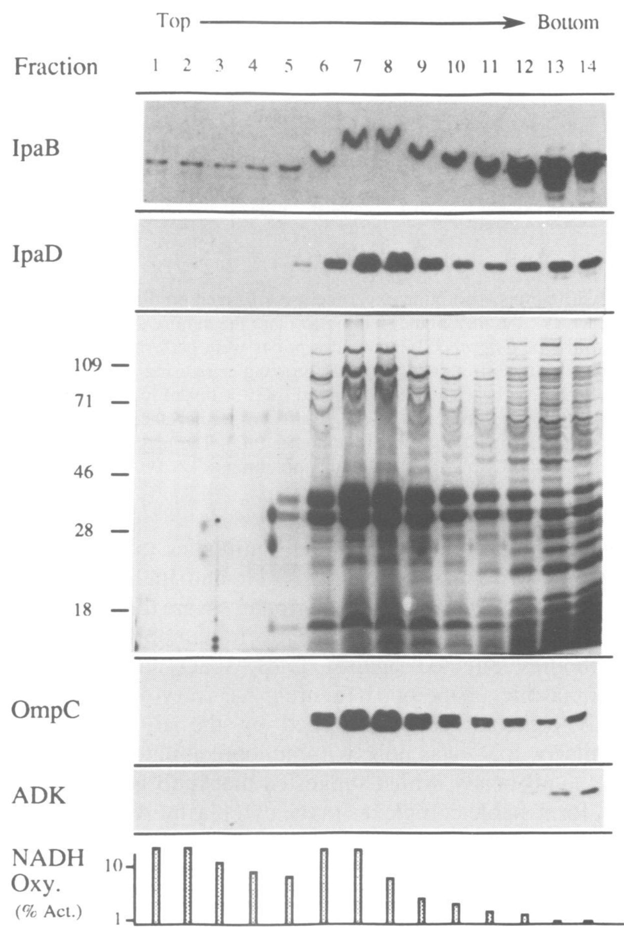

Fig. 4. Analysis of membrane preparations by sucrose gradient floatation. Following disruption of wild-type bacteria in a French pressure cell, the bacterial membranes were first collected by centrifugation of the lysate onto a sucrose cushion (62% sucrose), loaded at the bottom of a centrifuge tube and overlaid with successive solutions of decreasing sucrose density. After centrifugation, fractions were collected from the top of the tube and analysed by SDS–PAGE and Coomassie blue staining (central panel) or immunoblotting to determine the distribution of IpaB, IpaD, OmpC and adenylate kinase (ADK). The distribution of NADH oxidase was determined by an enzymatic activity assay.

cell-associated (containing both cytoplasmic and integral membrane proteins), periplasmic, outer membrane-associated and secreted proteins, IpaD was found mainly in the cell-associated fraction and only small amounts of the protein were detected in the medium (Figure 3A). Unlike alkaline phosphatase, IpaD could not be detected in the periplasmic extract. Moreover, treatment of the cells with xylene, which detaches proteins weakly associated with the external surface of bacteria (Michiels *et al.*, 1990), did not release detectable amounts of IpaD.

To localize IpaB and IpaD in the cell-associated fraction, wild-type bacteria were disrupted in a French pressure cell and soluble and membrane-associated proteins were separated and analysed by immunoblotting (Figure 3B). The membranes were found to contain both IpaB and IpaD, each in amounts ~100-fold lower than those detected in the cytoplasm. ADK was barely detected in these membrane fractions, indicating that they were free from cytoplasmic contamination. To determine whether IpaB and IpaD were indeed membrane-associated, we analysed the membrane fraction of wild-type bacteria by a floatation technique (Poquet *et al.*, 1993). Membranes from lysates obtained after disruption of bacteria were first centrifuged onto a sucrose cushion and the membrane fraction was then applied to a floatation sucrose gradient (see Materials and methods). Under these conditions, soluble and aggregated proteins that co-sediment with the membranes onto the sucrose cushion remain at the bottom of the gradient, whereas membrane-associated proteins float up the gradient. Fractions were collected from the top to the bottom of the gradient and the distributions of IpaB, IpaD, outer membrane porin C (OmpC) and the cytoplasmic ADK were determined by immunoblotting. The distribution of NADH oxidase (a cytoplasmic membrane protein) was determined by an enzymatic activity assay. As shown in Figure 4, a fraction of both IpaB and IpaD floated up the gradient, indicating that these two proteins were indeed membrane-associated. In contrast, the small amounts of IpaC that could be detected remained at the bottom of the floatation gradient (not shown), in fractions that also contained traces of ADK. Membrane-associated IpaB and IpaD co-fractionated mainly with the peak of OmpC, suggesting that they are associated with the outer membrane.

IpaB and IpaD do not associate in the cytoplasm or after secretion

To gain insight into the mechanisms by which IpaB and IpaD block the secretion apparatus, we investigated whether IpaB and IpaD interacted in the various compartments in which they had been detected. Immunoadsorption experiments were first performed on cytoplasmic extracts of the wild-type using either protein G–Sepharose beads

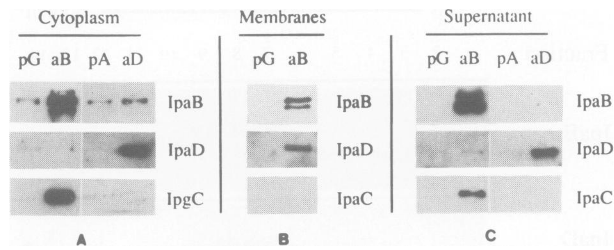

Fig. 5. Immunoadsorption experiments performed on the cytoplasmic extract (**A**), the membrane fraction (**B**) and the culture supernatant (**C**) of wild-type *S.flexneri*. Immunoadsorptions were performed on the various extracts from exponentially growing cells using protein G–Sepharose beads, either uncoupled (pG) or linked to anti-IpaB mAbs (aB), and protein A–Sepharose beads, either uncoupled (pA) or linked to anti-IpaD polyclonal antibodies (aD). The precipitates were analysed by SDS–PAGE and immunoblotting.

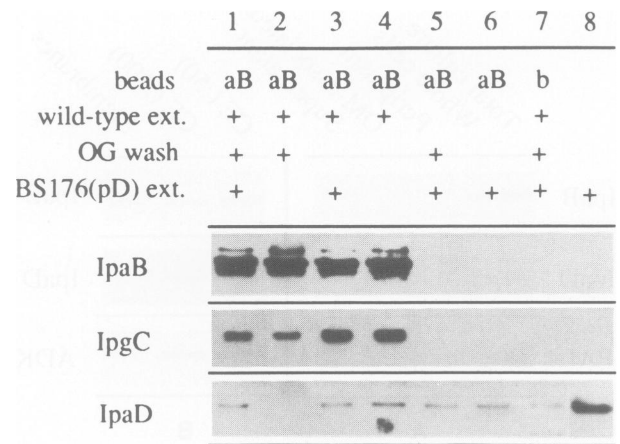

Fig. 6. IpaD does not bind to beads presenting either IpaB alone or IpaB–IpgC complexes. Protein G–Sepharose beads, either uncoupled (b) or covalently linked to anti-IpaB mAbs (aB beads) were incubated for 4 h at 4°C with soluble cytoplasmic extracts of the wild-type strain and washed either in PBS (lanes 3, 4 and 6) or in 50 mM octylglucoside (lanes 1, 2, 5 and 7). Beads from samples 2 and 4 were washed and resuspended in Laemmli sample buffer. Beads from samples 1, 3, 5, 6 and 7 were further incubated at 4°C for 4 h with soluble protein extracts of a derivative of BS176, the large plasmid-cured *S.flexneri* strain, containing plasmid pD (*ipaD*$^+$). The amounts of IpaD added in these samples were ~50-fold higher than those detected in lane 8. Beads from samples 1, 3, 5, 6 and 7 were then washed and resuspended in Laemmli sample buffer. The amounts of Ipa B, IpgC and IpaD in each of the samples were then compared by SDS–PAGE and immunoblotting.

linked to anti-IpaB monoclonal antibodies (aB beads) or protein A–Sepharose beads linked to anti-IpaD polyclonal antibodies (aD beads). The precipitates were then separated by SDS–PAGE and analysed by immunoblotting using antibodies directed against IpaB, IpaD and the IpgC cytoplasmic chaperone (Figure 5A). Very little, if any, IpaB protein was co-adsorbed by the aD beads and, similarly, IpaD was poorly immunoprecipitated with IpaB by the aB beads, which suggested that IpaB and IpaD did not form stable complexes in the cytoplasm. As previously reported (Ménard *et al.*, 1994), IpaB bound IpgC in the cytoplasm. In contrast, IpgC was not co-immunoadsorbed with IpaD by the aD beads, suggesting that IpaD did not interact with IpgC (Figure 5A).

We next investigated whether IpaB and IpaD associated in the extracellular medium. We performed immunoadsorptions on the culture supernatant of exponentially growing wild-type cells using the aB and aD beads and the precipitates were analysed by immunoblotting (Figure 5C). IpaB and IpaC were both adsorbed by the aB beads, but very low amounts of IpaD co-precipitated. Conversely, IpaD was readily adsorbed by the aD beads, whereas IpaB and IpaC could not be detected in the precipitates, suggesting that IpaD was not associated with the IpaB–IpaC extracellular complex. Moreover, IpaD was the only protein detected by Coomassie blue staining of the precipitates recovered with the aD beads (not shown). It thus appears that, whereas IpaB associates with IpgC in the cytoplasm and with IpaC in the extracellular medium, IpaD does not interact with any of these factors in either the cytoplasm or the extracellular milieu.

IpaB and IpaD associate in the bacterial membranes

In view of the presence of a fraction of IpaB and IpaD in the bacterial membranes, the most likely explanation for the oversecretion phenotype of the *ipaB* and *ipaD* mutants was that IpaB and IpaD interacted in the secretion apparatus to prevent secretion. To test this hypothesis, the membrane preparations that had been subjected to immunoblotting (Figure 3B) were used in immunoprecipitation experiments (Figure 5B). The membranes were solubilized in 0.4% Triton X-100 and incubated with either the protein G or the aB beads. The precipitates obtained with the aB beads contained IpaD in amounts significantly higher than those immunoadsorbed with IpaB in the cytoplasmic or extracellular compartments. In contrast, IpaC, whose absence does not affect secretion through the Mxi–Spa translocon, could not be precipitated from the membranes with IpaB by the aB beads. This suggests that IpaB and IpaD form, or are part of, a complex in the membranes, possibly linked to the secretion apparatus, that might be responsible for the low level secretion of *in vitro* cultured *S.flexneri*.

We then investigated whether dissociation of IpgC from IpaB was sufficient to allow interaction of IpaB with IpaD. To do this, we tested the capacity of IpaD to bind either IpaB molecules or IpaB–IpgC complexes fixed on beads (Figure 6). Large amounts of free IpaD (lane 8) were provided by the cytoplasmic extracts of strain BS176(pD), which lacks the large virulence plasmid and contains plasmid pD (*ipaD*$^+$). The aB beads presenting IpaB–IpgC complexes (lanes 3 and 4) were obtained after immunoadsorption of soluble extracts of the wild-type with the aB beads and the aB beads presenting free IpaB (lanes 1 and 2) were derived from the latter after treatment with octylglucoside, which removes IpgC from IpaB (Ménard *et al.*, 1994). None of these beads immunoadsorbed significant amounts of IpaD. In a similar experiment performed with soluble extracts of a strain producing large amounts of free IpaC, the latter was found to significantly bind IpaB after dissociation of IpgC (Ménard *et al.*, 1994). These results further suggest that the IpaB–IpaD membrane-associated complex does not result from mere aggregation of the two and indicate that dissociation of IpgC from IpaB is not sufficient to allow interaction of IpaB with IpaD.

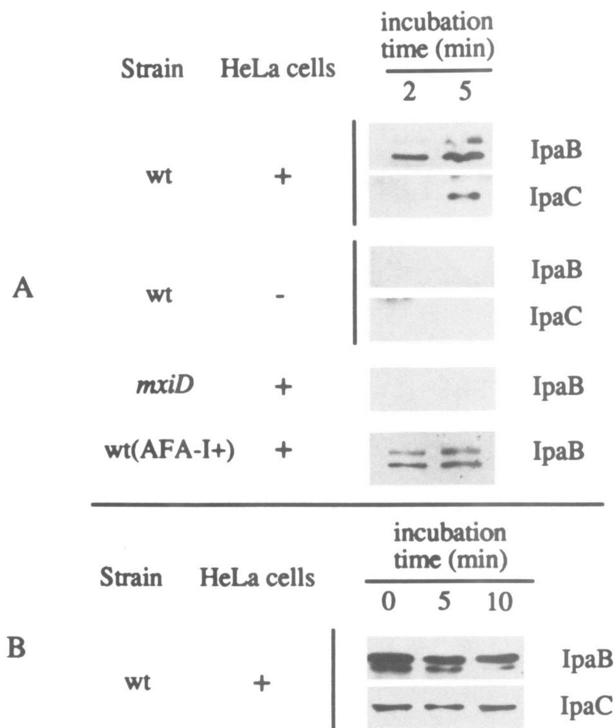

Fig. 7. Induced secretion of IpaB and IpaC upon contact of *S.flexneri* with HeLa epithelial cells. The wild-type strain (wt), the *mxiD* mutant and a derivative of the wild-type expressing the *E.coli* afimbrial adhesin AFA-I were centrifuged in dishes with or without HeLa cells grown to near confluency and transferred to 37°C (zero time). (**A**) At the indicated times, the soluble IpaB and IpaC proteins present in the medium were immunopurified and the precipitates were analysed by SDS–PAGE and immunoblotting. (**B**) At the indicated times, the total amounts of IpaB and IpaC present in the dishes were analysed by SDS–PAGE and immunoblotting.

Interaction with epithelial cells induces the release of pre-synthesized Ipa proteins

We next tested the hypothesis that secretion of the entry-promoting Ipa proteins is enhanced upon interaction with epithelial cells. Wild-type bacteria were centrifuged in dishes with or without HeLa cells treated with cytochalasin D, which inhibits *Shigella* entry, and subsequently transferred to 37°C (zero time). At indicated incubation times, soluble IpaB and IpaC present in the medium were immunopurified with either aB or aC beads and analysed by immunoblotting (Figure 7A). Higher amounts of both IpaB and IpaC were recovered from the medium in the presence of mammalian cells. To ensure that the increased extracellular levels of Ipa proteins in dishes containing mammalian cells did not arise from bacterial lysis but from secretion through the specific translocon, the experiment was carried out with the *mxiD* mutant, which is impaired in secretion of Ipa proteins *in vitro*. In this case, the aB beads did not precipitate detectable amounts of IpaB from the medium (Figure 7A). These results suggest that secretion of IpaB and IpaC through the Mxi–Spa translocon is induced upon interaction with epithelial cells.

Secretion of the YopE cytotoxin of *Yersinia* species, which is driven by the Ysc–Spa system, highly related to the Mxi–Spa system of *Shigella* species (Bergman *et al.*, 1994), has been described as a host cell-induced polarized transfer of the molecule into the cytosol of the target cell but not into the medium (Rosqvist *et al.*, 1994). In contrast to *Yersinia*, *Shigella* are poorly adhesive to epithelial cells (Pal and Hale, 1989). We thus wondered whether the Ipa secretory process was also polarized and whether the Ipa proteins detected in the medium were secreted from bacteria that had adhered to the cells but then subsequently lost adherence. We thus used a derivative of the wild-type *S.flexneri* that expresses the *E.coli* afimbrial adhesin AFA-I (Clerc and Sansonetti, 1987) and strongly adheres to HeLa cells. Bacteria were centrifuged in dishes containing non-permissive HeLa cells, i.e. treated with cytochalasin D, transferred to 37°C and IpaB was immunoprecipitated from the medium after interaction times of 2 or 5 min. As shown in Figure 7A, IpaB was detected in the medium as rapidly as 2 min after host–pathogen interaction. This suggests that, even when tightly attached to the target cell, *S.flexneri* release Ipa proteins into the environment.

To determine whether the increased level of extracellular Ipa proteins was a result of an enhanced production of the Ipa proteins by adherent bacteria, we first investigated whether transcription of the *ipa* operon was increased upon interaction with epithelial cells. A *lacZ* transcriptional fusion in the *ipaA* gene, which is the last gene of the *ipa* operon and is dispensable for entry *in vitro*, was used to compare expression of the *ipa* genes in the presence or absence of epithelial cells. Derivatives of wild-type *S.flexneri* containing the *ipaA–lacZ* transcriptional fusion were centrifuged in dishes with or without non-permissive HeLa cells and the levels of β-galactosidase activity produced by the strain prior to and after 5 and 15 min incubation at 37°C were determined. The levels of β-galactosidase activity were similar in the presence or in the absence of epithelial cells and remained constant during the course of the experiment in each case (data not shown). We also analysed the total amounts of IpaB and IpaC by immunoblotting before and after interaction of wild-type bacteria with non-permissive mammalian cells (Figure 7B). The total levels of IpaB and IpaC proteins remained similar during the course of the experiment. These results suggest that the host cell-induced secretion of IpaB and IpaC results neither from enhanced transcription of the *ipaB* and *ipaC* genes nor from increased production in IpaB and IpaC, but rather from the release of pre-formed Ipa proteins stored in the cytoplasm.

To test this hypothesis, the fate of Ipa proteins present in the bacterial cytoplasm prior to contact with epithelial cells was examined. Since the amounts of soluble Ipa proteins present in the medium might underscore the total Ipa proteins secreted by *Shigella* cells, secretion was analysed by measuring the residual Ipa proteins in the bacterial cytoplasm obtained after proteolytic degradation of extracellular proteins and removal of the eukaryotic cell-associated proteins. Wild-type bacteria were pulse-labelled with [^{35}S]methionine for 15 min, chased for 10 min with excess unlabelled methionine, centrifuged onto non-permissive HeLa cells and immediately placed at 37°C. At the indicated times, the cytoplasmic IpaB and IpaC proteins from adherent bacteria were immunopurified with either the aB or aC beads (Figure 8). The amounts of total bacterial proteins collected at each point of the experiment were similar. Upon interaction with epithelial cells, the amounts of labelled IpaB and IpaC present in

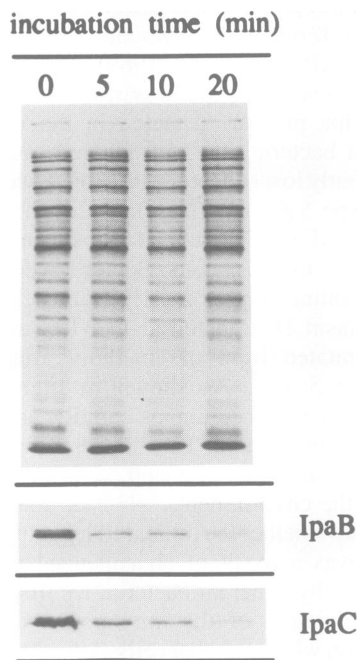

Fig. 8. Release of cytoplasmic IpaB and IpaC upon contact of *S.flexneri* with epithelial cells. Wild-type bacteria were radiolabelled with [^{35}S]methionine for 15 min, chased with unlabelled methionine for 10 min, centrifuged in dishes containing HeLa cells grown to near confluency and transferred to 37°C (zero time). At the indicated times, the total cytoplasmic proteins from adherent bacteria were collected and either directly loaded on a gel (upper part) or used for immunopurification of IpaB and IpaC. The samples were analysed by SDS–PAGE and autoradiography.

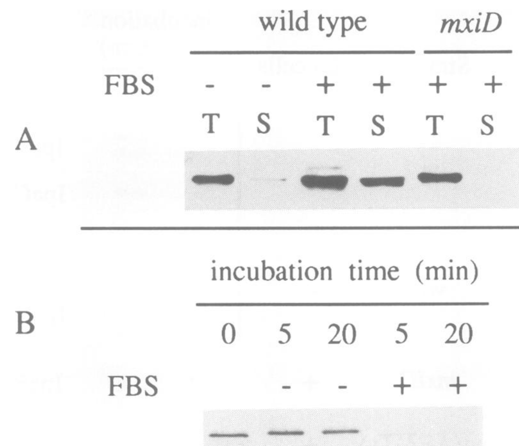

Fig. 9. Induced secretion of IpaB in the presence of fetal bovine serum. (**A**) The wild-type strain or the *mxiD* mutant were grown in TCS medium with or without 1% fetal bovine serum (FBS) and the amounts of total (T) or secreted (S) IpaB were compared by SDS–PAGE and immunoblotting. (**B**) Wild-type bacteria were radiolabelled with [^{35}S]methionine for 15 min, chased with unlabelled methionine for 2 min, resuspended in MEM medium with or without 10% FBS and incubated at 37°C. At the indicated times, the cultures were treated with proteinase K, cytoplasmic IpaB was immunopurified and the precipitates were analysed by SDS–PAGE and autoradiography.

the bacterial cytoplasm were lower. Furthermore, most of the IpaB and IpaC proteins were released within the first 5 min after contact with mammalian cells. This result indicates that *S.flexneri* accumulates the Ipa proteins in the cytoplasm and releases them upon interaction with epithelial cells. It also suggests that secretion, although rapidly induced, does not lead to the release of all the cytoplasmic Ipa proteins.

Secretion of Ipa proteins is induced in the presence of serum

We then investigated whether the Ipa secretory process could be enhanced by some factor other than interaction with epithelial cells. Wild-type bacteria were grown in TCS medium with or without 1% fetal bovine serum (FBS) and total and extracellular amounts of IpaB of exponentially growing cultures were compared by immunoblotting (Figure 9A). Although growth in the presence of 1% FBS did not lead to a significantly increased yield of IpaB, higher amounts of extracellular IpaB were obtained in the presence of serum. In contrast, the *mxiD* strain grown in the presence of 1% FBS did not secrete IpaB into the medium, suggesting that FBS enhanced secretion of IpaB through the specific translocon. The fate of radiolabelled Ipa proteins was also examined by measuring the residual Ipa proteins in the cytoplasm after addition of FBS. Wild-type bacteria were pulse-labelled with [^{35}S]methionine for 15 min and chased for 2 min with excess unlabelled methionine, resuspended in MEM with or without 10% FBS and placed at 37°C. At various incubation times the extracellular proteins were degraded by treating the cultures with proteinase K, bacteria were collected and the cytoplasmic IpaB was immunopurified with the aB beads (Figure 9B). Comparable amounts of IpaB were purified prior to and after 20 min incubation in MEM devoid of serum. Upon incubation in MEM supplemented with serum, the amount of labelled IpaB present in the cytoplasm was significantly lower. However, as observed upon interaction with epithelial cells, most of the labelled IpaB was released within the first 5 min of incubation with serum, comparable amounts of this protein being recovered after 5 or 20 min incubation with serum. This result suggests that a soluble factor in FBS enhances secretion of the Ipa proteins by triggering release of a proportion of the cytoplasmic Ipa proteins.

Discussion

Bacteria have evolved sophisticated signal transduction systems to sense and adapt to their environment. The field of microbial pathogenesis offers many examples of coordinate expression of virulence determinants controlled at the transcriptional level in response to physico-chemical changes in the various habitats encountered by the pathogen (Mekalanos, 1992). There is also accumulating evidence that some virulence traits may be induced by more specific signals resulting from host–pathogen cross-talk. For example, upon interaction with epithelial cells, enteropathogenic *E.coli* (EPEC) express an inducible 'localized adherence' phenotype associated with *de novo* protein synthesis and expression of bundle-forming pili (Vuopio-Varkila and Schoolnik, 1991; Giron *et al.*, 1991). Similarly, contact between *Yersinia pseudotuberculosis* and mammalian cells induces expression of *yopE* and the subsequent polarized transfer of the YopE cytotoxin into the cytosol of the eukaryotic cell (Rosqvist *et al.*, 1994). In such cases,

the first consequence of the host–pathogen interaction is the transcriptional activation of the genes that specify the inducible virulence phenotype.

Shigella species are enteropathogens that have the capacity to enter epithelial cells of the human colon (Hale, 1991). The genes required for invasion include regulatory genes (Sakai *et al.*, 1988; Adler *et al.*, 1989), the *mxi–spa* secretion loci (Allaoui *et al.*, 1992, 1993; Venkatesan *et al.*, 1992; Sasakawa *et al.*, 1993) and four genes that belong to the same transcription unit: *ipgC*, *ipaB*, *ipaC* and *ipaD* (Sasakawa *et al.*, 1989; Ménard *et al.*, 1993, 1994). IpaB, IpaC and IpaD, unlike IpgC, are secreted into the medium and none of these Ipa proteins is necessary for secretion of the other two, which suggests that these three Ipa proteins are the effectors of *S.flexneri* entry (Ménard *et al.*, 1993). However, little is known about the specific role of each of these extracellular factors. When grown in culture, *Shigella* cells accumulate most of the Ipa proteins in the cytoplasm (Andrews *et al.*, 1991; Ménard *et al.*, 1994). We show here that Ipa proteins delivery relies on the host cell-induced release of preformed molecules stored in the cytoplasm. The cell-induced response of *Shigella* differs markedly from those described above in that production of the Ipa effectors is not enhanced upon sensing the epithelial cell and the secreted effectors are synthesized prior to the host–pathogen interaction. Moreover, the observation that soluble Ipa proteins can be recovered from the medium upon interaction of strongly adhering *S.flexneri* with HeLa cells suggests that Ipa secretion is not only directed into the host cell but also into the medium. Although *Yersinia* and *Shigella* use highly conserved secretion machineries (Groisman and Ochman, 1993; Sasakawa *et al.*, 1993; Bergman *et al.*, 1994; Woestyn *et al.*, 1994), they appear to have evolved distinct strategies to deliver the effector molecules. Whereas the host cell might provide *Yersinia* with a signal for derepression of transcription of *yopE* and of the translocon-encoding genes (Rosqvist *et al.*, 1994), the target cell might provide *Shigella*, which constitutively express the effector- and translocon-encoding genes, with a signal for the rapid release of pre-synthesized Ipa proteins into the environment. Therefore, *Yersinia* may direct assembly of the translocons at the host–pathogen contact zones, driving polarized transfer of the newly synthesized YopE. *Shigella*, on the other hand, may secrete the Ipa proteins in all directions via pre-assembled translocons. The *Shigella* strategy is probably best adapted to processes that necessitate the delivery of high levels of effectors to the host–pathogen interface. This may account for the rapid internalization of *Shigella*, which gain access to the eukaryotic cytoplasm within <10 min after contact (Sansonetti *et al.*, 1986).

S.flexneri were found to release the Ipa proteins efficiently in response to both interaction with epithelial cells and to a factor present in FBS. Secretion was qualitatively similar in both cases; it was rapid (within 5 min), but some Ipa proteins remained cytoplasmic. The total release of the preformed Ipa proteins was never obtained within the time scale normally needed for internalization, suggesting that *Shigella* might retain part of the Ipa proteins within the cytoplasm. However, the exact secretion response of *Shigella* during entry into epithelial cells *in vivo* remains unknown. Indeed, secretion of only a portion of the cytoplasmic Ipa proteins upon interaction with HeLa cells might be due to the failure of some of the bacteria to adhere correctly to HeLa cells. Similarly, secretion induced by 10% FBS, although probably corresponding to the response of each bacterial cell, might have been limited by the amount or efficiency of the inducer. The nature of the inducer present in FBS and at the cell surface remains to be elucidated. *Shigella* may sense a unique secretion inducer produced by the cells, which would be both associated with, and released from, cells. It remains a formal possibility that the inducer found at the cell surface has been adsorbed during cell growth in the presence of serum. Whatever the nature of the factor present in FBS, the main conclusion we draw from these experiments is that induction of the Ipa secretory process might not be restricted to, or even require, interaction with a eukaryotic cell-associated receptor, but might also be triggered by a soluble factor sensed by *Shigella*. Ipa proteins secreted close to epithelial cells in response to a signal released by these cells might initiate cytoskeletal rearrangements that lead to rapid engulfment of bacteria in cell protrusions. One might even envision that *Shigella* secrete Ipa proteins in all directions before interacting with the target cell so that they can enter the endocytic vacuole without contacting the cell surface. This would be consistent with the fact that *Shigella* adhere only loosely to epithelial cells (Pal and Hale, 1989).

The inducible Ipa release process suggests that the Mxi–Spa secretion system acts as a tap that can be switched from an 'off' to an 'on' position upon sensing a signal. Insights into the mechanisms that control the secretory process were provided by analysis of the *ipaB* and *ipaD* mutants. In the absence of the signal, the lack of either IpaB or IpaD causes the translocon to remain fully open, inasmuch as virtually all the remaining Ipa proteins are extracellular in the absence of either one of these products. The lack of IpaB or IpaD affects neither the production of the other Ipa proteins nor the expression of the genes encoding the secretion machinery. Therefore, besides their presumed role in the invasion process as secretory products, IpaB and IpaD also directly contribute to secretion control by blocking the translocon in the 'off' position in the absence of the signal. We also conclude that, in agreement with the fact that the cell-induced release of Ipa proteins does not increase expression of the *ipa* genes, the synthesis of the Ipa proteins *in vitro* does not depend on their further localization inside or outside of the bacterial cytoplasm.

In *S.flexneri* grown in culture, IpaB and IpaD are found mainly in the cytoplasm and only small amounts of them are present in the membranes. Theoretically, IpaB and IpaD might prevent secretion either by titrating a secretion factor in the cytoplasm or by acting directly as anti-secretion factors in the translocon. The former implies that cytoplasmic IpaB and IpaD each associate with the putative factor that favours secretion. However, cytoplasmic IpaB associates only with IpgC, whereas cytoplasmic IpaD does not bind IpgC. Moreover, the latter is not required for normal secretion of IpaC or of other Mxi–Spa-translocated factors (Ménard *et al.*, 1994; our unpublished results). Thus, we favour the possibility that IpaB and IpaD act as anti-secretion factors by directly blocking the secretion apparatus. In agreement with this

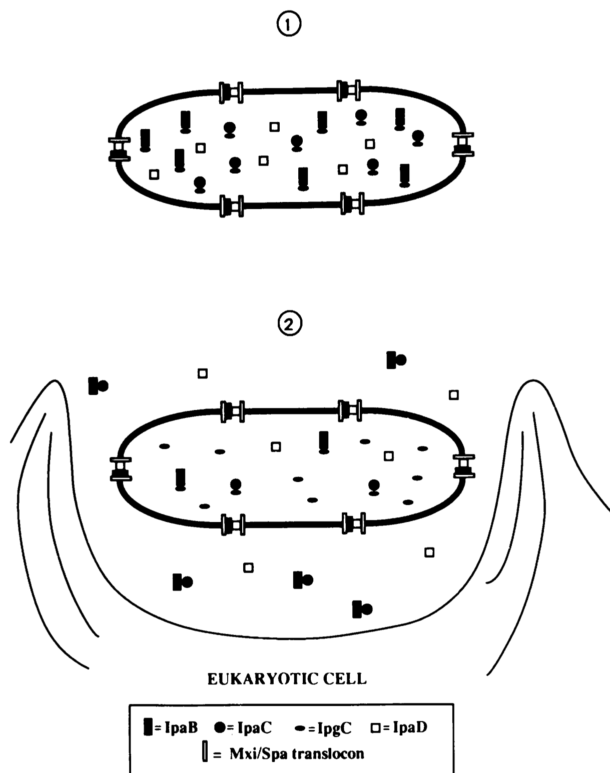

Fig. 10. Schematic representation of the various interactions between IpaB, IpaC, IpaD and IpgC in *S.flexneri*. In *in vitro* grown bacteria (**1**), cytoplasmic IpaB and IpaC independently bind the IpgC molecular chaperone; in addition, a small proportion of IpaB and IpaD interact with the Mxi–Spa secretion apparatus to block secretion. In the presence of eukaryotic cells (**2**), the activity of the Mxi–Spa translocon is induced, part of the Ipa proteins is secreted and IpaB and IpaC, but not IpaD, associate in the medium.

hypothesis, stable complexes containing both IpaB and IpaD, but not IpaC, whose absence does not affect secretion through the translocon, were recovered from the bacterial membranes. *In vitro* experiments have indicated that dissociation of IpgC from IpaB is not sufficient to promote association of IpaB and IpaD, but does allow IpaB–IpaC association (Ménard *et al.*, 1994). A likely candidate for triggering IpaB–IpaD association would be some component of the secretion machinery itself. The IpaB–IpaD association would be triggered by the translocon which, in turn, would be blocked by the complex in the absence of the signal.

The results of subcellular localization and immunoprecipitation experiments on Ipa proteins performed here and elsewhere (Ménard *et al.*, 1994) suggest the following model of the Ipa secretory process (Figure 10). IpaB and IpaC independently bind the IpgC molecular chaperone which impedes the formation of IpaB–IpaC complexes in the cytoplasm. Secreted IpaB and IpaC directly interact in a complex that probably contains other proteins, such as IpaA. On the other hand, IpaD does not interact with any of these factors in the cytoplasm or with the IpaB–IpaC extracellular complex. Secretion of the Ipa proteins through the specific translocon is controlled by IpaB and IpaD, which are proposed to form a complex acting as the plug of the system in the absence of a secretion signal. This IpaB–IpaD interaction represents a transient intermediate in the IpaB secretory pathway. This transient interaction might be important not only to block secretion in the absence of the signal but also to control secretion in the presence of the signal. Indeed, the levels of secretion obtained upon contact with epithelial cells or in the presence of FBS were lower than those observed *in vitro* in the absence of either IpaB or IpaD. It thus seems plausible that IpaB and IpaD, by interacting in the secretion apparatus, act as modulators of the secretion process by either blocking or reducing secretion through the translocon.

The virulence-associated system described here, composed of a specific translocon acting as a tap controlled by two of the secretory products, appears to be unique. The system is induced either by contact with the target cell or by a soluble factor. The two secretory proteins that act as braking or blocking molecules might provide an efficient means to modulate the secretion process, particularly in allowing a rapid shut-off of the secretion system upon disappearance of the signal. This might be designed to enable the bacterium to keep a proportion of the Ipa proteins in reserve for use in subsequent steps of the virulence process, such as lysis of the endocytic vacuole. Such a system would ensure the release of the proper amounts of Ipa effectors which must be delivered at the appropriate time and place to allow *Shigella* to proceed on the virulence pathway.

Materials and methods

Bacterial strains, plasmids and growth conditions

M90T, the *S.flexneri* wild-type strain serotype 5, M90T-Sm, a spontaneous streptomycin-resistant derivative of M90T, BS176, a plasmidless derivative of the wild-type strain, SF620 (*ipaB2*), SF621 (*ipaC2*), SF622 (*ipaD2*) and SF401 (*mxiD1*) have been previously described (Allaoui *et al.*, 1993; Ménard *et al.*, 1993). *E.coli* strains MC1061 and DH5α were used for plasmid constructions and SM10λpir for the transfer of suicide plasmids to *S.flexneri*. Plasmid pD has been previously described (Ménard *et al.*, 1993). *S.flexneri* strains were grown in trypticase soy (TCS) broth or on TCS agar plates. When appropriate, ampicillin (100 μg/ml), kanamycin (50 μg/ml) and streptomycin (100 μg/ml) were added.

Construction of the mxiD ipaD double mutant and transcriptional fusions

The *mxiD ipaD* double mutant was constructed by integration of plasmid pGPD3 into the *ipaD* locus of SF401, the *mxiD1* mutant (Allaoui *et al.*, 1993). Plasmid pGPD3 is a derivative of the suicide vector pGP704 (Miller and Mekalanos, 1988), carrying the 390 bp *Eco*RV–*Hin*dIII internal fragment of *ipaD*. Plasmid pGPD3 was transferred to SF401 (*mxiD*) by conjugal mating and, amongst the transconjugants, one clone in which plasmid pGPD3 had integrated into the *ipaD* locus was selected. The derivatives of the wild-type strain carrying the *ipaA–lacZ* and the *mxiD–lacZ* transcriptional fusions have been described elsewhere (Allaoui *et al.*, 1993; Ménard *et al.*, 1993).

Proteinase K experiments

Stationary phase cultures of the wild-type strain and the *ipaB* or *ipaD* mutants were diluted in fresh TCS medium and 3 ml aliquots were incubated at 37°C for 3 h. Proteinase K was added to each tube to a final concentration of 10 μg/ml and the tubes were incubated at 20°C. At the indicated times, phenylmethylsulfonylfluoride (PMSF, 2 mM final concentration) and 1 ml of Laemmli sample buffer (4×) were added and the tubes were heated at 100°C for 5 min. Samples were then analysed by SDS–PAGE and immunoblotting.

Infection assay

To compare the secretion of the Ipa proteins in the presence or in the absence of epithelial cells, wild-type bacteria were centrifuged in 35 mm diameter plastic dishes with or without HeLa cells. HeLa cells were cultured in minimum essential medium (MEM; GIBCO laboratories, Grand Island, NY) supplemented with 10% fetal calf serum (FCS;

GIBCO). Non-confluent HeLa cell monolayers (5–6×10^5 cells/ml) were treated with cytochalasin D (0.5 μg/ml) for 45 min prior to the infection assay. *S.flexneri* strains were cultured in TCS broth, harvested in the exponential phase (A = 0.5) by centrifugation at 5000 *g* for 10 min and resuspended at the appropriate density (~100 bacteria/HeLa cell) in 1.5 ml MEM containing 0.5 μg/ml cytochalasin D. The bacteria were then placed in dishes with or without HeLa cells, centrifuged for 10 min at 2000 *g* at room temperature and incubated for the indicated times at 37°C. To obtain the extracellular proteins, the medium (1.5 ml) was collected, centrifuged at 12 000 *g* for 3 min to remove non-adherent bacteria (~50% of the total bacteria present in dishes, irrespective of the presence of epithelial cells) and the supernatant was further clarified by centrifugation at 20 000×*g* for 30 min at 4°C. The final supernatant was then used in immunoprecipitation experiments using 20 μl of a slurry of protein G beads coupled to either H16 (anti-IpaB) or K24 (anti-IpaC) monoclonal antibodies (mAbs). After incubation with gentle agitation for 16 h at 4°C, beads were collected, washed three times in MEM and resuspended in 40 μl of Laemmli sample buffer. To determine the total amounts of Ipa proteins in the dishes, 0.5 ml of Laemmli sample buffer (4×) was added to the dishes. Following incubation at 100°C for 5 min, samples were subjected to SDS–PAGE and analysed by immunoblotting.

To evaluate the release of cytoplasmic Ipa proteins in the presence of HeLa cells, bacterial proteins were radiolabelled with [^{35}S]methionine. Cultures were grown at 37°C to logarithmic phase in M9 minimal medium supplemented with nicotinic acid, glucose and amino acids except methionine. Cells were pulse-labelled for 15 min (30 μCi/10^8 colony forming units) and chased by the addition of an excess of non-radioactive L-methionine for 10 min. Bacteria were then centrifuged at 6000 *g* for 10 min at room temperature, resuspended in MEM containing 0.5 μg/ml cytochalasin D, centrifuged at 3000 *g* for 10 min in plastic dishes and immediately transferred to 37°C. The time of the temperature shift was taken as time zero. At designated times the medium was gently removed and discarded and 1 ml of protease medium containing 250 μg/ml trypsin and 50 μg/ml proteinase K in phosphate-buffered saline was added to the dishes which were incubated at 37°C for 10 min in order both to detach bacteria from HeLa cells and to degrade extracellular proteins. The medium was then transferred in 10 ml of MEM containing 1 mM PMSF, 30 μg/ml trypsin inhibitor, 0.7 μg/ml pepstatin and 1 mM EDTA, centrifuged at 700 *g* for 4 min to remove HeLa cells and subsequently centrifuged at 6000 *g* for 10 min to pellet bacteria. The bacterial pellet was then resuspended in 1% SDS (300 μl), heated at 100°C for 5 min, immediatly placed on ice for 5 min and centrifuged at 13 000 *g* for 30 min at 4°C. Immunoprecipitation was performed with a portion (100 μl) of the supernatant of the samples diluted in 1 ml of TTS buffer (2% Triton X-100, 25 mM Tris–HCl, pH 7.5, 100 mM NaCl) using 20 μl of a slurry of protein G beads coupled to either H16 (anti-IpaB) or J22 (anti-IpaC) mAbs. After incubation for 16 h at 4°C, beads were collected by centrifugation, washed three times in 1 ml of TTS buffer and resuspended in 40 μl of Laemmli sample buffer. Following incubation at 100°C for 5 min, samples were subjected to SDS–PAGE. Dried gels were then exposed for autoradiography.

FBS-induced secretion

Experiments were performed with decomplemented FBS (GIBCO). To evaluate the FBS-induced release of the cytoplasmic IpaB protein, bacteria were labelled with [^{35}S]methionine for 15 min as indicated above and chased by the addition of an excess of non-radioactive L-methionine for 2 min. Bacteria were then centrifuged at 6000 *g* for 5 min at room temperature and resuspended in MEM with or without 10% FBS. Aliquots of 1 ml were then treated with proteinase K (100 μg/ml) for 10 min. Bacteria were then collected by centrifugation at 13 000 *g* for 5 min, washed twice in MEM and cytoplasmic IpaB was immunoprecipitated as described above.

Cellular fractionation

Total bacterial extracts were obtained by adding Laemmli sample buffer to a fraction of the total culture. Periplasmic extracts were obtained by an osmotic shock procedure (Neu and Heppel, 1965). Outer membrane-associated proteins were prepared by xylene extraction (Michiels *et al.*, 1990). Whole-cell extracts of bacteria harvested by centrifugation at 14 000 *g* for 10 min were obtained after solubilization in Laemmli sample buffer. The supernatant was obtained after centrifuging bacteria at 27 000 *g* for 30 min and was either precipitated with trichloracetic acid (TCA) or used directly for immunoadsorption experiments.

For disruption in a French pressure cell, bacteria were harvested, washed and resuspended in a 0.05 volume of phosphate-buffered saline and broken by two passages through the French press cell at 41 000 kPa. The lysates were centrifuged at 5000 *g* for 10 min to remove intact bacteria and the clarified lysates were then centrifuged at 30 000 *g* for 30 min at 4°C. The pellet containing the bacterial membranes was then washed twice in PBS and used in either immunoblot or immunoadsorption experiments. Soluble intracellular proteins were obtained after clarification of the bacterial extracts by two successive centrifugations at 27 000 *g* and 450 000 *g* for 30 min each.

The procedure used for sucrose gradient floatation centrifugation was as described by Poquet *et al.* (1993). Briefly, after disruption of bacteria in the French press cell, the membranes were collected by centrifugation onto a 65% sucrose cushion for 2 h at 90 000 *g*. The material sedimented onto the sucrose cushion (membrane fraction) was then loaded at the bottom of a centrifuge tube and overlaid with successive solutions of decreasing sucrose density (from 62 to 30% sucrose). The gradient was centrifuged at 245 000 *g* for 24 h at 10°C. Fractions were collected from the top of the tube and aliquots were assayed for NADH oxidase activity (Osborn *et al.*, 1972). Fractions were then precipitated by addition of TCA and analysed by SDS–PAGE and either Coomassie blue staining or immunoblotting.

SDS–PAGE and immunoblotting

Electrophoresis of the proteins in 10 or 12% polyacrylamide gels in the presence of sodium dodecyl sulfate (SDS–PAGE) was performed as described by Laemmli (1970). After electrophoresis, proteins were either stained with Coomassie brilliant blue or transferred onto a nitrocellulose membrane. Immunoblotting procedures were carried out with mouse mAbs H16 and J22, which recognize IpaB and IpaC respectively (Phalipon *et al.*, 1992; Barzu *et al.*, 1993) or rabbit polyclonal antisera raised against IpgC, adenylate kinase, OmpC or alkaline phosphatase. Horseradish peroxidase-labelled goat anti-mouse or goat anti-rabbit antibodies were used as secondary antibodies and visualized by enhanced chemiluminescence (Amersham International, Amersham, UK). The distribution of the antigens in the cellular fractions was estimated on the basis of the relative intensities of the signal produced on immunoblots by serial dilutions of the extracts.

Preparation of protein G–Sepharose beads covalently linked to mAbs

The procedure used for binding and coupling the H16 (anti-IpaB) and K24 (anti-IpaC) mAbs to protein G–Sepharose beads was similar to that described for protein A beads (Harlow and Lane, 1988). Briefly, 3 mg of a purified mAb were bound to 500 μl of protein G beads (Pharmacia, Uppsala, Sweden) by overnight incubation in PBS at 4°C, covalently attached to the beads with dimethylpimelimidate and stored at 4°C in PBS containing 0.02% sodium azide.

Co-immunoadsorption experiments

Immunoadsorption experiments on intracellular soluble proteins were carried out as follows. Beads (10 μl) were added to 1 ml of protein preparation and rocked gently for 4 h at 4°C. The immunoprecipitates were washed in PBS, resuspended in 20 μl of Laemmli sample buffer and heated to 100°C for 3 min before SDS–PAGE. Immunoadsorption of extracellular proteins was performed in the same way except that the beads were added to 50 ml of culture supernatant and incubated for 16 h at 4°C. Immunoprecipitation experiments performed with membrane preparations were as follows. The bacterial extracts obtained after disruption of bacteria were centrifuged at 30 000 *g* for 30 min, the pellet was rinsed twice in PBS containing 1 mM PMSF, 0.5 μg/ml leupeptin and 0.7 μg/ml pepstatin and disolved in 0.4% Triton X-100 in PBS for 4 h at 4°C. The extracts were then centrifuged at 30 000 *g* for 30 min and the supernatant was used in the immunoprecipitation experiments with 20 μl of beads. Incubation was carried out for 16 h at 4°C.

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