

# gC1q-R/p32, a C1q-binding protein, is a receptor for the InlB invasion protein of *Listeria monocytogenes*

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**InlB is a *Listeria monocytogenes* protein that promotes entry of the bacterium into mammalian cells by stimulating tyrosine phosphorylation of the adaptor proteins Gab1, Cbl and Shc, and activation of phosphatidylinositol (PI) 3-kinase. Using affinity chromatography and enzyme-linked immunosorbent assay, we demonstrate a direct interaction between InlB and the mammalian protein gC1q-R, the receptor of the globular part of the complement component C1q. Soluble C1q or anti-gC1q-R antibodies impair InlB-mediated entry. Transient transfection of GPC16 cells, which are non-permissive to InlB-mediated entry, with a plasmid-expressing human gC1q-R promotes entry of InlB-coated beads. Furthermore, several experiments indicate that membrane recruitment and activation of PI 3-kinase involve an InlB–gC1q-R interaction and that gC1q-R associates with Gab1 upon stimulation of Vero cells with InlB. Thus, gC1q-R constitutes a cellular receptor involved in InlB-mediated activation of PI 3-kinase and tyrosine phosphorylation of the adaptor protein Gab1. After E-cadherin, the receptor for internalin, gC1q-R is the second identified mammalian receptor promoting entry of *L.monocytogenes* into mammalian cells.**

**Keywords:** gC1q-R/InlB/invasion/*Listeria*/signaling

## Introduction

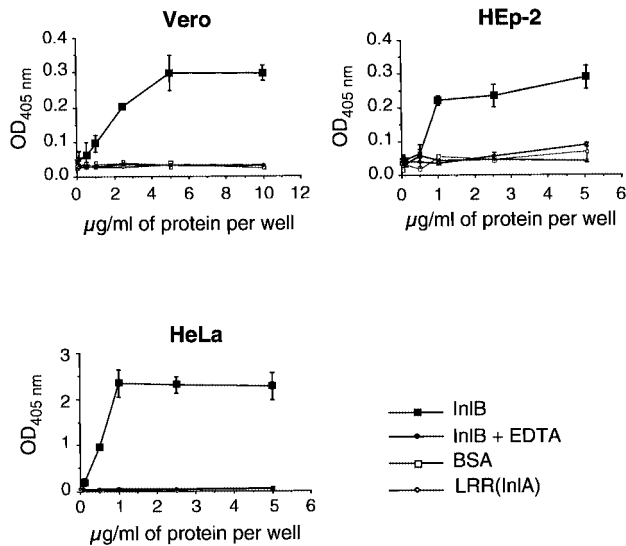
Most infectious diseases involve binding of a microorganism to a host cell as a critical step in tissue colonization, resulting in either localization of the microorganism on the surface of the host cell or internalization into an intracellular niche. Some intracellular pathogens have the ability to induce their own entry into mammalian cells that are non-phagocytic. A variety of strategies to promote their entry into host cells have been described that often imply participation of both bacterial factors and host cell surface components. In the case of *Salmonella* or *Shigella*, invasion is a multifactorial process (Galan, 1996; Nhieu and Sansonetti, 1999), and increasing evidence suggests that bacterial factors directly translocated in the host cell play a critical role. Some receptors of these proteins have been identified (Watarai *et al.*, 1996; Pier *et al.*, 1998). In contrast, entry of *Yersinia* or *Listeria* into cultured cells

implies direct interaction between a bacterial ligand and a mammalian receptor (reviewed in Finlay and Cossart, 1997; Ireton and Cossart, 1998). For *Yersinia*, the outer membrane protein invasin binds to  $\beta_1$  integrin receptors, leading to bacterial entry (Isberg and Leong, 1990). *Listeria monocytogenes* has developed a similar strategy to enter the human enterocyte-like epithelial cell line Caco-2 and some other epithelial cells. In these cells, E-cadherin, a cell surface molecule normally involved in calcium-dependent cell–cell adhesion, is the receptor for the bacterial protein internalin (InlA) (Mengaud *et al.*, 1996a; Lecuit *et al.*, 1999). Interestingly, entry of *L.monocytogenes* into most cell lines is not promoted by internalin but requires InlB, a bacterial protein that does not use E-cadherin as a receptor (Cossart and Lecuit, 1998).

InlB is a 630 amino acid surface protein that promotes bacterial internalization into a wide variety of cultured cell lines including Vero, HEp-2, HeLa and some hepatocytes and endothelial cells (Dramsi *et al.*, 1995; Lingnau *et al.*, 1995; Ireton *et al.*, 1996; Parida *et al.*, 1998). InlB is not only associated with the bacterial surface, but also found in culture supernatants of *L.monocytogenes*, indicating that a fraction of this protein is secreted or released from the bacterial surface (Lingnau *et al.*, 1995; Braun *et al.*, 1997; Jonquière *et al.*, 1999). The loose association of InlB with the bacterial surface is mediated by the so-called GW repeats located in the 232 amino acid C-terminal region of InlB, which bind to the bacterial membrane component lipoteichoic acid (Braun *et al.*, 1997; Jonquière *et al.*, 1999). InlB, present on the surface of non-invasive bacteria or on latex beads, is sufficient to induce uptake (Braun *et al.*, 1998). The contribution of released InlB to the entry process is unknown. While the role of InlA in virulence remains to be established, several reports indicate that InlB plays a role in the hepatic phase of the infection (Dramsi *et al.*, 1995; Gaillard *et al.*, 1996; Gregory *et al.*, 1997).

The InlB-mediated entry of *L.monocytogenes* into cultured cells requires bacterial stimulation of phosphatidylinositol (PI) 3-kinase (Ireton *et al.*, 1996). Activation of this lipid kinase appears to occur through tyrosine phosphorylation of three adaptor proteins Gab1, Cbl and Shc that may help recruitment of the kinase to the InlB receptor (Ireton *et al.*, 1999). InlB is sufficient to activate PI 3-kinase in mammalian cells since a recombinant InlB protein stimulates accumulation of the lipid products of this kinase and tyrosine phosphorylation of the three adaptor proteins.

gC1q-R is a ubiquitous protein, originally identified as a membrane protein that binds to the globular ‘heads’ of C1q (Ghebrehiwet *et al.*, 1994). This receptor now appears as a multifunctional protein with affinity for diverse ligands (reviewed in Ghebrehiwet and Peerschke, 1998). Here we identify gC1q-R as a receptor for InlB and



**Fig. 1.** Mammalian cells bind to purified InIB. Microtiter plates were coated with increasing concentrations of InIB, LRR(InIA) or BSA, and a suspension of Vero, HEp-2 or HeLa cells was added in either the absence or the presence of 10 mM EDTA. After incubation for 1 h, wells were washed and the number of bound cells was determined by assaying for lysosomal hexosaminidase. Values represent the mean  $\pm$  SEM of three independent experiments.

demonstrate that it plays a role in bacterial invasion of non-phagocytic cells.

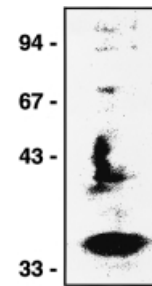
## Results

### Mammalian cells bind to InIB

We have shown previously that InIB bound to non-invasive bacteria or latex beads promoted entry into InIB-permissive cell lines and that pre-treatment of Vero cells with purified InIB inhibited entry of *L.monocytogenes*, indicating that interaction between InIB and mammalian cells is essential for the entry process (Braun *et al.*, 1998, 1999). A simple hypothesis for how this entry process could be induced would be that InIB binds to a cellular receptor. To address this issue, we determined whether mammalian cells were able to bind purified InIB. Microtiter plates (96-well) were coated with increasing concentrations of InIB and incubated with Vero cells. The number of bound cells was quantitated by assaying lysosomal hexosaminidase (Figure 1). Vero cells were able to bind to wells coated with InIB. In contrast, no cells bound to uncoated wells or wells coated with bovine serum albumin (BSA) or LRR(InIA), a recombinant protein containing the LRR region of InIA (Lecuit *et al.*, 1997). Binding was concentration dependent and saturable and was totally inhibited with 10 mM EDTA, suggesting that divalent cations were required for interaction of InIB with Vero cells. Similar results were obtained with HEp-2 and HeLa cells, two cell lines permissive to InIB-mediated entry (Figure 1).

### InIB binds to a 33 kDa protein

In order to detect a putative InIB receptor, we used a ligand overlay assay. Solubilized Vero cell proteins separated by SDS-PAGE and transferred onto a nitrocellulose membrane were probed with purified InIB. Bound InIB was detected with the InIB-specific monoclonal antibody H15.1 (Braun *et al.*, 1999) and horseradish peroxidase (HRP)-



**Fig. 2.** InIB binding to electrophoretically separated soluble proteins from Vero cells by ligand overlay assay. Soluble proteins from Vero cells were separated on an 8% polyacrylamide gel and transferred onto a nitrocellulose membrane. Proteins on nitrocellulose were then probed with 50 µg/ml InIB and subsequently detected with the InIB-specific monoclonal antibody H15.1 (Braun *et al.*, 1999).

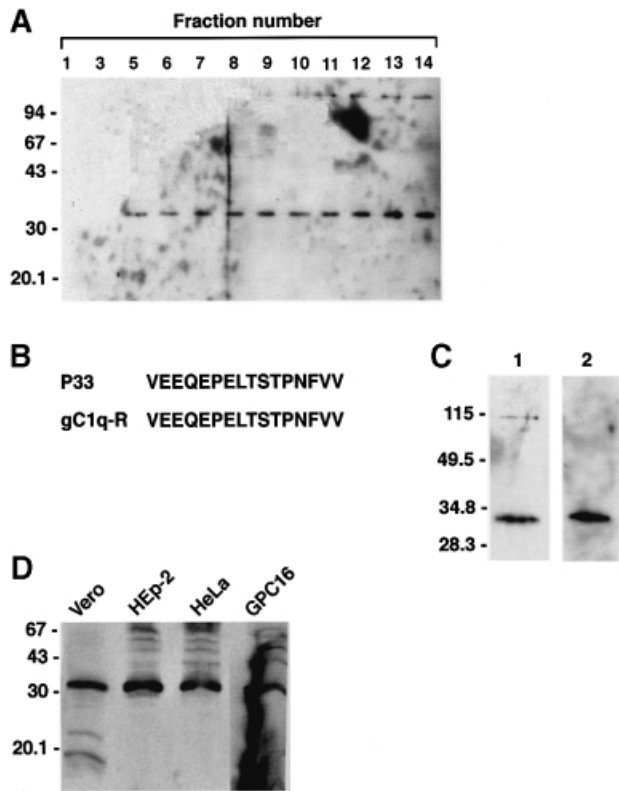
conjugated secondary antibody. InIB bound predominantly to a protein with an apparent mol. wt of ~33 kDa (Figure 2). We verified that H15.1 antibody did not cross-react with Vero components and that no binding was observed when the membrane was overlaid with LRR(InIA) (data not shown).

### gC1q-R binds to InIB

To identify the InIB receptor, we used a purification protocol based on preparation of affinity matrices of purified InIB (Isberg and Leong, 1990; Mengaud *et al.*, 1996a). Vero cell surface proteins were labeled using the membrane-impermeant biotinylation reagent NHS-LC-biotin. After labeling, detergent-soluble proteins were extracted with *n*-octyl glucoside, and loaded onto an InIB agarose column. After extensive washes, proteins bound to InIB were eluted with EDTA, separated by electrophoresis, transferred onto a nitrocellulose membrane and detected using streptavidin covalently coupled to HRP. This technique revealed a major biotinylated protein with an apparent mol. wt of 33 kDa (P33) (Figure 3A). As a control, we used a column of BSA and passed Vero cell extracts over it. No biotinylated protein was detected after EDTA elution. Moreover, when the flow-through of unbound proteins from the BSA column was loaded onto the InIB column, the same protein of 33 kDa was eluted, indicating that this protein interacts specifically with InIB (data not shown).

In order to identify the 33 kDa protein, the pooled EDTA eluate was loaded on a 12% polyacrylamide gel. The 33 kDa polypeptide was then subjected to internal amino acid sequencing. A 16 amino acid sequence was obtained. A search in the protein databanks showed that this sequence was identical to an internal sequence of a known protein, gC1q-R, the receptor for the globular heads of the complement component C1q, which has an apparent mol. wt of 33 kDa (Ghebrehiwet *et al.*, 1994) (Figure 3B). In Western blot experiments, the 33 kDa protein reacted specifically with a polyclonal antibody directed against gC1q-R (Figure 3C), indicating that this protein is antigenically related to gC1q-R. Taken together, these results demonstrate that InIB interacts with a cellular receptor named gC1q-R.

Since InIB-mediated entry takes place in a wide variety of cultured cell lines, and since Vero, HEp-2 and HeLa cells bind to InIB, it was essential to verify that gC1q-R

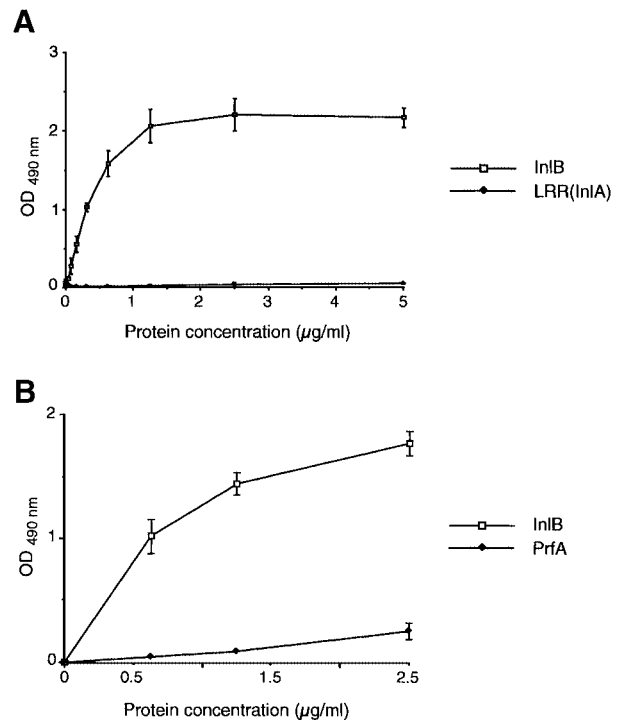


**Fig. 3.** gC1q-R is a ligand for InlB. (A) *N*-octyl glucoside extracts were prepared from surface biotin-labeled Vero cell extracts and loaded onto an InlB affinity column. After extensive washing, proteins were eluted with 10 mM EDTA, and 200  $\mu$ l fractions were collected. Fraction samples (10  $\mu$ l) were analyzed by SDS-PAGE, transferred onto nitrocellulose and probed with streptavidin coupled to peroxidase to detect biotinylated proteins by chemiluminescence. (B) Amino acid sequence comparison of internal sequences from P33 isolated on the displayed column and gC1q-R encoded by human lymphocytes (Ghebrehiwet *et al.*, 1994). (C) Western blot analysis of an elution fraction (15  $\mu$ l) with streptavidin (1) or with a polyclonal antibody directed against gC1q-R (2). (D) Western blot analysis of the cellular expression of gC1q-R. A 100  $\mu$ g aliquot of solubilized Vero, HEP-2, HeLa or GPC16 cell membrane proteins was separated by SDS-PAGE and transferred onto nitrocellulose. gC1q-R was revealed with an anti-gC1q-R polyclonal antibody.

was expressed in these cells. Membrane protein extracts of Vero, HEP-2 and HeLa cells were prepared and analyzed by Western blotting using an anti-gC1q-R polyclonal antibody. For the three cell lines, the antibody recognized the same protein with an approximate mol. wt of 33 kDa (Figure 3D). These results indicate that gC1q-R is expressed in the InlB-permissive Vero, HEP-2 and HeLa cells, in agreement with the reported ubiquitous expression of this protein (Ghebrehiwet *et al.*, 1994).

#### InlB interacts with purified gC1q-R

Interaction of InlB with gC1q-R was established definitively by an enzyme-linked immunosorbent assay (ELISA). Wells of microtiter plates were coated with recombinant gC1q-R and incubated with increasing concentrations of purified InlB or LRR(InlA) used as a control. Bound protein was detected using the InlB-specific monoclonal antibody B4.6 (Braun *et al.*, 1999) for InlB and the InlA-specific monoclonal antibody G6.1 (Mengaud *et al.*, 1996b) for LRR(InlA) (Figure 4A). InlB was able to bind to gC1q-R in a concentration-dependent and saturable



**Fig. 4.** InlB binds to gC1q-R. Wells of a microtiter plate were coated with a solution of 1  $\mu$ g/ml gC1q-R. After blocking with a 1% BSA solution, wells were incubated with increasing concentrations of purified proteins, either InlB, LRR(InlA) (A) or PrfA (B), and then analyzed by ELISA as described in Materials and methods.

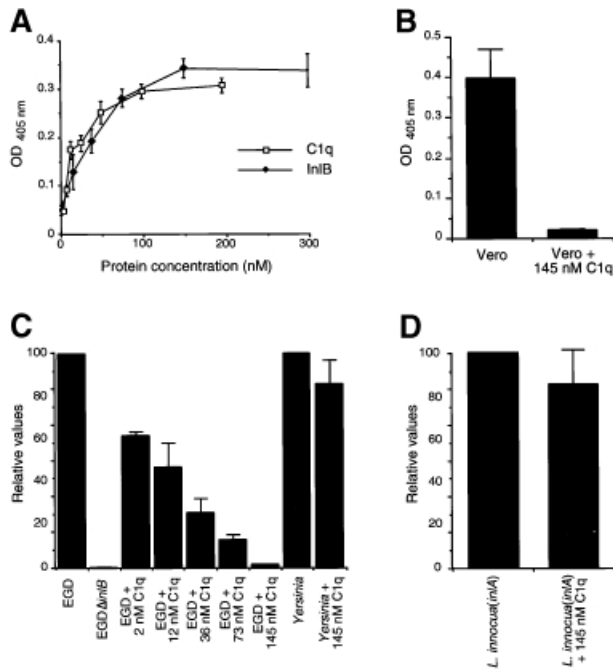
manner. In contrast, LRR(InlA) was unable to bind gC1q-R. To analyze further the specificity of the interaction between InlB and gC1q-R, gC1q-R-coated wells were incubated with different concentrations of PrfA, a cytoplasmic protein of *L.monocytogenes* with an isoelectric point close to that of InlB (9.1 versus 9.8, respectively). PrfA was unable to bind gC1q-R efficiently (Figure 4B). Taken together, these results indicate that the interaction between InlB and gC1q-R is direct and specific.

#### C1q competes with InlB for binding to gC1q-R and inhibits entry of *L.monocytogenes* into mammalian cells

To gain further insight into the relevance of gC1q-R in the InlB-mediated entry process, we used soluble C1q, a ligand of gC1q-R, as a potential competitive inhibitor. We first studied the ability of Vero cells to bind to C1q-coated wells using the hexosaminidase assay (Figure 5A). Vero cells were able to bind to wells coated with C1q in a saturable and C1q concentration-dependent manner, as was observed with InlB-coated wells.

Pre-treatment of a Vero cell suspension for 5 min at 37°C with 145 nM soluble C1q, a concentration determined to be maximal, resulted in a 95% reduction of Vero cell binding to InlB (Figure 5B). Similar results were obtained with HEP-2 and HeLa cells (data not shown), suggesting that InlB and C1q interact with the same sites, or sites located close by in gC1q-R.

We then studied the effect of C1q on entry of *L.monocytogenes* strain EGD into Vero cells. Pre-treatment of Vero cells with different concentrations of C1q for 5 min at 37°C prior to infection inhibited entry of EGD

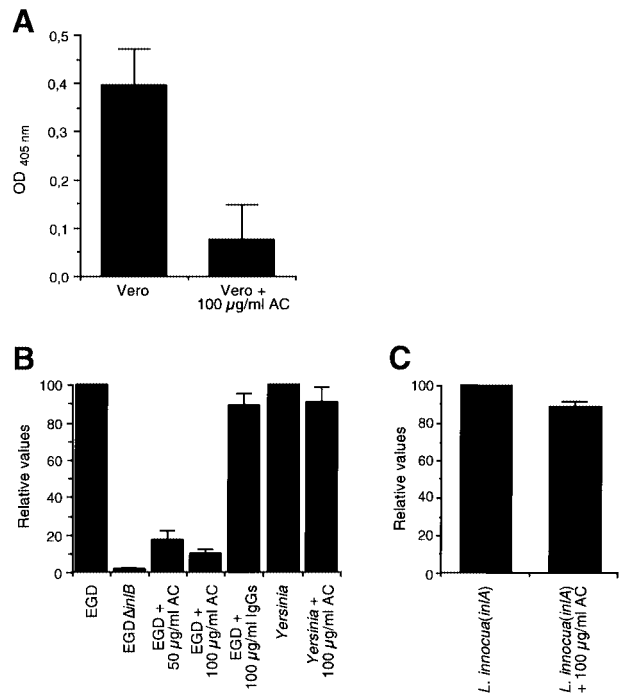


**Fig. 5.** C1q inhibits entry of EGD into Vero cells. (A) Comparison of the binding of Vero cells to wells coated with increasing concentrations of InIB or C1q using the colorimetric hexosaminidase assay. (B) Effect of C1q on the binding of Vero cells to InIB. Microtiter wells coated with a 10  $\mu\text{g/ml}$  concentration of InIB were incubated with a Vero cell suspension that had been treated or not for 5 min at 37°C with 145 nM C1q. After allowing 1 h for attachment of the Vero cells to immobilized InIB, wells were washed, and cell attachment was quantified using the colorimetric hexosaminidase assay. (C and D) Effect of C1q on entry of *L.monocytogenes* EGD and *Yersinia* into Vero cells (C) or on entry of *L.innocua*(inA) into Caco-2 cells (D). The inhibitory effect of C1q was tested by incubating the cells with different concentrations of C1q, 5 min before adding the bacteria. The number of intracellular bacteria was determined using gentamicin survival assays as described in Materials and methods. Values along the vertical axis are given relative to efficiencies of invasion of *L.monocytogenes* EGD in Vero cells or *L.innocua*(inA) in Caco-2 cells (D), which are fixed arbitrarily at 100.

(Figure 5C). The inhibition was concentration dependent and maximal at 145 nM (98% inhibition). At this concentration, C1q has no effect on entry of a *Yersinia pseudotuberculosis* strain YPIIIc cured of its virulence plasmid and which is internalized due to the interaction between invasin and its cellular receptor of the integrin  $\beta_1$  family (Isberg and Leong, 1990). The same results were obtained with HEp-2 and HeLa cells (data not shown). In contrast, pre-treatment of Caco-2 cells, which express gC1q-R (data not shown) and in which entry is mostly InIA dependent, with 145 nM C1q had no inhibitory effect on entry of *Listeria innocua*(inA) (Figure 5D), indicating that C1q inhibition of *Listeria* entry is specific for the InIB-mediated entry. Taken together, these results indicate that the binding of InIB to gC1q-R is required for InIB-mediated entry.

#### **Antibodies directed against gC1q-R inhibit entry of *L.monocytogenes* into cells**

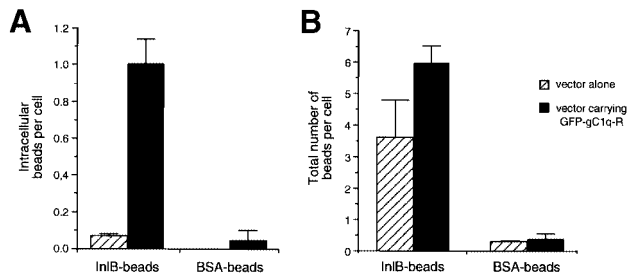
To investigate further the role of gC1q-R in bacterial internalization, we examined the ability of a polyclonal antibody directed against the 18 amino acid N-terminal peptide of gC1q-R to inhibit the InIB-mediated entry of *L.monocytogenes* EGD. We first analyzed whether binding



**Fig. 6.** Anti-gC1q-R antibodies inhibit entry of EGD into Vero cells. (A) Effect of anti-gC1q-R antibodies on the binding of Vero cells to InIB. Microtiter wells coated with a 10  $\mu\text{g/ml}$  concentration of InIB were incubated with a Vero cell suspension that had been treated or not with 100  $\mu\text{g/ml}$  antibodies (AC) for 60 min at 37°C. Cell attachment to InIB was quantitated using the colorimetric hexosaminidase assay. (B and C) Effect of anti-gC1q-R antibodies on entry of EGD and *Yersinia* into Vero cells (B) or *L.innocua*(inA) into Caco-2 cells (C). Vero or Caco-2 cells were incubated with anti-gC1q-R antibodies or pre-immune rabbit IgGs for 60 min before adding the bacteria. The inhibitory effect of the antibodies was tested by incubating the cells with the antibodies for 60 min before adding the bacteria. The number of intracellular bacteria was determined using gentamicin survival assays as described in Materials and methods. Values along the vertical axis are given relative to efficiencies of invasion of EGD in Vero cells or *L.innocua*(inA) in Caco-2 cells (C), which are the reference (value of 100).

of Vero cells to InIB was gC1q-R dependent by examining whether anti-gC1q-R antibodies had inhibitory effects using the microtiter plate binding assay. Pre-treatment of a Vero cell suspension for 60 min at 37°C with 100  $\mu\text{g/ml}$  antibodies resulted in an 81% reduction of Vero cell binding (Figure 6A), indicating that the binding to gC1q-R is required for Vero cell attachment to InIB.

In a second step, we determined whether these antibodies that reduce attachment to InIB could also interfere with InIB-mediated entry. Incubation of Vero cells with anti-gC1q-R antibodies reduced entry of wild-type *L.monocytogenes* (Figure 6B). Inhibition of entry was specific since it was observed neither with the *Y.pseudotuberculosis* strain YPIIIc nor when Vero cells were pre-treated with rabbit IgGs used at the same concentration (Figure 6B). Similar results were obtained with HEp-2 and HeLa cells (data not shown). This inhibition was specific for the InIB-mediated pathway of entry since pre-treatment of Caco-2 cells with these antibodies had no effect on entry of *L.innocua*(inA) (Figure 6C). These results strongly suggest that gC1q-R is acting as a receptor for the InIB protein of the *L.monocytogenes* strain EGD.



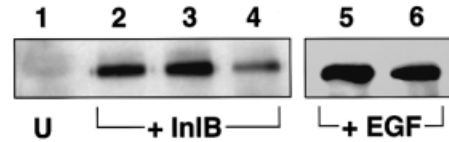
**Fig. 7.** InlB-dependent entry into gC1q-R-transfected GPC16 cells. GPC16 cells were transiently transfected with a control plasmid (hatched bars) or with a plasmid carrying a GFP-gC1q-R fusion protein (black bars) at a multiplicity of ~100 beads per cell for 1 h. Internalized (A) and associated (B) beads were identified and quantified using immunofluorescence as described previously (Braun *et al.*, 1998). The efficiency of entry is expressed as the average number of intracellular beads per cell. Association corresponds to the number of beads either bound or internalized. The results are expressed as the mean  $\pm$  SD of three independent experiments.

### Adhesion and entry of InlB-coated beads into gC1q-R-transfected GPC16 cells

GPC16 cells are guinea-pig cells of epithelial origin, which exhibit a high level of InlA-dependent entry but no significant InlB-dependent entry (Lecuit *et al.*, 1999; M.Lecuit and P.Cossart, unpublished results). In order to determine whether the absence of InlB-dependent entry was due to the absence of gC1q-R, we analyzed the presence of gC1q-R in membrane protein extracts of GPC16 cells by Western blotting using anti-gC1q-R polyclonal antibodies. A cross-reacting protein was present in these extracts, in amounts similar to those found in Vero, HEP-2 or HeLa cells (Figure 3D), suggesting that gC1q-R is not accessible or is not in the proper conformation for InlB-mediated entry or that the residues critical for interaction with InlB are absent or that other factors are required. To investigate whether expression of human gC1q-R in GPC16 cells would affect adhesion and entry of InlB-coated beads, we transfected GPC16 cells with a plasmid expressing a green fluorescent protein (GFP)-tagged human gC1q-R. The GFP intrinsic fluorescence allows direct visualization of transfected cells. Transfected cells were tested for their ability to promote adhesion and entry of InlB-coated beads. Results reported in Figure 7A provide evidence that transfected GPC16 cells promote entry of InlB-coated beads. Entry was 14 times more efficient in transfected cells than in cells transfected with the vector alone. Interestingly, adhesion of InlB-coated beads was not significantly affected in cells expressing the GFP-gC1q-R fusion protein ( $p = 0.122$ ) (Figure 7B). This effect of GFP-gC1q-R fusion protein on entry seemed to be InlB specific since it was not observed with BSA-coated beads (Figure 7A and B). Taken together, the transfection experiments indicate that human gC1q-R plays a critical role in the internalization step.

### Antibodies against gC1q-R reduce association of p85 with tyrosine-phosphorylated proteins

Entry of *L.monocytogenes* into Vero cells activates the p85/p110 PI 3-kinase and tyrosine phosphorylation of three mammalian adaptor proteins, Gab1, Cbl and Shc (Ireton *et al.*, 1999). InlB plays a direct role in this process since purified soluble InlB is able to activate this kinase (Ireton *et al.*, 1999). In order to determine whether gC1q-R



**Fig. 8.** Anti-gC1q-R antibodies decrease the InlB-dependent association of p85 with tyrosine-phosphorylated proteins. Vero cells were pre-treated for 1 h with DMEM (lanes 1, 2 and 5), with 100  $\mu$ g/ml rabbit IgGs (lane 3) or with 100  $\mu$ g/ml anti-gC1q-R antibodies (lanes 4 and 6). Cells were then left untreated (U) (lane 1), or treated with 3 nM InlB (lanes 2–4) or 17 nM EGF for 1 min (lanes 5 and 6). The cells were then washed with PBS and lysed in the immunoprecipitation buffer. Tyrosine-phosphorylated proteins were immunoprecipitated with anti-P-tyr antibodies, separated by SDS-PAGE, and p85 was detected by immunoblotting with anti-p85 antibodies. The anti-gC1q-R antibodies decrease the InlB-induced association of p85 with tyrosine-phosphorylated proteins (lane 4), but had no effect on EGF-induced association of p85 with tyrosine-phosphorylated proteins (lane 6). Similar amounts of material were used for each immunoprecipitation. The results are representative of three experiments.

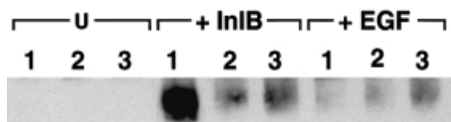
was implicated in the PI 3-kinase activation pathway, we analyzed the effect of anti-gC1q-R antibodies on the InlB-dependent stimulation of association of p85 with tyrosine-phosphorylated proteins in Vero cells. Rabbit IgGs were used as a control. As shown in Figure 8, the amount of p85 decreased when cells were pre-treated with the antibodies (compare lanes 2 and 4). In contrast, rabbit IgGs used at the same concentration had no effect (lane 3). Moreover, anti-gC1q-R antibodies had no significant effect on the association of p85 in anti-phosphotyrosine immunoprecipitates prepared from epidermal growth factor (EGF)-treated cells (compare lanes 5 and 6). These results suggest that recruitment of p85 involves an interaction between InlB and gC1q-R.

### Interaction of gC1q-R with the adaptor protein Gab1

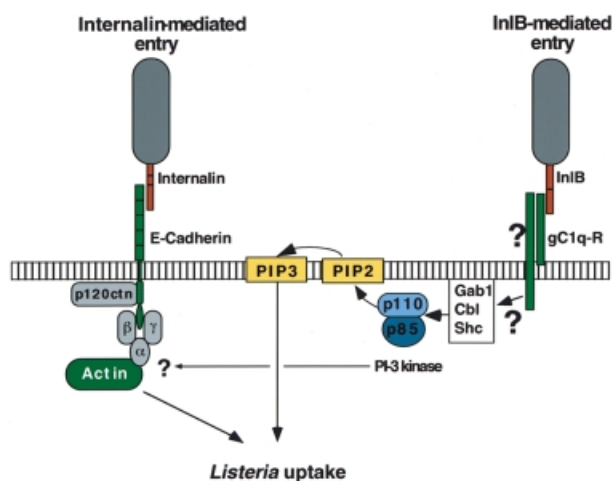
The adaptor protein Gab1 was shown to be tyrosine phosphorylated and to co-precipitate with p85 in response to InlB in Vero cells (Ireton *et al.*, 1999). In order to examine the possible association of gC1q-R with Gab1, we investigated the ability of a recombinant His<sub>6</sub>-gC1q-R protein to associate with Gab1. Extracts from Vero cells treated with InlB or EGF used as a control were incubated with His<sub>6</sub>-gC1q-R and protein complexes were precipitated with agarose-nickel beads. The presence of tyrosine-phosphorylated Gab1 in the precipitates was then analyzed by 're-immunoprecipitation' experiments with anti-Gab1 antibodies as described previously (Ireton *et al.*, 1999). As shown in Figure 9, tyrosine-phosphorylated Gab1 was able to co-precipitate with gC1q-R upon stimulation with InlB but not upon stimulation with EGF. Tyrosine-phosphorylated Gab1 was barely detectable when cell lysates were incubated without recombinant protein or with LRR(InlA)-His<sub>6</sub>, used as a control (Figure 9). These results suggest that there is a significant gC1q-R-Gab1 association in Vero cells stimulated with InlB.

### Discussion

This study is the first report describing the identification of an InlB receptor. After E-cadherin, the receptor for internalin, gC1q-R is the second identified cellular protein



**Fig. 9.** Association of gC1q-R with tyrosine-phosphorylated Gab1. Vero cells were left untreated (U), or were treated with 3 nM InIB or 17 nM EGF for 1 min. Solubilized lysates were then prepared by addition of immunoprecipitation buffer, and 2.5 mg of lysate were incubated for 2 h at 4°C in the presence of 5 µg His<sub>6</sub>-gC1q-R (1), 5 µg LRR(InIA)-His<sub>6</sub> (2) or buffer alone (3). After addition of Ni-NTA-agarose beads, the adsorbed proteins were dissociated by boiling in the presence of 0.5% SDS/5 mM dithiothreitol, diluted in re-immunoprecipitation buffer and re-precipitated with antibodies against Gab1. Tyrosine-phosphorylated proteins in the final immunoprecipitates were detected with anti-P-Tyr antibodies. Similar amounts of starting material were used for each re-precipitation. The results are representative of two experiments.



**Fig. 10.** The two pathways of entry of *L.monocytogenes* into mammalian cells.

used by *L.monocytogenes* for entry into cells (Figure 10). This 33 kDa protein (also called p32) is a highly acidic and ubiquitous cell membrane protein that binds to the globular heads of C1q, the first component of complement present in the serum. Although gC1q-R was first identified as a protein displaying affinity for the globular 'heads' of C1q, it is also able, as shown recently, to bind multiple ligands including thrombin, vitronectin, high molecular weight kininogen and the HIV-1 Tat transactivator (Fridell *et al.*, 1995; Yu *et al.*, 1995; Herwald *et al.*, 1996; Joseph *et al.*, 1996; Lim *et al.*, 1996). InIB represents a new 'ligand' for gC1q-R. We have demonstrated that gC1q-R binds to InIB in a divalent cation-dependent manner. However, a requirement for divalent cations is not a general feature of the binding of gC1q-R to its various ligands. Indeed, binding of C1q to gC1q-R is not metal ion dependent (Ghebrehwet *et al.*, 1994) while the binding of high molecular weight kininogen to gC1q-R on endothelial cells is strictly zinc dependent (Joseph *et al.*, 1996). A requirement for divalent cations seems to be a common theme in bacterial induced phagocytosis. Receptors for the *Y.pseudotuberculosis* invasin, i.e.  $\beta_1$  integrins, and for the *L.monocytogenes* internalin, E-cadherin, also require divalent cations.  $\beta_1$  integrins and E-cadherins are members of large families and are found on a variety of cell types. gC1q-R is not a member of a large family but is expressed on a wide range of cell types including

lymphocytes, neutrophils, hepatocytes and endothelial cells (Ghebrehwet *et al.*, 1994; Eggleton *et al.*, 1995; Peerschke *et al.*, 1996). No cell line devoid of gC1q-R has yet been reported. This ubiquitous distribution correlates well with the role of InIB for entry of *L.monocytogenes* into a wide range of cell types.

Binding to gC1q-R appears to be critical for InIB-dependent entry into mammalian cells because both C1q, a ligand of gC1q-R, and a polyclonal anti-gC1q-R antibody were able to inhibit attachment of mammalian cells to purified InIB. In line with these results, the wild-type *L.monocytogenes* strain EGD was unable to enter Vero, HEp-2 or HeLa cells pre-treated with C1q or anti-gC1q-R antibody. These data are consistent with a direct interaction between InIB and gC1q-R during entry, and illustrate that a first requirement for entry is bacterial attachment to gC1q-R. That InIB-coated beads enter into GPC16 cells transfected with a GFP-gC1q-R fusion protein definitively establishes the critical role of gC1q-R in the entry process. Why the endogenous gC1q-R of the GPC16 cells was unable to promote entry remains unclear. It is possible that InIB could interact with other surface structures in addition to gC1q-R and that these other factors could be required for entry. The hypothesis of an additional receptor is highly probable in view of the gC1q-R amino acid sequence itself. Indeed, gC1q-R is devoid of a typical hydrophobic transmembrane-spanning region or of a consensus site for glycosylphosphatidylinositol anchoring. The mode of attachment of gC1q-R to the surface membrane is unclear (Ghebrehwet *et al.*, 1994). The crystal structure of human gC1q-R has recently been determined (Jiang *et al.*, 1999) but did not shed light on the understanding of cell surface attachment. In addition, gC1q-R localizes not only to the cell surface (Eggleton *et al.*, 1995) but also to intracellular compartments (Dedio *et al.*, 1998). However, the functional role of cytosolic gC1q-R, if any, remains to be determined. It has been proposed that gC1q-R would be tethered to the cell surface by as yet unknown intrinsic membrane proteins. These docking proteins, by interacting with gC1q-R, could also be implicated in InIB-dependent entry and could also associate with InIB. The possibility of multiple protein-protein interactions is well served by the structure of the InIB LRR domain, whose crystal structure has been determined recently (Marino *et al.*, 1999). Its elongated shape could provide an extended surface for protein-protein interactions. Identification of an eventual InIB co-receptor is currently under investigation.

The regions of gC1q-R and that of InIB involved in the gC1q-R-InIB interaction are unknown. The highly acidic N-terminal part of gC1q-R has been shown to contain a binding site for C1q (Ghebrehwet *et al.*, 1994) and to mediate the binding of gC1q-R to the heparin-binding forms of vitronectin (Lim *et al.*, 1996). It probably also contains a binding site for InIB since C1q or anti-gC1q-R antibodies directed against this region inhibit InIB-dependent entry into mammalian cells.

Activation of PI 3-kinase and tyrosine phosphorylation of the adaptor proteins Gab1, Cbl and Shc are among the early signal transduction events that take place during InIB-mediated entry of *L.monocytogenes* (Ireton *et al.*, 1996, 1999). In this work, we show that anti-gC1q-R antibody decreases association of PI 3-kinase with

tyrosine-phosphorylated proteins induced by InIB and that gC1q-R is associated with tyrosine-phosphorylated Gab1. Whether this association is direct or not remains to be determined. These findings favor the hypothesis for a role of gC1q-R in the signaling pathway leading to activation of PI 3-kinase. Signal transduction events associated with gC1q-R responses including inositol triphosphate production on platelets (Peerschke *et al.*, 1993), chemotaxis on fibroblasts (Oiki and Okada, 1988) or Fc-mediated phagocytosis by neutrophils (Bobak *et al.*, 1987) are poorly understood. It will be important to analyze PI 3-kinase activation during these events.

The identification of gC1q-R as an InIB receptor provides an important insight into understanding the different steps leading to *L. monocytogenes* uptake by mammalian cells. Whether other receptors in addition to gC1q-R are required for InIB-mediated entry and, if any, how these molecules are involved in the infectious process of *L. monocytogenes* remain challenging issues.

## Materials and methods

### Bacterial strains and cell lines

*Listeria* strains were cultured in brain heart infusion broth or plates at 37°C. For invasion assays, we used the wild-type *L. monocytogenes* strain EGD (BUG 600) and its  $\Delta$ inIB isogenic mutant (BUG 947), and a recombinant *L. innocua* strain expressing internalin (BUG 991) and its isogenic counterpart harboring the plasmid vector alone (BUG 994) (Dramsi *et al.*, 1995). *Yersinia pseudotuberculosis* strain YPIIIC was grown in Luria-Bertani broth.

The African green monkey kidney cell line Vero, the human laryngeal epithelial cell line HEP-2, the human cervical epithelial cell line HeLa and the human enterocyte-like cell line Caco-2 were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL) supplemented with 10% fetal calf serum (FCS; Valbiotech), 2 mM L-glutamine (Sigma) and 1% non-essential amino acids (Gibco-BRL) at 37°C in 10% CO<sub>2</sub>. The guinea-pig epithelial cell line GPC16 (ATCC CCL-242) was cultured in minimal essential medium (MEM) with non-essential amino acids supplemented with 10% FCS, 2 mM L-glutamine (Sigma) and 1 mM sodium pyruvate (Gibco-BRL).

### Antibodies and purified proteins

Polyclonal antiserum against rat p85 $\alpha$  was purchased from Upstate Biotechnology. Rabbit pre-immune IgG was from Sigma. Monoclonal anti-Tyr(P) antibodies (clone RC20) coupled to peroxidase (E120H) were from Transduction Laboratories. Streptavidin covalently linked to HRP was purchased from Bioss. Monoclonal antibodies against phosphotyrosine (clone 4G10) used for immunoprecipitation were purified using Affi-Prep protein A support from Bio-Rad following the manufacturer's instructions. Polyclonal rabbit antiserum against gC1q-R was raised as described below. The InIA-specific monoclonal antibodies I4.4 and G6.1 and the InIB-specific monoclonal antibody H15.1 have been described previously (Mengaud *et al.*, 1996b; Braun *et al.*, 1999). Recombinant InIB, internalin LRRs [LRR(InIA)] and PrfA were purified as described previously (Sheehan *et al.*, 1996; Braun *et al.*, 1997; Lecuit *et al.*, 1997). Recombinant gC1q-R was purified as described below. Recombinant human EGF was from Upstate Biotechnology. BSA and complement component C1q were purchased from Sigma.

### Purification of gC1q-R

A 648 bp PCR fragment encoding the mature form of human gC1q-R (amino acids 74–282) was produced using pBluescript containing the gC1q-R cDNA insert (Ghebrehwet *et al.*, 1994) as a template and the primers 5'-(CTA)GCTAGC.CTGCACCGACGGAGAC-3' and 5'-(CGC)GGATCC.TTTCAGCATCTGTCTGCTCTA-3' with Vent DNA polymerase (Biolabs). The purified amplified fragment was cloned in-frame downstream from the His tag sequence, between the *NheI* and *BamHI* sites of the pET28a expression vector (Novagen), yielding plasmid pET28a-5. The nucleotide sequence of the amplified fragment was verified by sequencing. For expression of the recombinant protein, pET28a-5 was transformed into *Escherichia coli* strain BL21(DE3). The

recombinant protein was purified as described previously (Braun *et al.*, 1997) except that, after the elution step with imidazole, purified recombinant protein was dialyzed against 20 mM Tris pH 8.5, 0.15 M NaCl and loaded onto a HiTrap Q column. The protein was eluted by a continuous salt gradient from 0.15 to 1 M NaCl. Pure fractions were concentrated to the desired volume using Centriprep 10 devices (Amicon), and then stored at –20°C.

### Cell-binding assays

Binding of InIB-permissive cells to InIB was determined by the hexosaminidase assay as described previously (Landegren, 1984; Mengaud *et al.*, 1996a).

To determine the effect of C1q, 145 nM protein was added to the cell suspension for 5 min at 37°C before and during the cell-binding assay. The effect of anti-gC1q-R antibodies was tested by adding 100  $\mu$ g/ml antibodies or 100  $\mu$ g/ml rabbit IgG used as a control to the cell suspension for 60 min at 37°C before and during the cell-binding assay.

### Ligand overlay assays

Vero cell components recognized by InIB were detected using a ligand overlay of Western blotted proteins. Approximately  $8 \times 10^5$  Vero cells were seeded in 75 cm<sup>2</sup> tissue culture flasks and grown for 48 h. Cells were washed three times with 10 ml of phosphate-buffered saline (PBS), and then scraped into 1.5 ml of PBS, spun down (60 s at 4000 g) and lysed in 0.2 ml of lysis solution [1% NP-40, 50 mM Tris pH 7.5, 150 mM NaCl, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1  $\mu$ g/ml pepstatin, 2.5  $\mu$ g/ml leupeptin, 2  $\mu$ g/ml aprotinin]. The suspension was incubated for 15 min on ice and spun (5 min at 15 000 g). The supernatant was mixed with Laemmli sample buffer and boiled for 5 min. Solubilized proteins were separated by SDS-PAGE on a 10% polyacrylamide gel, electroblotted onto nitrocellulose membrane and then blocked overnight at room temperature in TBS-T (50 mM Tris pH 7.5, 100 mM NaCl, 0.1% Tween-20) containing 2% BSA, 3% milk and 1% ovalbumin. The nitrocellulose was incubated overnight at 4°C with the purified proteins [50  $\mu$ g/ml InIB or LRR(InIA)] diluted in blocking solution. Following extensive washes, the membrane was incubated with primary antibody, followed by washing, and a second incubation with HRP conjugate. Bound proteins were subsequently detected using the chemiluminescent ECL detection kit (Amersham).

### Preparation of cell membrane proteins from Vero, HEP-2 and HeLa cells

Approximately  $1 \times 10^7$  cells were washed three times and resuspended in 1 ml of 5 mM phosphate buffer pH 7.5 containing 0.5 mM EDTA, 150 mM NaCl and 0.5 mM PMSF. Cell membranes were prepared by freeze-thawing (five times) at –80°C and centrifugation at 30 000 g for 1 h at 4°C. The pelleted membranes were then solubilized by suspension in 0.5 ml of the above buffer containing 1% NP-40 and stirred for 20 h at 4°C. The solubilized membrane proteins were freed from insoluble material by centrifugation at 30 000 g for 60 min at 4°C. After dialysis against 5 mM phosphate buffer pH 7.5 containing 0.5 mM EDTA, 20 mM NaCl, 0.5 mM PMSF and 0.1% NP-40, 100  $\mu$ g of the dissolved membrane proteins were applied to a 12% polyacrylamide gel.

### Purification of the InIB receptor

InIB or BSA was covalently coupled to Affi-gel 10 (Bio-Rad) in 50 mM HEPES pH 7.5, following the manufacturer's instructions. A total of  $5 \times 10^8$ – $5 \times 10^9$  Vero cells were grown to 90% confluence. Biotinylation, extraction of surface proteins and affinity chromatography of the InIB receptor(s) were performed as described (Isberg and Leong, 1990; Mengaud *et al.*, 1996a). Proteins eluted with EDTA were analyzed by SDS-PAGE, transferred to nitrocellulose membrane and detected with streptavidin covalently coupled to HRP and the chemiluminescent ECL detection kit (Amersham).

### Protein sequencing

EDTA-eluted proteins were pooled and loaded on a 10% polyacrylamide gel. Protein bands were visualized by Amido black staining of the gel. The 33 kDa band present on the gel was cut out and subjected to internal sequencing using an Applied Biosystems 473A sequencer.

### ELISA

A standard ELISA was performed using microtiter plates (MaxiSorb, Nunc) coated overnight at 4°C with 50  $\mu$ g/well of 1  $\mu$ g/ml gC1q-R diluted in 50 mM carbonate buffer pH 9.5. After blocking the unreacted sites with 1% BSA in TBS (20 mM Tris-HCl pH 7.5, 150 mM NaCl), the plates were incubated with 50  $\mu$ l of various concentrations of either



InIB, LRR(InIA) or PrfA in TBS containing 0.1% BSA. Bound InIB and LRR(InIA) proteins were detected using the monoclonal antibodies B4.6 and G6.1, respectively (Mengaud *et al.*, 1996b; Braun *et al.*, 1999), while PrfA protein was detected using an anti-PrfA polyclonal antibody (Sheehan *et al.*, 1996). Antibodies were detected using HRP-conjugated anti-mouse or anti-rabbit antibody and the chromogenic substrate 1,2-phenylenediamine dihydrochloride (Dako). The absorbance of the resulting color development was measured at 490 nm. Washes between all reactions were carried out three times in TBS containing 0.05% Tween-20.

#### Production of anti-gC1q-R antibodies

Antibodies to an 18 amino acid synthetic peptide (TDGDKAFV-DFLSDEIKKEE), spanning residues 76–93 of gC1q-R, were generated by injection of keyhole limpet hemocyanin (KLH)-conjugated peptide following the immunization protocol described previously (Friederich *et al.*, 1995). Antibodies were affinity purified as described (Friederich *et al.*, 1995).

#### Invasion assays

Invasion assays in Vero, HEp-2, HeLa and Caco-2 cells were performed in 24-well plates using the gentamicin survival assay as described previously (Braun *et al.*, 1998). Invasion assays were carried out in three independent experiments, each time in duplicate.

To test the ability of anti-gC1q-R antibodies to inhibit entry of the *L.monocytogenes* strain EGD, antibodies were added to cells in 0.5 ml of DMEM in 24-well tissue culture dishes for 1 h before and during the cell infection step. To test the ability of C1q to inhibit entry of the *L.monocytogenes* strain EGD, different concentrations of purified C1q were added to cells in 0.5 ml of DMEM in 24-well tissue culture dishes for 5 min before and during the cell infection step. At the concentrations used, anti-gC1q-R antibodies and C1q had no effect on the viability of cells, as determined by staining with Trypan blue.

#### Transient transfection experiments, immunofluorescence labeling and quantification of entry of InIB-coated beads

gC1q-R was fused to the C-terminus of EGFP by cloning human gC1q-R full-length cDNA at *Xho*I and *Bam*HI sites in pEGFP-C1 (Clontech), giving rise to pEGFP-gC1q-R. Expression of the fusion protein was verified by Western blotting experiments using the gC1q-R polyclonal antibody. pEGFP-gC1q-R was purified using the Nucleobond AX kit (Macherey-Nalge) and transfections were carried out using the Lipofectamine Plus Reagent (Gibco-BRL) with  $5 \times 10^4$  GPC16 cells, grown for 36 h on coverslips. At 24 h post-transfection, cells were incubated with  $2 \times 10^7$  InIB- or BSA-coated beads, prepared as described previously (Braun *et al.*, 1998), with centrifugation at 200 g for 1 min. Cells were then incubated at 37°C in 10% CO<sub>2</sub> for 1 h, washed in PBS and fixed with 3% paraformaldehyde in PBS. Adhesion and entry of beads coated with InIB or BSA were analyzed as described previously (Braun *et al.*, 1998). Briefly, extracellular beads were labeled with an anti-InIB polyclonal antibody or anti-rabbit BSA antiserum, followed by a second labeling step with fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit IgG antibodies. Cells were then permeabilized and intracellular beads were allowed to react with an anti-InIB polyclonal antibody or anti-rabbit BSA antiserum. Cells were then incubated with rhodamine-conjugated antibodies against rabbit IgGs, a step that labels both extra- and intracellular beads.

For enumeration of total and intracellular beads in transfected cells, four or five coverslips were observed. On each coverslip, 50 transfected cells were selected randomly by immunofluorescence. The number of total beads per transfected cell was evaluated under phase contrast observation and the number of intracellular beads by enumerating among these beads those that were rhodamine labeled.

#### Immunoprecipitation

Approximately  $1.5 \times 10^5$  Vero cells were seeded in 35 mm tissue culture dishes and grown for 40 h. Cells were then pre-treated with 100 µg/ml anti-gC1q-R antibodies for 1 h before the addition of 3 nM (200 ng/ml) of purified InIB or 17 nM (100 ng/ml) of EGF for 1 min. Cells were then washed with cold PBS, and incubated in lysis buffer [50 mM NaCl, 50 mM Tris pH 8, 1% NP40, 1 mM EDTA, 1 mM aminoethylbenzenesulfonyl fluoride (AEBSF), 3 mM sodium vanadate, and 5 µg/ml aprotinin, leupeptin, pepstatin and chymostatin]. Pre-clearance and immunoprecipitations with anti-phosphotyrosine antibody 4G10 were performed as described (Ireton *et al.*, 1996). After the pre-clearance step, protein concentrations were determined using the BCA system (Pierce) and equal quantities of total protein were used for immunoprecipitations.

Samples were subjected to electrophoresis (7.5% SDS-PAGE), and p85 was detected using immunoblotting with a polyclonal antibody to p85α.

#### Pull-down assay

Vero cells were starved for 5 h in serum-free DMEM and cell lysates were prepared from untreated, InIB- or EGF-treated cells in lysis buffer as described above. The extracts were clarified by centrifugation at 15 000 g for 15 min at 4°C. The supernatants were pre-cleared for 16 h at 4°C with Ni-NTA-agarose (Qiagen). Protein concentrations of the lysates were determined using the BCA system and equal quantities of total protein were incubated with His<sub>6</sub>-gC1q-R, LRR(InIA)-His<sub>6</sub> or buffer alone, followed by addition of Ni-NTA-agarose beads for 16 h. The beads were then washed and proteins retained on beads were dissociated by boiling for 2 min in the presence of buffer containing 50 mM Tris-HCl pH 7.5, 0.5% SDS and 5 mM dithiothreitol. 'Re-immunoprecipitation' experiments with antibodies against Gab1 were performed as described (Ireton *et al.*, 1999).

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