**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 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; Jonquières 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; Jonquières 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 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 β1 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).

**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

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InlB binds to a 33 kDa protein

In order to detect a putative InlB 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 InlB. Bound InlB was detected with the InlB-specific monoclonal antibody H15.1 (Braun et al., 1999) and horseradish peroxidase (HRP)-conjugated secondary antibody. InlB 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(InlA) (data not shown).

gC1q-R binds to InlB

To identify the InlB receptor, we used a purification protocol based on preparation of affinity matrices of purified InlB (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 InlB agarose column. After extensive washes, proteins bound to InlB 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 InlB column, the same protein of 33 kDa was eluted, indicating that this protein interacts specifically with InlB (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 digestion by trypsin and analyzed by mass spectrometry. The peptide sequence obtained 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.

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(InlA) (data not shown).
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**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 μl fractions were collected. Fraction samples (10 μl) 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 μl) 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 μg 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 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...
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 InlB-mediated entry of L. monocytogenes EGD. We first analyzed whether binding of Vero cells to InlB 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 with 100 μg/ml antibodies resulted in an 81% reduction of EGD entry (Figure 6B). Inhibition of entry was specific since it was observed neither with the L. innocua strain 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 InlB-mediated pathway of entry since pre-treatment of Caco-2 cells with these antibodies had no effect on entry of L. innocua (inlA) (Figure 6C). These results strongly suggest that gC1q-R is acting as a receptor for the InlB protein of the L. monocytogenes strain EGD.
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 (Ireten et al., 1999). InLB plays a direct role in this process since purified soluble InLB is able to activate this kinase (Ireten et al., 1999). In order to determine whether gC1q-R 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 (Ireten et al., 1999). In order to examine the possible association of gC1q-R with Gab1, we investigated the ability of a recombinant His6-gC1q-R protein to associate with Gab1. Extracts from Vero cells treated with InLB or EGF used as a control were incubated with His6–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 (Ireten 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)-His6 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
gC1q-R is a receptor for InlB of L. monocytogenes

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). InlB represents a new ‘ligand’ for gC1q-R. We have demonstrated that gC1q-R binds to InlB 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 (Ghebrehiwet 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. β1 integrins, and for the L. monocytogenes internalin, E-cadherin, also require divalent cations. β1 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 (Ghebrehiwet 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 InlB for entry of L. monocytogenes into a wide range of cell types.

Binding to gC1q-R appears to be critical for InlB-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 InlB. 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 InlB and gC1q-R during entry, and illustrate that a first requirement for entry is bacterial attachment to gC1q-R. That InlB-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 InlB 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 (Ghebrehiwet 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 InlB-dependent entry and could also associate with InlB. The possibility of multiple protein–protein interactions is well served by the structure of the InlB 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 InlB co-receptor is currently under investigation.

The regions of gC1q-R and that of InlB involved in the gC1q-R–InlB interaction are unknown. The highly acidic N-terminal part of gC1q-R has been shown to contain a binding site for C1q (Ghebrehiwet et al., 1994) and to mediate the binding of C1q-R to the heparin-binding forms of vitronectin (Lim et al., 1996). It probably also contains a binding site for InlB since C1q or anti-gC1q-R antibodies directed against this region inhibit InlB-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 InlB-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 InlB 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) and 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 InlB 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 ΔinlB isogenic mutant (BUG 947), and a recombinant *L.*monocaca strain expressing internal (BUG 991) and its isogenic counterpart harboring the plasmid vector alone (BUG 994) (Dramsi et al., 1995). *Yersinia pseudotuberculosis* strain PI1103 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% CO₂. 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 p85α was purchased from Upstate Biotechnology. Rabbit pre-immune IgG was from Sigma. Monoclonal anti-p85α antibodies (clone RC20) coupled to peroxidase (E120H) were from Transduction Laboratories. Streptavidin covalently linked to HRP was purchased from Biosys. Monoclonal antibodies against 5 mM phosphate buffer pH 7.5, 100 mM NaCl, 0.1% Tween-20) containing 2% BSA, 3% milk and 1% ovalbumin. The nickel-nucleosome was incubated overnight at 4°C with the purified proteins [50 μg/ml InlB or LRR(InlA)] 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 × 10⁸ 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 pellet 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% PMSF and 0.1% NP-40, 100 μg of the dissolved membrane proteins were applied to a 12% polyacrylamide gel.

**Purification of the InlB receptor**

InlB 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 × 10⁸–5 × 10⁹ Vero cells were grown to 90% confluence. By centrifugation, extraction of surface proteins and affinity chromatography of the InlB receptor(s) were performed as described (Isberg and Leong, 1990; Mauget 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 sequence. ELISA

A standard ELISA was performed using microtiter plates (MaxiSorb, Nunc) coated overnight at 4°C with 50 μg/well of 1 μg/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 μl of various concentrations of either

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InB, LKR(InlA) or PrfA in TBS containing 0.1% BSA. Bound InB and LKR(InlA) proteins were detected using the monoclonal antibodies B4.6 and G6.1, respectively (Mengaud et al., 1996b; Braun et al., 1999), while PrfA 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 (TTDGDKAFV-DFLSDEIKEE), 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).

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

gC1q-R was fused to the C-terminus of EGFP by cloning human gC1q-R full-length cDNA at Xhol and BamHI 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-Nagel) and transfections were carried out using the Lipofectamine Plus Reagent (Gibco-BRL) with 5 × 10⁶ GPC16 cells, grown for 36 h on coverslips. At 24 h post-transfection, cells were incubated with 2 × 10⁷ InlB- or BSA-coated beads, prepared as described previously (Braun et al., 1998), with 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 His6-gC1q-R, LKR(InlA)-His6, 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 diithiorethiol. 'Re-immunoprecipitation' experiments with antibodies against Gab1 were performed as described (Breton et al., 1999).

Pull-down assay

Vero cells were starved for 5 h in serum-free DMEM and cell lysates were prepared from untreated, InlB- 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 His6-gC1q-R, LKR(InlA)-His6, 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 diithiorethiol. 'Re-immunoprecipitation' experiments with antibodies against Gab1 were performed as described (Breton et al., 1999).

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