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GW domains of the *Listeria monocytogenes* invasion protein InlB are required for potentiation of Met activation

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Summary

The *Listeria monocytogenes* protein InlB promotes intracellular invasion by activating the receptor tyrosine kinase Met. Earlier studies have indicated that the LRR fragment of InlB is sufficient for Met activation, but we show that this is not the case unless the LRR fragment is artificially dimerized through a disulphide bond. In contrast, activation of Met proceeds through monomers of intact InlB and, at physiologically relevant concentrations, requires coordinated action *in cis* of both InlB N-terminal LRR region and C-terminal GW domains. The GW domains are shown to be crucial for potentiating Met activation and inducing intracellular invasion, with these effects depending on association between GW domains and glycosaminoglycans. Glycosaminoglycans do not alter the monomeric state of InlB, and are likely to enhance Met activation through a receptor-mediated mode, as opposed to the ligand-mediated mode observed for the LRR fragment. Surprisingly, we find that gC1q-R, a host protein implicated in InlB-mediated invasion, specifically antagonizes rather than enhances InlB signalling, and that interaction between InlB and gC1q-R is unnecessary for bacterial invasion. Lastly, we demonstrate that HGF, the endogenous ligand of Met, substitutes for InlB in promoting intracellular invasion, suggesting that no special properties are required of InlB in invasion besides its hormone-like mimicry of HGF.

Introduction

A number of intracellular bacterial pathogens invade cells of their host that are normally not phagocytic by modulating signalling pathways and inducing phagocytic behaviour in these cells. An example is the Gram-positive bacterium *Listeria monocytogenes*, which is a cause of meningitis, gastroenteritis, septicaemia and abortions in humans (Lorber, 1997; Aureli *et al*., 2000). This facultative intracellular pathogen invades a broad variety of cell types, including epithelial, endothelial, hepatocyte and fibroblast-like cell lines, through the action of the 67 kDa bacterial protein InlB (Cossart *et al*., 2003). InlB is an exogenous agonist of the receptor tyrosine kinase Met (also known as hepatocyte growth factor receptor, HGFR) (Shen *et al*., 2000), and elicits signalling events culminating in induced phagocytosis of the bacterium. Phagocytic uptake is an unusual outcome for Met stimulation, as the endogenous ligand of Met, hepatocyte growth factor (HGF), usually evokes only growth and motility responses.

As with other receptor tyrosine kinases, ligand binding to Met is thought to bring about its activation through receptor dimerization, or more generally, oligomerization (Schlessinger, 2000). Dimerization of Met leads to autophosphorylation of tyrosines in its cytoplasmic domain, some of which are responsible for turning on Met tyrosine kinase activity fully and others for acting as binding sites for signalling proteins (Maina *et al*., 1996; Trusolino and Comoglio, 2002). Recently, association of Met with $\alpha6\beta4$ integrin, a heterodimeric glycoprotein which binds the extracellular protein laminin, has been found to enhance signal transduction (Trusolino *et al*., 2001). This effect does not depend on extracellular ligand binding by $\alpha6\beta4$, but does depend on tyrosine phosphorylation of the cytoplasmic region of $\alpha6\beta4$ by Met. InlB signalling through Met elicits activation of a number of cytoplasmic signalling proteins, including phosphoinositide (PI) 3-kinase, Ras, MAPK, PLC-γ, Akt and NF-κB (Ireton *et al*., 1996; Bierne *et al*., 2000; Mansell *et al*., 2001; Copp *et al*., 2003). Activation of PI 3-kinase and consequent rearrangement of the actin cytoskeleton are crucial events in the induction of phagocytosis, as shown by inhibitor studies (Ireton *et al*., 1996). The importance of other proteins activated by InlB is not known, although it is possible that these may influence events following intracellular invasion.

InlB is a highly elongated molecule with several discrete structural domains (Fig. 1A) (Marino *et al*., 2002). The N-

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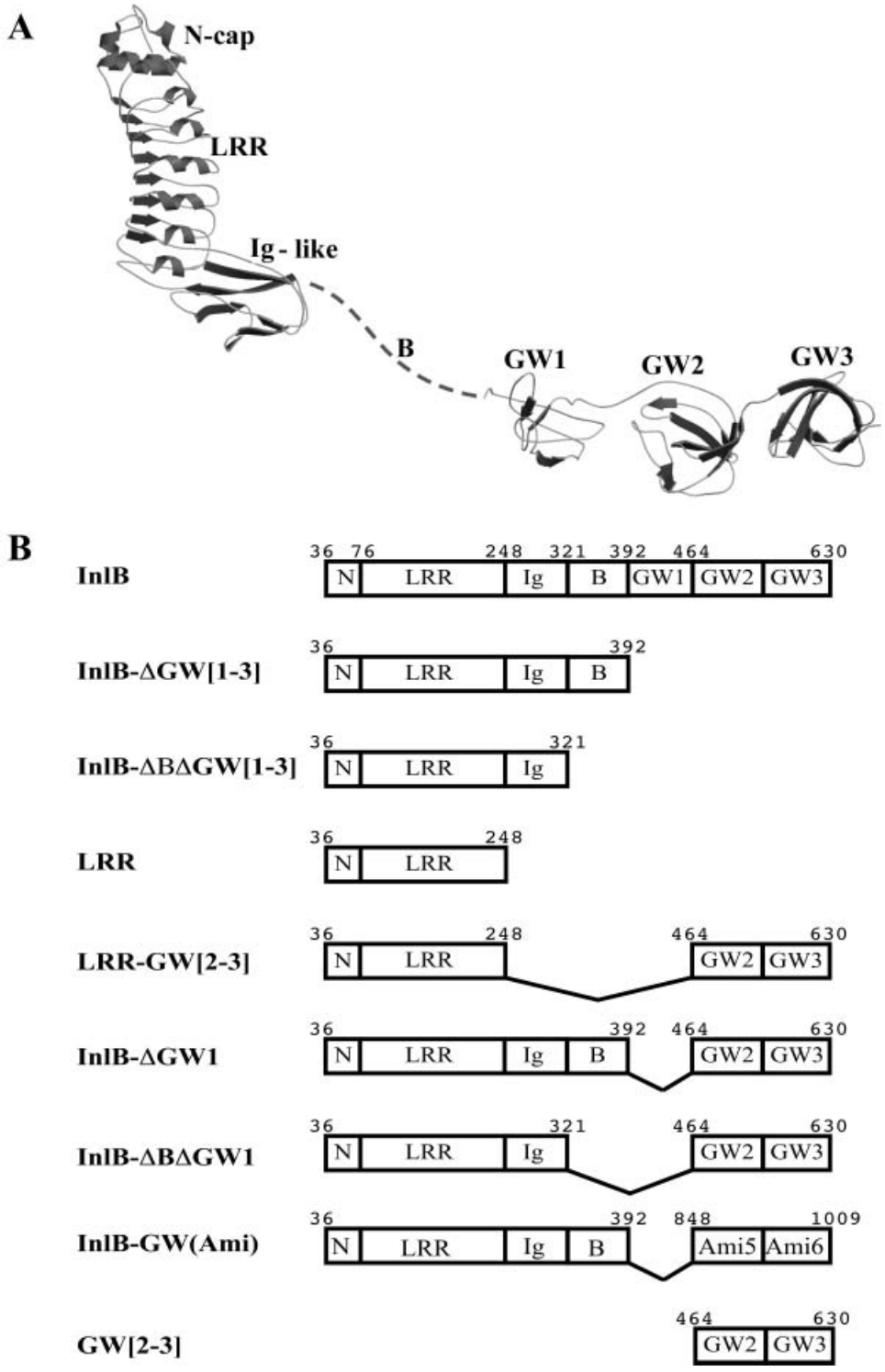


Fig. 1. InlB domains and deletion fragments.
A. Domains of InlB in ribbon representation**.** The N-terminal domain consists of N-cap, leucine rich repeat (LRR), and immunoglobulin (Ig)-like regions. The B-repeat domain connects the N-terminal domain to three C-terminal GW domains.
B. Deletion fragments of InlB used in this study. All protein constructs have C-terminal histidine tags, except for LRR, which has an N-terminal histidine tag.

terminal domain of InlB consists of three continuous structural regions, known as N-cap, leucine rich repeat (LRR), and immunoglobulin (Ig)-like (Schubert *et al*., 2001; Marino *et al*., 2002). The LRR fragment (24 kDa), composed of just the N-cap and LRR regions (Marino *et al*., 1999), is reported to be necessary and sufficient to bind and activate Met (Shen *et al*., 2000). The extent of Met activation evoked by this LRR fragment is less than that evoked by intact InlB, suggesting that other regions of the protein are also involved in activating Met. The LRR fragment is also reported to be necessary and sufficient to promote internalization of bacteria or latex beads into mammalian cells (Braun *et al*., 1999). These experiments suggest that the mode of Met activation by the LRR fragment is likely to be representative of the mode of Met activation by intact InlB. The N-terminal LRR region is followed by the B-repeat domain, which appears to be involved in mammalian cell signalling as well. The B-repeat is required for superactivation of the Ras-MAPK pathway by InlB (Copp *et al*., 2003). Although equal concentrations of InlB and HGF elicit equivalent levels of Met activation, InlB is observed to activate the Ras-MAPK pathway to a considerably greater extent than HGF. Deletion of the B-repeat domain abrogates superactivation. The B-repeat domain is flexible in the crystallographic structure of InlB and has not yet been characterized at atomic resolution (Marino *et al*., 2002).

InlB localizes both to the cell surface of *L. monocytogenes* and to the extracellular medium. InlB is partially retained on the bacterial cell wall through interaction between its C-terminal GW domains and lipoteichoic acid (LTA) (Jonquières *et al*., 1999). This interaction is noncovalent and dissociable, such that approximately half of InlB is released from the cell wall into soluble form (Jonquières *et al*., 1999). Once released, InlB is able to interact through its GW domains with two host components implicated in invasion, glycosaminoglycans and the protein gC1q-R (Braun *et al*., 2000; Jonquières *et al*., 2001; Marino *et al*., 2002). Glycosaminoglycans are known to be important to HGF signalling. They increase the efficiency of Met activation by HGF (Zioncheck *et al*., 1995; Lyon *et al*., 2002), and are critical for signalling by splice variants of HGF, the NK1 and NK2 fragments (Schwall *et al*., 1996; Sakata *et al*., 1997). Glycosaminoglycans have been shown to dimerize the NK1 fragment and to lead to oligomerization of HGF (Zioncheck *et al*., 1995; Schwall *et al*., 1996; Sakata *et al*., 1997; Chirgadze *et al*., 1999), although the functional importance of oligomerization has recently been challenged (Lyon *et al*., 2002; Gherardi *et al*., 2003). The precise role of glycosaminoglycans in InlB-mediated intracellular invasion is not yet clear (Jonquières *et al*., 2001). Similarly, the role of gC1q-R in InlB-mediated invasion is not understood and, indeed, the physiological role and subcellular localization of gC1q-R are in dispute (Peterson *et al*., 1997; Dedio *et al*., 1998; van Leeuwen and O'Hare, 2001). gC1q-R has been shown to bind a large number of substrates, including the globular heads of the complement protein C1q (gC1q), vitronectin, *Staphylococcus aureus* protein A, and hepatitis C virus core protein among others (Lim *et al*., 1996; Kittlesen *et al*., 2000; Nguyen *et al*., 2000).

To understand how InlB activates Met, we studied the biochemical and signalling properties of intact InlB and InlB deletion mutants. Surprisingly, we find that the mode of Met activation by the LRR fragment does not resemble the mode of Met activation by intact InlB. Our studies show that the LRR fragment is able to activate Met only when this fragment is artificially dimerized through a disulphide-bond that involves a fortuitously exposed cysteine in this truncated fragment. In striking contrast, this cysteine residue is not involved in Met activation by intact InlB, and unlike the LRR fragment, no prior dimerization of InlB is required for Met activation. Met activation by intact InlB instead proceeds through InlB monomers and involves concerted action *in cis* between the N-terminal domain and the C-terminal GW domains. The GW domains greatly enhance signalling potency by directly binding glycosaminoglycans, providing evidence for a functional parallel between InlB and HGF. Interactions with glycosaminoglycans do not alter the monomeric state of intact InlB or cause the GW domains to bind Met. We discuss the implications of these results in terms of ligand- and receptor-mediated modes of Met activation. Finally, we show that interaction between InlB and gC1q-R is required neither for Met activation nor for InlB-mediated bacterial invasion, and that HGF is able to substitute for InlB in promoting invasion.

Results

Regions of InlB required for Met activation

The InlB LRR fragment (composed of N-cap and LRR regions, Fig. 1) is reported to be sufficient to activate Met (Shen *et al*., 2000). As a result of the discrepancies in reproducing this result, we reassessed which portions of InlB are necessary and sufficient for Met activation. We constructed, expressed, and purified a series of InlB deletion mutants (Fig. 1B), and analysed their ability to activate Met. Vero cells were stimulated with 1.5 nM intact InlB or InlB deletion mutants, Met was immunoprecipitated from cell lysates, and the level of Met tyrosine phosphorylation was detected by Western blotting with an anti-phosphotyrosine antibody (Fig. 2A). Although 1.5 nM intact InlB is seen to elicit marked Met tyrosine phosphorylation, the LRR fragment, even at 100 nM or 1 μM, elicits a weak response at best. Mutants lacking the GW domains or lacking both the B-repeat and GW domains, respectively, InlB-ΔGW[1–3] and InlB-ΔBΔGW[1–3]

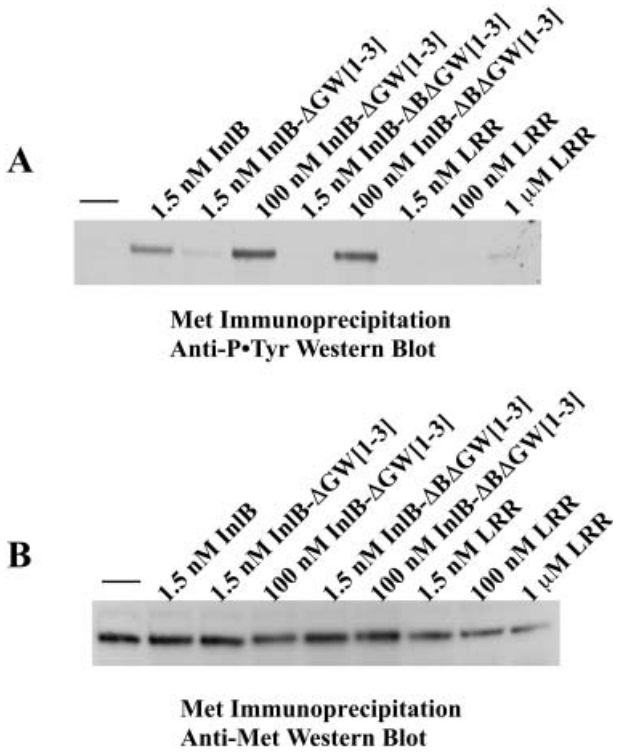


Fig. 2. Tyrosine phosphorylation of Met stimulated by InlB and InlB deletion mutants.
A and B. Vero cells were either not stimulated or stimulated with the indicated concentrations of InlB or InlB deletion mutants. Met was immunoprecipitated from these cells using the anti-Met antibody DO-24, and (A) Met tyrosine phosphorylation was detected by Western Blot using the anti-phosphotyrosine antibody 4G10, and (B) the total amount of Met was detected by Western blot using the anti-Met antibody DL-21.

(Fig. 1B), retain the ability to activate Met when used at 100 nM. However, when used at a concentration of 1.5 nM, these constructs show barely detectable activity compared to 1.5 nM InlB. These differences are directly attributable to differences in Met activation and not Met expression levels, because Met expression levels are seen to be equivalent across all samples, as detected by Western blotting with an anti-Met antibody (Fig. 2B). These results indicate that the LRR fragment is insufficient to activate Met, and that a larger region including the Ig-like portion is required. However, this larger region, which corresponds to the N-terminal domain of InlB (Fig. 1A, N-cap, LRR and Ig-like regions), is seen to have lower signalling potency than intact InlB.

LRR dimers, but not monomers, activate Met

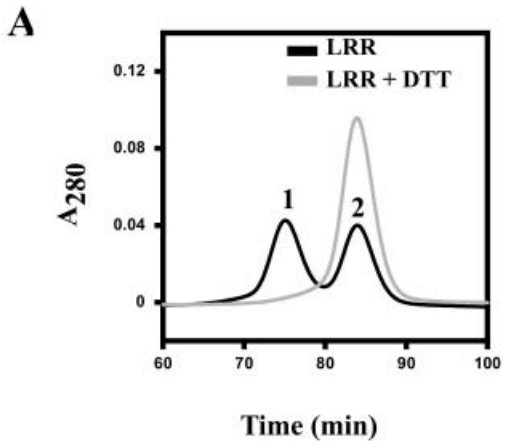
We noted that our purification of the LRR fragment uses dithiothreitol (DTT) throughout (Marino *et al*., 2002) and yields a protein that is incapable of activating Met. In contrast, other protocols do not include reducing agents (Braun *et al*., 1999; Shen *et al*., 2000), and are reported to produce LRR fragments capable of activating Met (Shen *et al*., 2000). We find that in the presence of reducing agents the LRR fragment elutes by molecular sizing chromatography as a single, homogenous fraction, and in the absence of reducing agents the LRR fragment elutes as two separable fractions (Fig. 3A). The first of these two fractions is composed of disulphide-linked dimers (Mr 57 kDa) and the second of monomers (Mr 22 kDa), as shown by non-reducing SDS–PAGE (Fig. 3B). Consistent with this assignment, the monomer fraction is seen to be coincident with the single fraction observed in the presence of reducing agents. Regardless of the method of purification, LRR dimers readily convert to monomers by incubation with reducing agents, and monomers convert to dimers under oxidizing conditions (Fig. 3A). Most significantly, disulphide-linked dimers of the LRR fragment at 0.75 nM (dimer concentration) are found to activate Met to the same level as intact InlB, whereas monomers are unable to activate Met (Fig. 3C).

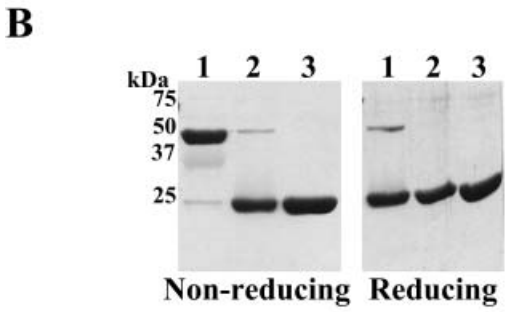
To verify that dimers are required for Met activation, a mutant of the LRR fragment incapable of forming disulphide-linked dimers was created by alanine substitution of Cys-242, the only cysteine in the LRR fragment. LRR C242A was purified in the absence of reducing agents, and molecular sizing chromatography and non-reducing SDS–PAGE confirm that it exists as monomers and does not form disulphide-linked dimers (Fig. 3B and D). To

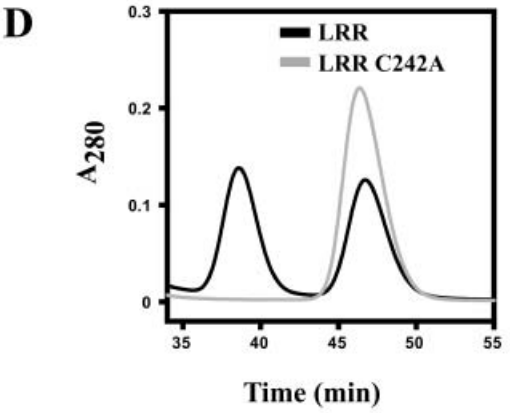
Fig. 3. Disulphide-linked dimers of the LRR fragment activate Met.
A. Molecular sizing chromatography of LRR in the absence (black) or presence (grey) of reducing agent (dithiothreitol, DTT). LRR in the absence of reducing agent appears in two fractions, labelled 1 and 2.
B. Non-reducing (left) and reducing (right) SDS–PAGE of LRR molecular sizing fractions 1 and 2 from panel A, corresponding to lanes 1 and 2, respectively. Lane 3 contains LRR C242A.
C. Tyrosine phosphorylation of Met in Vero cells stimulated with InlB, InlB C242A, LRR dimer, LRR monomer or LRR C242A. Protein concentrations were 1.5 nM for all samples, except for LRR dimer, which was used at 0.75 nM (dimer concentration). Tyrosine-phosphorylated Met was visualized as in Fig. 2.
D. Molecular sizing chromatography of LRR (black) and LRR C242A (grey) carried out in the absence of reducing agent.
E. Association of Met-Fc with InlB, InlB C242A, LRR and LRR C242A detected by protein A-agarose bead pull-down assay. Bound proteins are visualized on 10% SDS–PAGE with Coomassie staining.
F. Sedimentation equilibrium analytical ultracentrifugation of InlB. Protein concentration, as detected by absorbance at 280 nm (circles), in the centrifugation cell is fit with a single exponential (line), which indicates a molecular weight of 70 271 ± 273 Da, and shown in the lower panel. Residuals for the fit are shown in the upper panel.

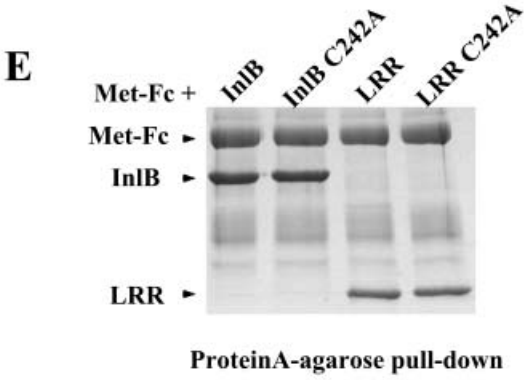
determine whether LRR C242A binds Met, protein A-agarose beads were used to precipitate Met-Fc (containing the ectodomain of Met fused to the Fc portion of an IgG) (Mark *et al*., 1992) along with associated wild type or C242A LRR. LRR C242A is observed to retain the ability to bind Met like wild-type LRR (Fig. 3E), but unlike the dimeric form of wild-type LRR, monomers of LRR C242A are unable to activate Met (Fig. 3C). These results confirm that dimers of the LRR fragment are required for Met dimerization and activation.

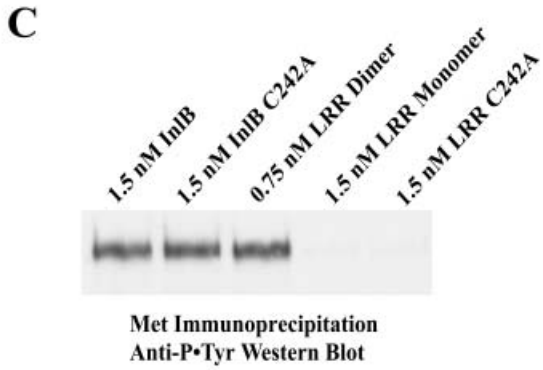
Although the LRR fragment is not produced physiologically, we wondered whether its dimeric mode of Met acti-

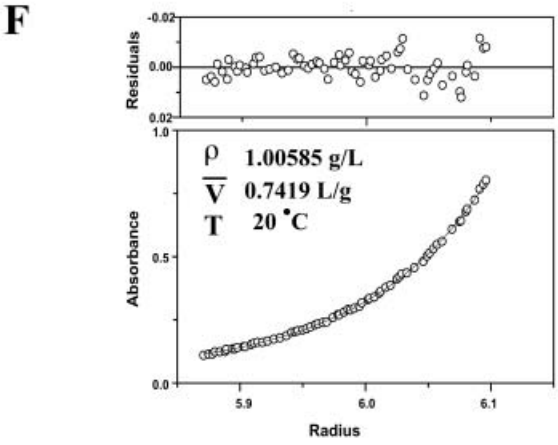












vation sheds light on the mechanism of action of intact InlB. To determine whether Cys-242 in intact InlB is involved in Met activation, the C242A substitution was introduced into intact InlB. InlB C242A binds to Met (Fig. 3E), and in striking contrast to LRR C242A, has the ability to activate Met (Fig. 3C). InlB C242A at 1.5 nM activates Met as potently as 1.5 nM wild-type InlB. This indicates that the mechanism of Met activation through Cys-242 disulphide-linked dimers is artificial and does not represent the physiological mechanism of Met activation by intact InlB. This result agrees with the observation that Cys-242 is solvent exposed and free to form intermolecular disulphides in the truncated LRR fragment (Marino *et al*., 1999), but is buried and unavailable for disulphide bond formation in intact InlB (Marino *et al*., 2002).

Unlike the LRR fragment, intact InlB is found to exist exclusively as monomers. Sedimentation equilibrium ultracentrifugation of InlB (in the absence of reducing agent) indicates a molecular weight of 70 271 ± 273 Da, consistent with a calculated monomer molecular weight of 68 391 Da (Fig. 3F). This conclusion is also supported by chemical cross-linking experiments, in which InlB is observed to exist only as monomers (data not shown).

GW domains enhance InlB signalling potency

Our data indicate that removal of the GW domains results in a loss of potency in InlB signalling (Fig. 2). To assess whether this loss is physiologically significant, we examined the level of Met activation elicited by *L. monocytogenes* during invasion. The wild-type *L. monocytogenes* strain EGD was incubated with Vero cells at a 100 : 1 multiplicity of infection, which is typical of invasion assays, and the extent of Met tyrosine phosphorylation was evaluated. This was then compared with the level of Met tyrosine phosphorylation elicited by purified InlB (in the absence of bacteria), using quantitative densitometry of Western Blots. We find that the lower limit for linear detection corresponds to the level of Met tyrosine phosphorylation evoked by 150 pM InlB (data not shown). By comparison, invading *L. monocytogenes* activates Met to a level somewhat lower than that elicited by 150 pM InlB (Fig. 4A). This activation by *L. monocytogenes* EGD is entirely caused by InlB as seen by the lack of Met activation by EGD (*ΔinlB*) under the same invasion conditions (Fig. 4A) (Dramsi *et al*., 1995; Shen *et al*., 2000).

This invasion experiment indicates that 150 pM InlB represents an experimentally tractable and physiologically meaningful standard by which to judge the potency of InlB fragments. Using this standard, we provide quantification of the loss of signalling potency resulting from deletion of the three, C-terminal GW domains (InlB-ΔGW[1–3]). A 10-fold higher concentration (1500 pM) of InlB-ΔGW[1–3] is required to yield Met activation equivalent to that yielded by 150 pM intact InlB (Fig. 4B). Further deletion from the C-terminus of the B-repeat domain (InlB-ΔBΔGW[1–3]) causes a further decrease in potency, with a 20-fold higher concentration (3000 pM) being required to yield Met activation comparable to that yielded by 150 pM intact InlB (Fig. 4C). These results indicate that the GW domains are crucial to the signalling potency of InlB, and that the B-repeat domain has a role in Met activation, if somewhat minor.

The second and third GW domains of InlB associate structurally as a pair, whereas the first GW domain is unpaired and flexible, consistent with the proteolytic sensitivity of this first domain (Marino *et al*., 2002). To find out whether all three GW domains are required for potency, a fragment lacking just the first GW domain was tested (InlB-ΔGW1, Fig. 1B). Deletion of the first GW domain has no effect on the potency of InlB, with 150 pM of InlB-ΔGW1 activating Met to the same extent as 150 pM of intact InlB (Fig. 4D). This potency is attributable entirely to the second and third GW domains, as shown by further deletion of the B-repeat (InlB-ΔBΔGW1). InlB-ΔBΔGW1 at 150 pM activates Met to the same extent as 150 pM intact InlB (Fig. 4E), indicating that the enhancement in signalling potency through the GW domains compensates for the minor loss in potency arising from deletion of the B-repeat domain.

The striking effect on potency of the second and third GW domains is best appreciated when these domains are fused to the LRR fragment (LRR-GW[2–3], Fig. 1B). LRR-GW[2–3] used at 3 nM activates Met to the same extent as intact InlB at 150 pM (Fig. 4F). Although full potency is not restored by the GW domains to the LRR fragment, it is remarkable that the GW domains promote signalling by a fragment that is otherwise unable to signal (unless artificially dimerized).

We next asked whether the second and third GW domains are required *in cis* with the N-terminal domain of InlB in order to confer potency. InlB-ΔGW[1–3] at 150 pM was supplemented with either 150 pM or 150 nM of a fragment composed of the second and third GW domains (Fig. 1B, GW[2–3]) in a Met activation assay. Whereas InB-ΔGW1, in which the second and third GW domains are located *in cis* with the N-terminal domain, is seen once again to potentiate Met activation, addition of these domains as a separate fragment (GW[2–3]) *in trans* with the N-terminal domain fragment (InlB-ΔGW[1–3]) does not result in Met activation (Fig. 4G). On its own, GW[2–3] does not activate Met either. These results indicate that the GW domains function only *in cis* in conferring potency.

gC1q-R is not involved in activation of Met by InlB

How do the GW domains potentiate InlB signalling? Although the GW domains do not bind Met (Shen *et al*.,

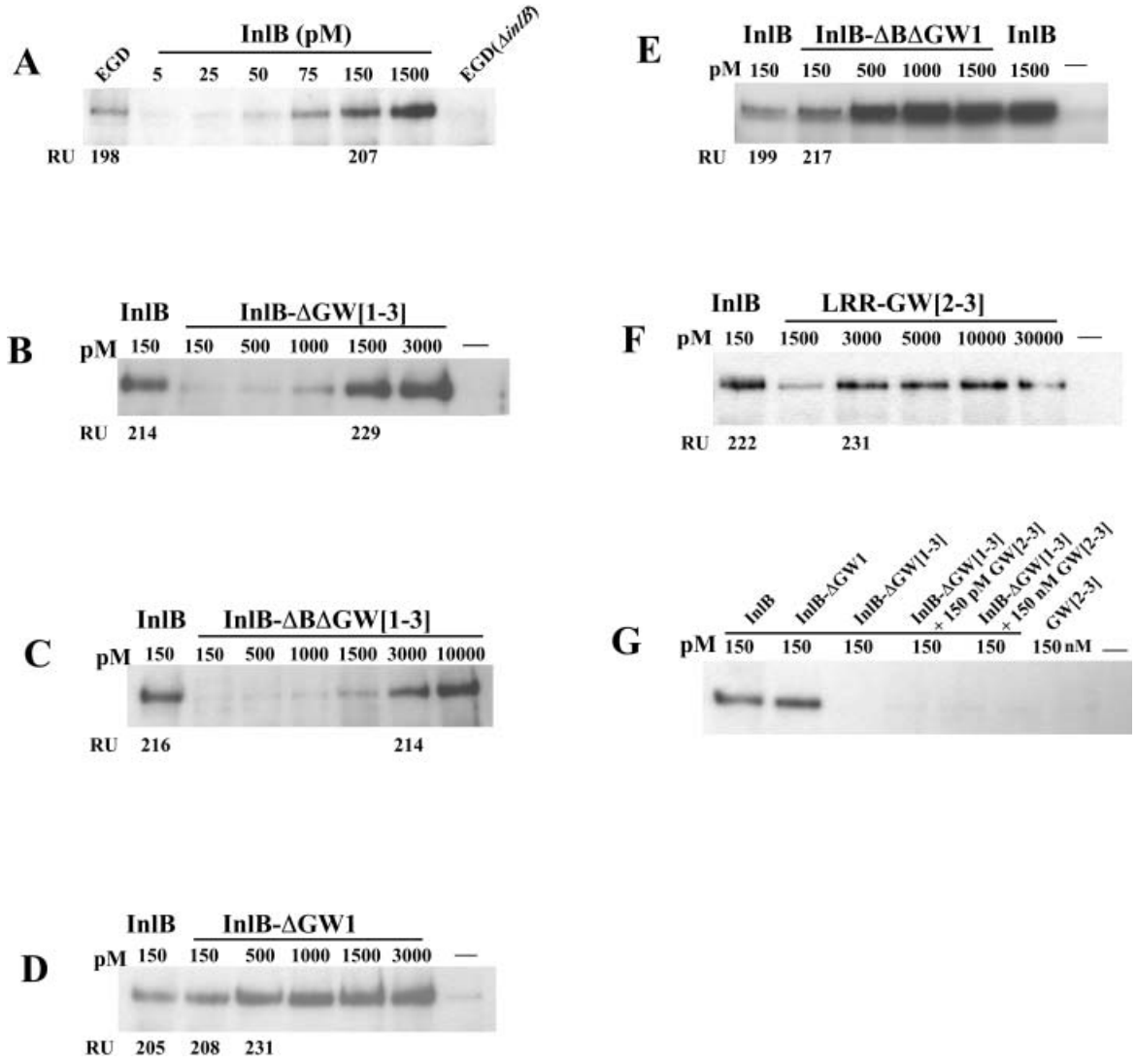


Fig. 4. GW domains are required for potency.
A. Tyrosine phosphorylation of Met in Vero cells elicited by wild type *L. monocytogenes* EGD and EGD (*ΔinlB*) at 100 : 1 multiplicity of infection is compared to that elicited by varying concentrations of purified InlB. Tyrosine-phosphorylated Met was visualized as in Fig. 2 for this and following panels. Quantitative densitometry was carried out for this panel and panels B–F. Values shown below bands represent the integrated intensity of the band in relative units (RU). Only values for bands that have intensities within the linear range of quantification are shown.
B–F. Signalling potency of InlB deletion fragments. Vero cells were stimulated with 150 pM InlB or varying concentrations of B, InlB-ΔGW[1–3]; C, InlB-ΔBΔGW[1–3]; D, InlB-ΔGW1; E, InlB-ΔBΔGW1 or F, LRR-GW[2–3].
G. GW domains function only in *cis*. Vero cells were stimulated with either 150 pM InlB-ΔGW[1–3] or 150 pM InlB-ΔGW[1–3] supplemented with varying concentrations (150 pM or 150 nM) of GW[2–3] to test for GW action in *trans*. For comparison, Vero cells were also stimulated with 150 pM InlB, 150 pM InlB-ΔGW1 or 150 nM GW[2–3].

2000), they are known to bind gC1q-R (Marino *et al.*, 2002), a host protein implicated in invasion (Braun *et al.*, 2000). To determine if gC1q-R is involved in signalling through Met, increasing concentrations of purified gC1q-R were added in combination with 1.5 nM InlB to Vero cells. This experiment shows that gC1q-R does not enhance InlB-mediated activation of Met, but instead antagonizes it in a dose-dependent manner (Fig. 5A). To determine whether this antagonistic effect of gC1q-R is specific to InlB, increasing concentrations of gC1q-R were added along with 1.5 nM HGF to Vero cells, as HGF and gC1q-R are not implicated to interact with one another. In contrast to the antagonism observed for InlB signalling, gC1q-R is not observed to antagonize HGF signalling, consistent with the gC1q-R antagonism being specific to InlB (Fig. 5B). Lastly, gC1q-R is not observed to activate Met by itself (Fig. 5B).

To probe the role of gC1q-R further, we replaced InlB GW domains 2 and 3 in InlB-ΔGW1 with GW domains 5 and 6 from the *L. monocytogenes* protein Ami, yielding InlB-GW(Ami) (Fig. 1B). Ami GW domains 5 and 6 are ~40% identical to InlB GW domains 2 and 3, but they do

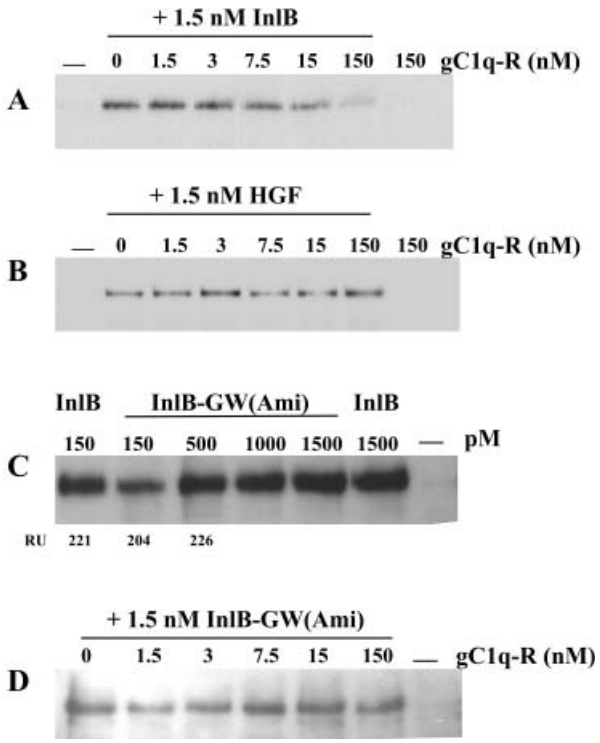


Fig. 5. gC1qR antagonizes Met activation by InlB but not by InlB-GW(Ami).
A and B. Levels of tyrosine-phosphorylated Met in Vero cells stimulated with either (A) 1.5 nM InlB or (B) 1.5 nM HGF in the presence of gC1q-R ranging from 0 to 150 nM. The first lane corresponds to no stimulation, and the last lane corresponds to stimulation with 150 nM gC1q-R alone. Tyrosine-phosphorylated Met in this and following panels was visualized as in Fig. 2.
C. Levels of tyrosine-phosphorylated Met in Vero cells stimulated with InlB-GW(Ami) ranging from 150 to 1500 pM. For comparison, cells were also stimulated with 150 or 1500 pM InlB, or not stimulated at all. The integrated intensity as measured in relative units (RU) is reported for those bands falling with the linear range of quantification.
D. Levels of tyrosine phosphorylation in Vero cells stimulated with 1.5 nM InlB-GW(Ami) in the presence of gC1q-R ranging from 0 to 150 pM.

not bind gC1q-R (Marino *et al*., 2002). We find that the Ami GW domains almost completely substitute for the InlB GW domains in potentiating signalling; InlB-GW(Ami) at 500 pM elicits Met tyrosine phosphorylation that is equivalent to that elicited by InlB at 150 pM (Fig. 5C). Furthermore, addition of exogenous gC1q-R does not inhibit Met activation by InlB-GW(Ami) (Fig. 5D), providing further evidence that specific antagonism of InlB by gC1q-R stems from its association with the GW domains of InlB. In summary, these experiments demonstrate that potentiation of InlB signalling via the GW domains is not mediated by gC1q-R.

HGF suffices for Listeria *invasion and interaction of InlB with gC1q-R is not required*

Even though gC1q-R is not required for Met activation, it remains possible that its association with InlB is required for invasion. To address this, the efficiency of invasion into Vero cells by *L. monocytogenes* EGD (*ΔinlB*) supplemented with purified InlB, InlB-ΔGW1, InlB-ΔBΔGW1, InlB-GW(Ami), GW[2–3], or HGF was assessed. First, the invasion efficiency of wild-type EGD was compared to that of EGD (*ΔinlB*) supplemented with varying concentrations of soluble InlB. It was found that supplementing EGD (*ΔinlB*) with 1.5 nM InlB yields an invasion efficiency similar to that of wild-type EGD (Fig. 6). Similarly, addition of 1.5 nM InlB-ΔGW1, InlB-ΔBΔGW1, or InlB-GW(Ami) to EGD (*ΔinlB*) yields invasion efficiencies resembling that of wild-type EGD and significantly greater than that of EGD (*ΔinlB*) without supplementation. The functionality of InlB-ΔBΔGW1 indicates that superactivation of the Ras-MAPK pathway, which is conferred by the B-repeat of InlB (Copp *et al*., 2003), is not required for intracellular invasion. As expected, supplementation of EGD (*ΔinlB*) with 1.5 nM GW[2–3] does not restore intracellular invasion. Lastly and most strikingly, we find that addition of 1.5 nM HGF to EGD (*ΔinlB*) is sufficient to trigger invasion, and that HGF promotes invasion as efficiently as InlB (Fig. 6). In summary, these results indicate that neither association between InlB and gC1q-R nor superactivation of the Ras-MAPK pathway are necessary for intracellular invasion, and that HGF can replace InlB for invasion.

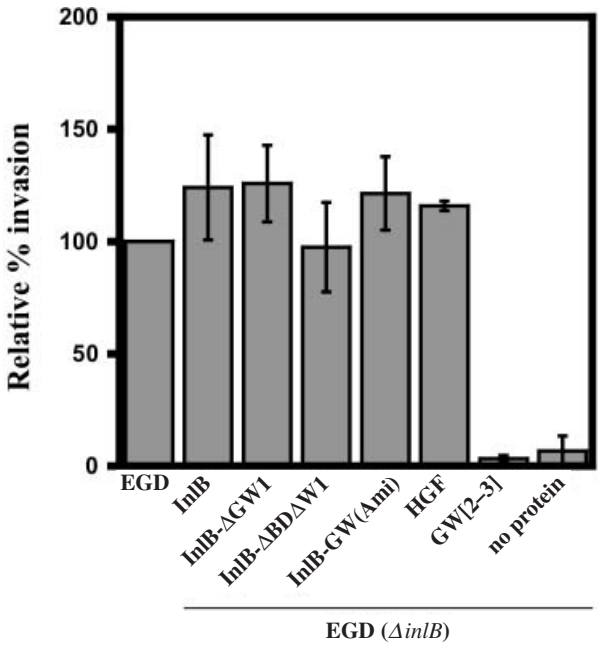


Fig. 6. InlB-GW(Ami) and HGF mediate Vero cell invasion by *L. monocytogenes*. Efficiency of bacterial invasion of Vero cells, as evaluated by a gentamicin survival assay, was determined for *L. monocytogenes* EGD and for EGD (*ΔinlB*), and for EGD (*ΔinlB*) to which 1.5 nM InlB, InlB-ΔGW1, InlB-ΔBΔGW1, InlB-GW(Ami), HGF, or GW[2–3] was added. A multiplicity of infection of 100 : 1 was used, and values are normalized to invasion by *L. monocytogenes* EGD.

Potency effect of GW domains is mediated by glycosaminoglycans

Cell surface glycosaminoglycans represent a second candidate for mediating the potency effect of the GW domains. We confirmed by affinity chromatography that the GW domains bind heparin with high affinity (data not shown) (Jonquières *et al*., 2001). To determine whether heparin converts monomers of InlB to dimers or oligomers, molecular sizing chromatography in the presence of heparin was performed. The NK1 fragment of HGF has been observed by sizing chromatography to switch from monomer to dimer in the presence of heparin (Chirgadze *et al*., 1999). InlB was incubated with varying heparin concentrations, and applied to a molecular sizing column in a buffer containing the same heparin concentration used in incubation. The molar ratio of heparin to InlB (7.5 μM) was varied from 1 : 1 (average heparin molecular weight 18 kDa, 0.135 mg ml^{-1}) to ~7.5 : 1 (Fig. 7A). In contrast to NK1, incubation of InlB with heparin does not result in a shift in elution time. Likewise, lower concentrations of heparin down to 10 μg ml^{-1} are not seen to result in a shift in elution time either. These results indicate that association with heparin does not cause oligomerization of InlB.

Whether heparin binding endows the GW domains with the ability to bind Met was next examined using a qualitative assay. Met-Fc was incubated with histidine-tagged GW[2–3] in the presence or absence of 10 μg ml^{-1} or 1 mg ml^{-1} heparin, and association was detected using a nickel-NTA agarose bead pull-down assay**.** Similar results were found at both heparin concentrations, and data from the 10 μg ml^{-1} heparin incubation are shown in Fig. 7B. GW[2–3] was not found to associate with Met-Fc in the presence or absence of heparin, and consistent with this observation, association of InlB and InlB-ΔGW[1–3] with Met-Fc was not qualitatively altered by heparin in a reproducible fashion.

However, heparin was seen to have a dramatic effect on Met activation. We used the glycosaminoglycan-deficient Chinese hamster ovary (CHO) cell line S745 to ask whether glycosaminoglycans are involved in InlB-mediated activation of Met. CHO-S745 cells lack xylosyltransferase, which is the enzyme responsible for the first step of glycosaminoglycan biosynthesis (Esko *et al*., 1985), and these cells have been used in earlier studies of HGF activation of Met (Sakata *et al*., 1997; Lyon *et al*., 2002). Activation of Met in CHO-S745 cells was followed by detection of tyrosine phosphorylated MAP kinase in whole cell lysates, as anti-hamster Met antibodies did not yield quantitative recoveries in immunoprecipitations. The MAP kinase pathway has been shown to be important for *L. monocytogenes* invasion, and activation of MAP kinase has been shown to be a marker for Met activation (Tang *et al*., 1998; Lyon *et al*., 2002; Copp *et al*., 2003). Importantly, our experiments show that while 0.15 pM InlB is insufficient to elicit detectable tyrosine phosphorylation of MAP kinase (ERK-1/2), addition of 10 μg ml^{-1} heparin (0.5 μM, average heparin molecular weight 18 kDa) along with 0.15 pM InlB brings about significant tyrosine phosphorylation of MAP kinase (Fig. 8A). Heparin enhances the potency of InlB signalling across the range of tested concentrations, while heparin alone does not induce MAP kinase phosphorylation.

To verify that heparin functions through the GW domains, signalling by InlB-ΔGW[1–3] was examined in the same cell line. As expected, the potency of InlB-ΔGW[1–3] is much reduced compared to that of intact InlB. Tyrosine phosphorylated MAP kinase is not detectable until 1500 pM InlB-ΔGW[1–3] is used. Notably, the level of tyrosine phosphorylation elicited by this fragment

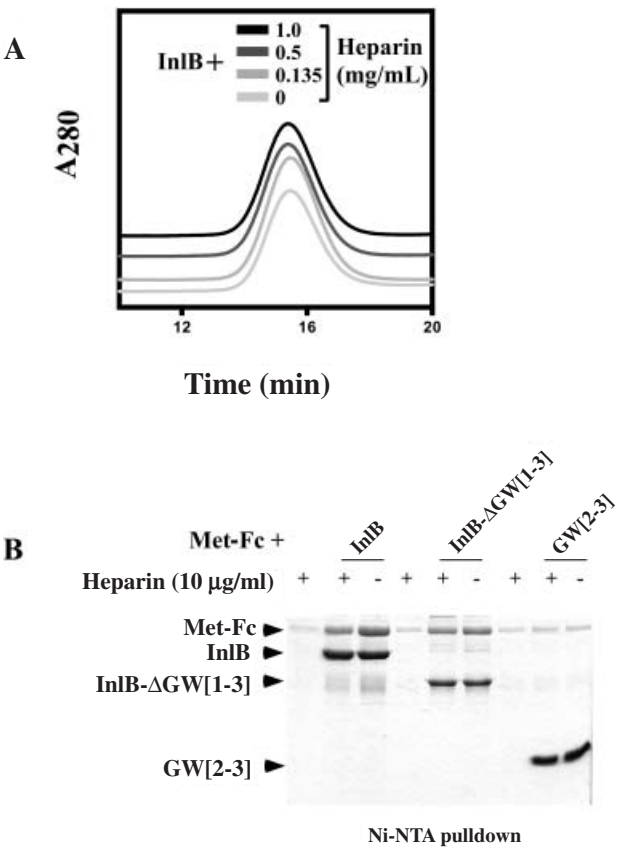


Fig. 7. Glycosaminoglycans do not oligomerize InlB or cause the GW domains to bind Met.
A. Molecular sizing chromatography of InlB at 0.5 mg ml^{-1} (~7.5 μM) with varying concentrations of heparin. InlB was incubated with 0.135 mg ml^{-1} (~7.5 μM), 0.5 mg ml^{-1} (~28 μM), or 1 mg ml^{-1} (~56 μM) heparin. Heparin at these concentrations was also included in the column elution buffer. The elution profiles are off-set vertically for clarity.
B. Association in presence or absence of 10 μg ml^{-1} heparin between Met-Fc and histidine-tagged InlB, InlB-ΔGW[1–3] and GW[2–3], as detected by nickel-NTA agarose bead pull-down. Bound proteins are visualized on 12% SDS–PAGE with Coomassie staining.

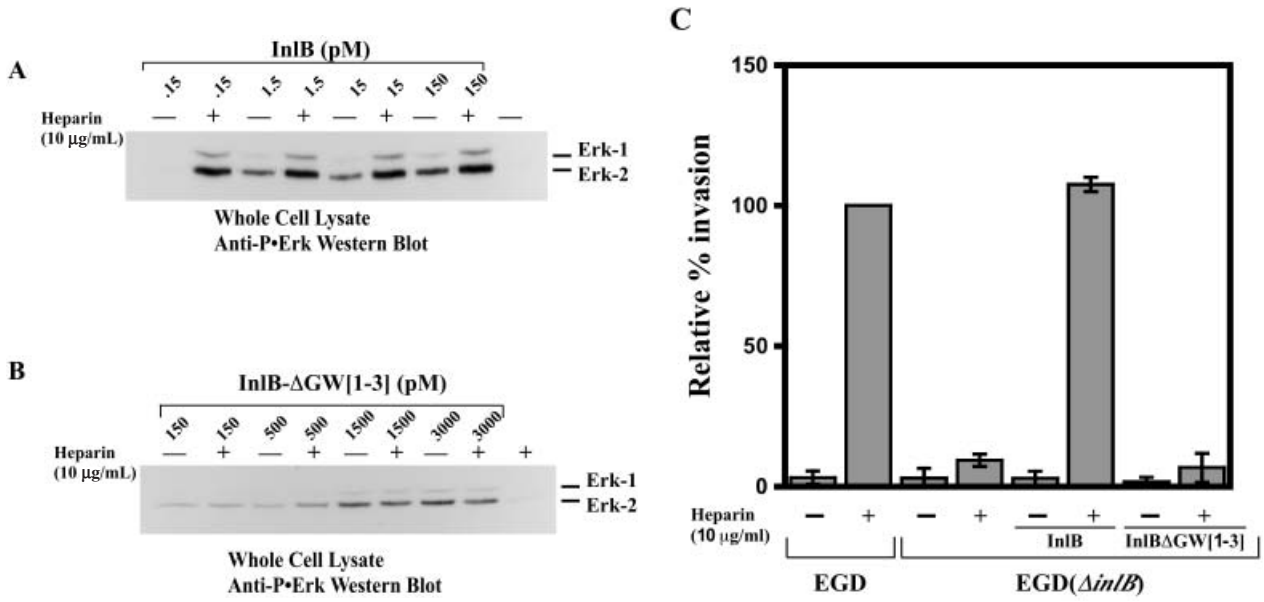


Fig. 8. Glycosaminoglycans mediate the potency effect of GW domains.
A and B. Levels of tyrosine phosphorylated ERK-1/2 in CHO-S745 cells stimulated with (A) InlB ranging from 0.15 to 150 pM or with (B) InlB-ΔGW[1–3] ranging from 150 to 3000 pM, in the absence or presence of 10 μg ml^{-1} heparin. Tyrosine phosphorylated ERK-1/2 was detected in whole cell lysates of CHO-S745 cells by Western Blot using a phospho-specific anti-ERK-1/2 antibody.
C. Efficiency of bacterial invasion of CHO-S745 cells, as evaluated by a gentamicin survival assay, was determined for *L. monocytogenes* EGD and EGD (*ΔinlB*), alone or supplemented with 10 μg ml^{-1} heparin. EGD (*ΔinlB*) was also supplemented with either 1.5 nM InlB or 1.5 nM InlB-ΔGW[1–3], in the presence or absence of 10 μg ml^{-1} heparin. Reported invasion efficiencies are normalized to the invasion efficiency of *L. monocytogenes* EGD supplemented with heparin.

lacking the GW domains is not altered by the addition of heparin (Fig. 8B). These results demonstrate that interactions between glycosaminoglycans and the GW domains are required for potentiation of InlB signalling.

Glycosaminoglycans potentiate L. monocytogenes *invasion into CHO-S745 cells*

Lastly, we determined whether glycosaminoglycan binding to the InlB GW domains is required for intracellular invasion. We found that *L. monocytogenes* EGD invades glycosaminoglycan-deficient CHO-S745 cells very inefficiently, unless 10 μg ml^{-1} heparin is added exogenously (Fig. 8C). The invasion efficiency is approximately 50-fold lower in the absence of heparin than it is in the presence of heparin. *Listeria monocytogenes* EGD (*ΔinlB*) also invades CHO-S745 cells very inefficiently, and requires the combined addition of 1.5 nM InlB and 10 μg ml^{-1} heparin to restore invasiveness. In contrast, heparin is seen not to restore invasiveness to EGD (*ΔinlB*) that is supplemented with InlB-ΔGW[1–3], consistent with glycosaminoglycan action being mediated through association with the GW domains. Taken together, these results demonstrate that activation of Met and subsequent intracellular invasion by *L. monocytogenes* is potentiated by interactions between glycosaminoglycans and the GW domains of InlB.

Discussion

Receptor tyrosine kinases are essential for control of cell growth, proliferation and differentiation. Met, for example, induces mitogenesis, morphogenesis and motility in response to its endogenous ligand, HGF. Similar to other receptor tyrosine kinases, dysfunction of Met is linked to a plethora of malignancies, such as kidney, breast, liver and gastric carcinomas (Danilkovitch-Miagkova and Zbar, 2002; Maulik *et al.*, 2002). To gain insight into mechanisms of Met activation, we examined signalling by *L. monocytogenes* InlB, an exogenous ligand of Met, and have dissected the role of individual InlB domains in this process.

Two general modes account for how growth factor receptors like Met are dimerized and thereby activated for signal transduction (Schlessinger, 2000). The first mode involves ligand-mediated dimerization, in which receptors are brought together and dimerized by binding to ligands that either are bivalent, as in the case of human growth hormone (de Vos *et al.*, 1992), or are dimeric, as in the case of vascular endothelial growth factor (Wiesmann *et al.*, 1997). In contrast, the second mode involves receptor-mediated dimerization, in which receptors are dimerized by binding to ligands that trigger conformational changes in the receptor. Ligands in this mode may be monomeric and univalent, but the key is that conforma-

tional changes induced in the receptor by ligand binding enable dimerization. Examples of receptor-mediated activation have been observed in epidermal growth factor (EGF) and transforming growth factor α binding to EGF receptor (Garrett *et al*., 2002; Ogiso *et al*., 2002).

Which of these mechanisms HGF uses to activate Met is controversial. The predominant view is that HGF dimers or oligomers activate Met in ligand-mediated fashion, and that oligomerization of HGF is aided by glycosaminoglycan binding (Zioncheck *et al*., 1995; Schwall *et al*., 1996; Sakata *et al*., 1997). A similar mode of action is posited for the NK1 and NK2 fragments of HGF. However, recent work shows that cross-linked complexes of HGF and heparin, that appear to be monomeric (i.e. 1 : 1 HGF:heparin), have the ability to activate Met (Lyon *et al*., 2002). Furthermore, examination of the sedimentation velocity behaviour of HGF-heparin-Met complexes is more consistent with a 1 : 1 : 1 stoichiometry than other stoichiometries (Gherardi *et al*., 2003). From these data, it appears that HGF dimers are not required for Met activation, and these results argue for a receptor-mediated mode of activation. There is precedence for glycosaminoglycans being involved in receptor-mediated activation in the case of the fibroblast growth factor receptor (FGFR). Heparin has been shown to stabilize interactions between fibroblast growth factor (FGF) and FGFR, resulting in 1 : 1 : 1 FGF:FGFR:heparin complexes. These 1 : 1 : 1 complexes are seen to associate primarily through receptor–mediated interactions to form 2 : 2 : 2 FGF:FGFR:heparin complexes, which are competent for receptor activation (Schlessinger *et al*., 2000). Consistent with this model, Met is observed to have some affinity, albeit weak, for heparin (Lietha *et al*., 2001; Lyon *et al*., 2002; Gherardi *et al*., 2003).

Our experiments with the exogenous ligand InlB suggest that Met can be activated either by ligand-mediated or receptor-mediated modes. We find that dimers but not monomers of the N-terminal LRR fragment are capable of activating Met, indicating a ligand-mediated mode of activation for the LRR fragment. However, the LRR fragment does not exist physiologically, and its dimerization through a disulphide bond at Cys-242 is found to be an artifact of truncation and purification in the absence of reducing agents. This is shown by alanine substitution of Cys-242 in the LRR fragment destroying dimer formation and Met activation but not Met binding, whereas the same mutation in intact InlB having no effect on Met binding or activation. In contrast to the LRR fragment, InlB exists exclusively as monomers, and its mechanism of Met activation requires concerted action *in cis* of both its N-terminal and C-terminal domains. These results provide evidence that the mechanism of Met activation differs significantly between InlB and its LRR fragment. The N-terminal LRR domain of InlB has previously been shown to bind Met (Shen *et al*., 2000), but the involvement of the C-terminal domain in mammalian cell signalling is surprising given its previously assigned role in tethering InlB to the bacterial cell wall (Jonquières *et al*., 1999).

We find that the GW domains greatly enhance the signalling potency of InlB and its capacity to promote invasion, effects which require interaction of these domains with glycosaminoglycans and indicate a functional parallel between InlB and HGF. As noted above, the effect of glycosaminoglycans on the oligomeric state of HGF is controversial, but with InlB, we find no change in its monomeric state upon association with heparin. It should be noted that heparin has been reported to cause InlB oligomerization as examined by chemical cross-linking (Jonquières *et al*., 2001). However, this effect appears to be quantitatively minor, and we observe no oligomerization of InlB, heparin-promoted or otherwise, by chemical cross-linking (data not shown). Is it possible that InlB is bivalent? Although the stoichiometry of InlB association with Met has not yet been determined, it seems unlikely for the following reasons. First, the N-terminal LRR fragment binds Met but requires dimerization for Met activation, indicating that this region binds Met univalently. This is in agreement with conclusions from a recent mutagenesis study of the Met-binding region of InlB (Machner *et al*., 2003). Second, the GW domains do not have the ability to bind Met in the presence or absence of heparin, making it unlikely that the GW domains recruit a second Met molecule. These findings implicate a receptor-mediated mode of Met activation for intact InlB, in which heparin binding to the GW domains might stabilize formation of a 1 : 1 : 1 InlB:heparin:Met complex that is conformationally competent to recruit a second complex and transduce a signal. An alternative hypothesis is that the GW domains might themselves interact with a cell surface receptor, for example, a heparan sulphate proteoglycan. The proteoglycans syndecan-1 and syndecan-4 have been shown to bind *Neisseria gonorrhoeae* and to be crucial to internalization of this bacterial pathogen, involving either signal transduction or cytoskeletal rearrangements through the cytoplasmic domains of these proteoglycans (Freissler *et al*., 2000). However, this hypothesis is less attractive for InlB, as we find that exogenously added heparin suffices to potentiate InlB signalling in CHO-S745 cells and to promote InlB-mediated invasion of the same cells.

The GW domains are critical for mammalian cell signalling at concentrations of InlB achieved physiologically during bacterial invasion. We find that at least 10-fold greater concentrations of InlB mutants lacking GW domains are required to activate Met to the same extent as intact InlB. This explains an earlier report that a GW-deficient mutant of InlB promotes bacterial invasion at 10–100 fold lower efficiencies than intact InlB in Vero cells (Braun *et al*.,

1998). Similarly, we find that InlB-ΔGW[1–3] and heparin together promote invasion at approximately 50-fold lower efficiency in CHO-S745 cells than does intact InlB and heparin. The GW-deficient mutants InlB-ΔBΔGW[1–3] and InlB-ΔGW[1–3], which retain some ability to activate Met, are observed to be monomeric (data not shown). This suggests that they too function by a receptor-mediated mode but are inefficient at triggering conformational changes in Met due to the loss of the potentiating GW domains. In contrast, the LRR fragment appears unable to trigger conformational changes required for receptor-mediated activation, as it signals only as a dimer.

Our results call into question the functional significance of interactions between InlB and the host protein gC1q-R (Braun *et al*., 2000). It has been previously reported that C1q, a ligand for gC1q-R, can reduce InlB-mediated invasion of Vero cells, as can an antibody directed against gC1q-R. Furthermore, transfection of gC1q-R into GPC16 cells was reported to make these cells permissive to InlB-mediated invasion, although puzzlingly, these cells contain a protein recognized by an anti-gC1q-R antibody before transfection (Braun *et al*., 2000). In contrast to these findings implicating a functional importance for interaction between InlB and gC1q-R, we demonstrate that interaction between InlB and gC1q-R is not required for Met activation in Vero cells or intracellular invasion of these same cells, as shown using the InlB-GW (Ami) variant of InlB. This variant of InlB lacks its native GW domains and instead contains Ami GW domains, which do not bind gC1q-R (Marino *et al*., 2002). We show that InlB-GW(Ami) is capable of activating Met and promoting intracellular invasion in Vero cells. These results are consistent with HGF, which does not interact with gC1q-R, also being able to promote invasion of Vero cells. Rather than enhancing Met signalling by InlB, gC1q-R is found to act as a specific antagonist of InlB, agreeing with the finding that gC1q-R competes with glycosaminoglycans for binding to the GW domains (Marino *et al*., 2002). Although our results indicate that gC1q-R is unlikely to have a functional role in invasion, it is possible that exogenously added gC1q-R behaves somehow differently from endogenously produced gC1q-R, or that gC1q-R has a role in invasion other than binding to InlB. Definitive conclusions regarding the functional role of gC1q-R in invasion await a gC1q-R knockout.

Interactions between the InlB GW domains and glycosaminoglycans are likely to occur following release of InlB from the bacterial surface. This is because glycosaminoglycans are found to compete with the bacterial cell wall for binding to the GW domains (Jonquières *et al*., 2001). Furthermore, this would reconcile the observation that InlB is deeply buried within the cell wall, and is probably inaccessible to the mammalian cell surface in the bacterial cell-attached form (Jonquières *et al*., 1999). The involvement of the GW domains in mammalian cell signalling provides evidence that InlB functions much like a hormone, in released and soluble form rather than as a bacterial cell-attached ligand. This idea is supported by the observation that HGF is able to substitute for InlB in promoting invasion, indicating that the only properties of InlB necessary for invasion are those that mimic the hormone HGF in its capacity to activate Met. Whether transient attachment of InlB to the bacterial surface serves a functional purpose in not clear, but it may spatially and temporally couple the release of InlB and consequent Met activation with bacterial proximity during invasion (Marino *et al*., 2002).

In summary, our results suggest that Met may be activated either through ligand- or receptor-mediated modes. For physiologically relevant activation of Met by monomeric InlB, concerted action *in cis* by both N-terminal and C-terminal domains is required. The N-terminal domain binds Met, whereas the C-terminal domains bind glycosaminoglycans and potentiate InlB signalling, most likely by stabilizing higher-order bacterial-mammalian signalling complexes during intracellular invasion.

Experimental procedures

Cloning, expression and protein purification

Procedures for intact InlB and LRR fragment have been described previously (Marino *et al*., 1999; 2002). In addition, purification of LRR and LRR C242A fragments in non-reducing conditions was carried out according to a published protocol (Braun *et al*., 1999). Proteins purified by this latter method were subsequently treated with 0.5% polyethyleneimine and centrifuged (31 000 *g*, 40 min) to pellet contaminating nucleic acids. The supernatant form this treatment, containing purified protein, was precipitated with 80% saturated $(NH_4)_2SO_4$ by centrifugation (31 000 *g,* 40 min), and resuspended and dialysed in 500 mM NaCl, 50 mM HEPES, pH 7.4, with or without 2 mM dithiothreitol (DTT).

Deletion mutants of InlB (InlB-ΔGW[1–3], InlB-ΔBΔGW[1–3], InlB-ΔGW1, InlB-ΔBΔGW1, LRR-GW[2–3] and GW[2–3]) were cloned by strand-overlap extension using the polymerase chain reaction (Higuchi *et al*., 1988). InlB-GW(Ami) was constructed similarly, and encodes InlB residues 36–392 fused to Ami residues 848–1009 (NP_446081). These proteins along with InlB C242A were purified as described for InlB (Marino *et al*., 2002). Protein concentrations were determined using absorption at 280 nm and calculated extinction coefficients.

Cell lines, antibodies and bacterial strains

Vero cells were maintained in minimum essential media supplemented with Earle's salts and 10% fetal bovine serum (FBS). CHO-S745 cells were generously provided by Dr Jeffrey Esko (University of California, San Diego). CHO wild-type and CHO-S745 cells were maintained in Ham's F-12 media (Gibco) supplemented with 10% FBS. Monoclonal

anti-Met antibodies DO-24 and DL-21 and anti-phosphotyrosine antibody 4G10 were from Upstate Biotechnology (Lake Placid, NY), as was horse radish peroxidase (HRP)-conjugated goat anti-mouse antibody. Phospho-specific anti-ERK1/2 antibody *was* obtained from Cell Signaling Technology (Beverly, MA). *Listeria monocytogenes* strains EGD and EGD (Δ*inlB*) (Dramsi *et al*., 1995) were generously provided by Dr Pascale Cossart (Institut Pasteur, Paris)

Molecular sizing chromatography

Molecular sizing chromatography of LRR fragments was performed using Superdex 75 or 200 (Amersham Pharmacia) media at a flow rate of 1.5 ml min^{-1} in 500 mM NaCl, 50 mM HEPES, pH 7.4, with or without 2 mM dithiothreitol (DTT). For experiments with heparin, 0.5 mg ml^{-1} (7.5 μM) InlB was incubated with heparin (Sigma, average molecular weight 18 kDa) varying in concentration from 10 μg ml^{-1} to 1 mg ml^{-1} for 2 h at 4°C. Chromatography was carried out using Superdex 200 media at a flow rate of 1 ml min^{-1} in 150 mM or 500 mM NaCl and 50 mM Tris, pH 8.0, with or without heparin at the incubating concentration. Apparent molecular weights of protein samples were determined from elution times, based on standards consisting of thyroglobulin (670 kDa), gamma globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and vitamin B12 (1.35 kDa) (Bio-Rad). Each experiment was repeated twice.

Met binding assay

InlB, InlB C242A, LRR or LRRC242A (10 μg) was incubated with 10 μg Met-Fc (Mark *et al*., 1992) at 4°C for 30 min, after which protein A-agarose beads (Repligen) were added and incubated with shaking at 4°C for 30 min. Unbound protein were removed from beads by washing four times in 300 mM NaCl, 50 mM phosphate buffer, pH 8.0, and 0.05% Tween-20. Bound proteins were removed from beads by boiling in SDS sample buffer and visualized by SDS–PAGE. The experiment was repeated twice.

Ni-NTA pull-down assay

Met-Fc fusion protein (10 μg) was incubated with 10 μg InlB, InlB-ΔGW[1–3], or GW[2–3] with or without 10 μg ml^{-1} or 1 mg ml^{-1} heparin (Sigma) at 4°C for 30 min, after which Ni-NTA agarose beads (Qiagen) were added and incubated with shaking at 4°C for 30 min. Unbound proteins were removed from beads by washing four times in 150 mM NaCl, 50 mM phosphate buffer, pH 8.0, and 20 mM imidazole. Bound protein were removed from beads by boiling in SDS sample buffer and visualized by SDS–PAGE.

Detection of tyrosine-phosphorylated proteins

Met tyrosine phosphorylation was detected in stimulated Vero cells as described (Copp *et al*., 2003). Briefly, Vero cells were starved overnight and stimulated with indicated concentrations of InlB, InlB mutants, or HGF (Sigma) for 1 min at 37°C, then rinsed twice with ice-cold Tris-buffered saline (TBS, 150 mM NaCl, 10 mM Tris, pH 8) and lysed. Whole cell lysates were immunoprecipitated using the anti-Met antibody DO-24, and immunoprecipitates were analysed by Western Blot using the anti-phosphotyrosine antibody 4G10, HRP-conjugated goat anti-mouse secondary antibody, and ECL reagent (Amersham Pharmacia). For analysis of CHO-S745 cells stimulated with InlB or InlB mutants in the absence or presence of exogenously added heparin (10 μg ml^{-1}), whole cell lysates were analysed by Western blot with the anti-phosphotyrosine antibody 4G10 *or* phospho-specific anti-ERK1/2 antibody (Cell Signaling Technology, Beverly, MA), as previously described (Copp *et al*., 2003). Each experiment was repeated 3–4 times.

For experiments with *L. monocytogenes* EGD or EGD (Δ*inlB*) (Dramsi *et al*., 1995), Vero cells were grown to confluence in 10 cm plates ($4–6 \times 10^5$ cells/plate) as described above, and then starved overnight in serum free media. *Listeria monocytogenes* EGD and EGD (Δ*inlB*) were grown overnight in Brain–Heart Infusion media (Difco) at 37°C without shaking. Overnight bacterial cultures were washed three times in phosphate-buffered saline (PBS) and added to Vero cells at a multiplicity of infection (MOI) of 100 : 1. After incubation with bacteria at 37°C for 5 min, Vero cells were washed twice with ice-cold TBS and lysed. Cell lysates were immunoprecipitated and analysed by Western Blot for Met tyrosine phosphorylation, as above. Each experiment was repeated 3–4 times.

Quantification of Western blots

Standards (varying quantities of the mouse anti-Met antibody DO-24) were blotted, probed with a goat anti-mouse secondary antibody, reacted with ECL reagent (Amersham Pharmacia), and exposed to X-ray film (Perkin-Elmer). Scanned films (300 dpi, UMAX Astra 1220S) were quantified densitometrically using NIH image (developed at the U.S. National Institutes of Health and available at http://rsb.info.nih.gov/nih-image/). A constant, rectangular integration area (0.1×0.07 $inches^2$) was used for measurement of mean grey level for each band, and values between 196.9 and 230.9 (0 white, 255 black) were found to be within the linear range of the X-ray film. Comparisons between bands were made only when intensities fell within this grey value range.

Invasion assays

Vero or CHO-S745 cells were grown at 37°C overnight, as described above, to confluence in 24-well tissue culture plates (10^5 cells/well). *Listeria monocytogenes* EGD and EGD (Δ*inlB*) (Dramsi *et al*., 1995) were grown in Brain–Heart infusion media overnight at 37°C without shaking. Overnight bacterial cultures (10^7 cells) were pelleted, washed twice in PBS, and resuspended and incubated for 20 min at room temperature in 100 μl PBS alone or PBS containing 1.5 nM purified InlB, InlB-ΔGW1, InlB-ΔBΔGW1, InlB-GW(Ami), GW[2–3], InlB-ΔGW[1–3], or HGF (Sigma). For certain samples, heparin at 10 μg ml^{-1} final concentration was added to CHO-S745 cells before addition of bacterial cultures. Bacterial cultures, supplemented with protein or not, were then incubated with cells at an MOI of 100 : 1 for 30 min. Cells were then washed three times with PBS and fresh medium was added. One hour later gentamicin (5 μg ml^{-1}) was added

to kill extracellular bacteria, and 3.5 h later mammalian cells were lysed in 0.2% Triton X-100. Cell lysates were plated on LB agar, and the number of invasive bacteria was counted. These assays were repeated three times.

Analytical ultracentrifugation

Sedimentation equilibrium measurements were performed on a Beckman Optima XL-A equipped with a UV-visible detection system (Beckman Instruments, Palo Alto, CA). Centrifugation of InlB (0.5 mg ml^{-1} in 150 mM NaCl, 50 mM Tris, pH 8.0) was carried out at 10, 12 and 14 Krpm at 20°C, and protein distribution was monitored by absorbance at 280 nm. Equilibrium was reached at 26 h at each speed. Absorbance data were analysed using the program Origin (Beckman). A calculated partial specific volume (0.7419 l g^{-1}) of InlB based on its amino acid sequence was used, and buffer density was found to be 1.00585 g l^{-1} using the program SEDNTERP (John Philo, Thousand Oaks, CA). The experiment was repeated twice.

Acknowledgements

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