MET-activating Residues in the B-repeat of the Listeria monocytogenes Invasion Protein InlB*

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The facultative intracellular pathogen Listeria monocytogenes causes listeriosis, a rare but life-threatening disease. Host cell entry begins with activation of the human receptor tyrosine kinase MET through the bacterial invasion protein InlB, which contains an internalin domain, a B-repeat, and three GW domains. The internalin domain is known to bind MET, but no interaction partner is known for the B-repeat. Adding the B-repeat to the internalin domain potentiates MET activation and is required to stimulate Madin-Darby canine kidney (MDCK) cell scatter. Therefore, it has been hypothesized that the B-repeat may bind a co-receptor on host cells. To test this hypothesis, we mutated residues that might be important for binding an interaction partner. We identified two adjacent residues in strand β2 of the β-grasp fold whose mutation abrogated induction of MDCK cell scatter. Biophysical analysis indicated that these mutations do not alter protein structure. We then tested these mutants in human HT-29 cells that, in contrast to the MDCK cells, were responsive to the internalin domain alone. These assays revealed a dominant negative effect, reducing the activity of a construct of the internalin domain and mutated B-repeat below that of the individual internalin domain. Phosphorylation assays of MET and its downstream targets AKT and ERK confirmed the dominant negative effect. Attempts to identify a host cell receptor for the B-repeat were not successful. We conclude that there is limited support for a co-receptor hypothesis and instead suggest that the B-repeat contributes to MET activation through low affinity homodimerization.

InlB is a virulence factor contributing to the pathogenicity of L. monocytogenes (2, 3). InlB binds to and activates the human receptor tyrosine kinase MET (4). MET downstream signaling then allows L. monocytogenes entering host cells. MET natively acts as receptor for hepatocyte growth factor/scatter factor (HGF/SF)² and is obligatory for normal embryonic development in vertebrates (5). InlB is able to mimic HGF/SF signaling (6, 7) and suffices to trigger normally non-phagocytic cells to take up InlB-coated latex beads (2).

InlB belongs to the bigger internalin protein family of the genus Listeria (8). Internalins are characterized by the presence of an N-terminal internalin domain containing a variable number of leucine-rich repeats (LRRs; see Fig. 1a). LRR motifs are frequently found in protein-protein interactions (9) and unsurprisingly account for most of the binding surface to the MET receptor in InlB (10). Additionally the InlB internalin domain might function as a weak homodimerization interface to initiate ligand-mediated MET dimerization (11). The center of the 595-amino acid mature InlB is constituted by the 70-amino acid B-repeat. C-terminally, InlB consists of three GW domains that are named for a conserved Gly-Trp sequence motif and structurally resemble SH3 domains. The GW domains anchor InlB non-covalently on the bacterial cell wall (12). They also bind to the host cell receptor gC1qR (13, 14) or heparan sulfate proteoglycans (14, 15) and enhance the ability of InlB to trigger MET phosphorylation and cellular phenotypes (10, 16).

We had previously determined the crystal structure of the InlB B-repeat (17). So far the B-repeat is the only structure representing Pfam domain Flg_new (PF09479) that occurs in more than 400 mostly secreted or cell surface-located proteins from over 300 eubacterial and few archaeal species (18). These proteins contain between one and 34 copies of the Flg_new domain that often occur in tandem arrays with up to more than 20 copies. Generally, the function of Flg_new domains and most proteins in which they occur are unknown. The DALI server (19) identifies β-grasp fold proteins as structurally most similar to the B-repeat.

The β-grasp fold is a motif found in a variety of small proteins or domains including ubiquitin, small ubiquitin-like modifiers (SUMOs), and protein L and protein G, two bacterial antibody-binding proteins (20). The core of the β-grasp fold consists of a
**Results**

**Mutations in Strand β2 of the B-repeat Impede the Ability of InlB to Stimulate Cell Motility**—Two servers predicting potential protein-protein interaction sites based on structures of monomeric proteins (30, 31) identified a likely binding hot spot in the B-repeat formed by the loop N-terminal of strand β4 (PINUP and ProMate) and the N terminus of strand β1 (PINUP only). In addition, we identified a potential interaction site in strand β2 of the B-repeat, because its sequence is least conserved among all secondary structure elements in Flg_new domains (17). Strands β1, β3, and β4 and the loop connecting strands β2 and β3 form the hydrophobic core of the domain, resulting in substantial sequence conservation. We speculated that strand β2 may have the freedom to evolve for binding of various interaction partners because of its high sequence variability. Interactions with strand β2 could either resemble the complex of protein L with antibody (β-sheet extension) or that of SUMOs bound to SUMO-interacting motifs (extended peptide in α/β groove).

To test the functional importance of the potential interaction sites, we introduced single or multiple substitutions (Table 1 and Fig. 1b) and tested their effect on the ability of InlB to stimulate cell motility of MDCK cells (Fig. 2a). The mutations were introduced into InlB392, a construct consisting of the internalin domain and the B-repeat that consistently stimulates MDCK cell colony scatter at a concentration of 10 nM (17). Mutations in the binding region predicted by the PINUP and ProMate servers had no effect on cell scattering (data not shown). The two variants with mutations in strand β2 behaved differently. Variant C (K335S/T336K/K337E) had no effect, whereas variant D (I334K/T336L) was unable to stimulate scattering (Fig. 2b). This encouraged us to investigate additional
variants with substitutions adjacent to Ile\textsuperscript{334} in strand β2. We generated three single-point mutations: T332E, V333E, and T336Y, to further pin down the interaction region of the B-repeat. InlB\textsubscript{392} V333E and InlB\textsubscript{392} T336Y elicited cell scattering at concentrations of 10 nM (Fig. 2b), whereas variant T332E abolished cell scattering. The loss of function in variant D can be solely attributed to the substitution of Ile\textsuperscript{334}, because mutation of Thr\textsuperscript{336} had no effect in variant C and in variant T336Y. Only mutation of residues whose side chains point to the top side of the β-sheet (Thr\textsuperscript{332} and Ile\textsuperscript{334}) resulted in loss of function. In contrast, substitution of neighboring amino acids whose side chains point to the exposed bottom side of strand /H\textsuperscript{9252}2, namely Val\textsuperscript{333} and Lys\textsuperscript{335}, had no effect. The side chains on the top side of strand /H\textsuperscript{9252}2 form a hydrophobic groove together with the long loop connecting strands /H\textsuperscript{9252}2 and /H\textsuperscript{9252}3. Thus, binding of the B-repeat to a hypothetical interaction partner may rather resemble the SUMO-1/Daxx\textsuperscript{20} interaction than that of protein L with antibodies.

**Mutations in the B-repeat Do Not Influence Protein Stability**—To test whether the observed loss of function is due to misfolding of the protein, we conducted biophysical characterization of the non-functional B-repeat variants. To this end, mutations T332E and I334K/T336L were introduced into an expression construct comprising only the B-repeat (residues 322–392). The purified protein variants were compared with the wild type B-repeat by size exclusion chromatography (SEC), CD spectroscopy, and differential scanning fluorimetry (DSF).

The SEC profiles of the wild type protein and both variants were virtually identical, illustrating that all three are well behaved proteins showing no signs of misfolding or aggregation (Fig. 3a). All proteins showed CD spectra typical for an overall β-sheet protein (Fig. 3b), revealing no (I334K/T336L) or only little (T332E) influence on the secondary structure. The calculated values for the wild type are mainly antiparallel β-sheet (43.1%) and other (41.4%) with only 4% helical content and 11.4% turn (32), which fits well to the crystal structure of the B-repeat. Melting point determination by DSF revealed no difference from wild type for the T332E variant and a small but significant (33) destabilization for the I334K/T336L variant (WT \(58.85 °C \pm 0.39 °C\), T332E \(60.45 °C \pm 0.29 °C\), and I334K/T336L \(52.69 °C \pm 0.56 °C\)). Still, the melting point of the I334K/T336L variant is well above the temperature at which the scatter assays were conducted. Taken together, these findings suggest that the effect on cell scatter is due neither to thermal instability nor to misfolding of the inactive protein variants.
Scatter Assays with Human HT-29 Cells Reveal a Dominant Negative Effect of Mutations in Strand β2—MDCK cells are canine cells. To examine the effect of the inactivating mutations on human cells, we tested the inactive InlB392 variants with HT-29 cells, a human cell line frequently used for colony scatter assays. We also probed InlB321, an InlB construct comprising only the internalin domain, to assess the effect of deleting the entire B-repeat. We analyzed InlB321 and the four InlB392 variants (T332E, V333E, I334K/T336L, and T336Y) for their ability to stimulate scatter at concentrations ranging from 1 to 1000 nM. As reference, we used InlB392 at 1 nM, a concentration that had previously been shown to be sufficient for the induction of cell motility in HT-29 cells (17). The activity pattern of the InlB392 variants with HT-29 cells was similar to that with MDCK cells. InlB392 T332E and InlB392 I334K/T336L were unable to induce cell scatter up to 1000 nM (Fig. 4). The other point mutations had no effect on cell motility (Fig. 4), once again demonstrating the site specificity that causes loss of function. Interestingly, InlB321 stimulated HT-29 cells to scatter at only a 10-fold higher concentration than InlB392. This is surprising, because InlB321 was unable to induce cell scatter up to 1 μM, the highest concentration that was tested (11). In contrast, HT-29 cells reproducibly scattered upon the addition of 10 nM InlB321 (Fig. 4). Thus, the mutations T332E and I334K/T336L are not only inactivating, but actually they dominantly impair InlB function beyond the effect observed upon deletion of the B-repeat. This behavior is interesting but not easily explained. Therefore, we introduced the T332E mutation into full-length InlB (InlB T332E) and additionally generated an internal deletion of the B-repeat, resulting in a construct in which the GW domains are directly linked to
the internalin domain (InlB ΔB-repeat; Fig. 1a). We then compared their ability to stimulate HT-29 cell scattering to that of wild type full-length InlB. As expected, wild type InlB was the most active protein among the tested constructs and induced scattering at concentrations down to 125 pM (Fig. 4). InlB T332E and InlB ΔB-repeat were less active by approximately a factor of 4 (Fig. 4). These results demonstrate that the GW domains can to some extent compensate the effect of either deletion of the B-repeat or mutation within strand 2o ft h e B-repeat.

**Effect of Inactivating Mutations on Phosphorylation of MET, AKT, and ERK**—Scattering is the visible phenotype at the end of an extensive cellular signaling cascade. Hence we were interested whether the results of the scattering assays are reflected by intracellular MET signaling. We tested the ability of different InlB variants to stimulate MET, AKT, and ERK phosphorylation in Vero cells, the cell line typically used to assess InlB-induced intracellular signaling pathways (4, 7, 16, 34). For comparison we used 500 pM full-length InlB, a concentration that repeatedly gave a well detectable but unsaturated level on our Western blots. InlB ΔB-repeat stimulated phosphorylation of MET, AKT, and ERK at a concentration of 1 nM or more (Fig. 5a), indicating a 2-fold reduced activity. Interestingly, this activity is comparable with that of the GW domain-truncated construct InlB392 (Fig. 5b), suggesting that truncation of the GW domains and deletion of the B-repeat have similar effects on MET activation. Mutation T332E reduced the activity of full-length InlB by a factor of 10 (Fig. 5a), highlighting that an inactivating mutation in the B-repeat has a stronger effect on activation of MET and its downstream signaling targets than the deletion of the whole domain. This effect was not clearly visible in the HT-29 scattering assays.

Once more, there was no difference between InlB392, wild type and the V333E (Fig. 5a) or T336Y (data not shown) variants. All three proteins induced phosphorylation of MET and its downstream targets at a ligand concentration of 1 nM. Consistent with the scattering assays, InlB392 variants with altered side chains on the top side of the β2-strand (InlB392 T332E and InlB392 I334K/T336L) were almost inactive. Only a little MET phosphorylation was visible at a concentration of 100 nM, and no further downstream signaling could be observed (Fig. 5a). InlB321 was ~5-fold less active than InlB392 and induced phos-
phorylation at a concentration of 5 nM (Fig. 5a). Generally, the results of the phosphorylation assays agreed well with those of the HT-29 scattering assay but additionally pointed out that the mutation of T332 in full-length InlB had more impact on MET activation than deletion of the entire B-repeat.

No Evidence for Binding of the B-repeat to Cell Surface Molecules in FACS Experiments—The observation that mutation of a single amino acid on the surface of the B-repeat abolishes MET activation corroborates the hypothesis of a co-receptor being involved in MET activation by InlB through interaction with the B-repeat. To test this hypothesis, we performed FACS experiments on human HT-29 and A549 cells and on MET-deficient T47D cells. Cells treated only with the antibody and cells incubated with InlB321 F104S variant that does not bind to MET were used as negative control.

The experimental data clearly showed binding of InlB321 and InlB392 to HT-29 (Fig. 6a) and A549 (Fig. 6b), whereas cells incubated with InlB321 F104S were indistinguishable from cells treated only with anti-HA antibody coupled to phycoerythrin. The fluorescence in experiments with InlB321 and InlB392 was due to specific binding of these proteins to the MET receptor, because MET-deficient T47D cells did not show any fluorescence signal (Fig. 6c). In the same experiments, no binding of the B-repeat was observed (Fig. 6, a and b). These results call into question a direct binding of the B-repeat to some co-receptor or any other molecule on the cellular surface. If there was any interaction, its affinity would be so low that it could not be detected by FACS.
Discussion

In this study we examined the function of the InlB B-repeat in the context of MET receptor activation. We identified a region in the β2-strand that is important for MET signaling. Interestingly, mutations in this region have a stronger effect than a deletion of the whole domain, which raises the question of how this works at the molecular level. We can exclude that the inactivating mutations impair binding of InlB to MET. Although MET is the primary interaction partner of InlB, the B-repeat most likely does not bind to MET, because no direct interaction was observed in surface plasmon resonance experiments (17).

The B-repeat was suggested to bind an additional receptor other than MET that would enhance activation of the mitogen activated protein kinase pathway downstream of MET (7). Based on our structural and functional data, we had also suggested that the B-repeat might bind a co-receptor to enhance MET activation (17). The fact that we could identify a functionally relevant site based on structural similarity to the SUMO-1 binding site (25) supports the hypothesis of a further receptor being involved. Indeed, there have been several co-receptors reported to participate in or enhance activation of MET by its physiological ligand HGF/SF, including neuropilin-1 (36), ICAM-1 (37), integrin αβ4 (38, 39), and CD44v6 (40). CD44v6 was also shown to participate in MET activation by InlB (41).

However, the effect of CD44v6 on InlB-mediated MET phosphorylation was also observed with the isolated internalin domain (InlB321), suggesting that it cannot be mediated by the B-repeat.

The fact that we were not able to detect binding of the B-repeat to two human cell lines in FACS experiments indicates that either there is no surface-exposed receptor on host cells or binding is too weak to be detected. If there was only very weak binding of the B-repeat to such a co-receptor, it would be difficult to address and unlikely to be seen in FACS experiments. The observation that a single point mutation in the B-repeat has a stronger effect on MET activation than deletion of the whole domain makes the theory of co-receptor binding even less favorable. In line with this, recent literature reports also suggest that plain receptor dimerization without participation of any co-receptor is sufficient for full MET activation. Monoclonal antibodies, for instance, are able to fully trigger MET phosphorylation and cellular responses (42). Even small artificial MET binding macrocycles and DNA aptamers, which are unlikely to bind further co-receptors, are able to elicit full MET activation when dimerized (43, 44).

How could the B-repeat enhance MET activation, if it binds neither to MET nor to a cell surface co-receptor? Our current hypothesis is that it might form one of several low affinity homodimerization contacts contributing to MET dimerization (Fig. 7). This idea would be consistent with the contemporary models of how other RTKs are activated. Newer data suggest that RTK dimerization can occur even in the absence of ligand, constituting a first step toward activation of the receptor. Binding of the ligand stabilizes the dimeric tyrosine kinase complex and thereby changes the probability of phosphorylation (45). It has been known for over 20 years that the transmembrane helices of receptor tyrosine kinases including MET bear a common sequence, the GXXXG motif, which might self-dimerize the transmembrane helices (46). The full-length structures of KIT and PDGF receptor bound to their ligands stem cell factor and PDGF-B, respectively, revealed that in the case of class III receptor tyrosine kinases, multiple regions of the extracellular receptor contribute to the actively dimerized kinase complex (47–50). Several oncogenic mutations of the KIT receptor have been analyzed, implying that normal receptor activation is an interplay between ligand binding, formation of homotypic receptor contacts, and transphosphorylation (49). A common factor of all referenced models is that proper receptor activation is a cooperative process involving several matched weak interactions along the length of the receptor. Could we apply these models to MET receptor activation by InlB?

InlB392 and also the complex of InlB392 bound to the Met ectodomain are strictly monomeric in solution (17). Nevertheless, InlB392 is a potent MET activator. Recently, ligand-independent MET receptor dimers were confirmed on the cellular surface (51, 52). It is also known that a covalently linked dimer of the internalin domain, structurally resembling a non-covalent InlB321 dimer that frequently occurs in crystal structures, is a highly potent MET activator (11). Disrupting this homodimerization interface of InlB led to a strong reduction or complete loss of activity in full-length InlB and InlB321, respectively. Thus, this very low affinity dimerization interface clearly appears to be of biological relevance (11). It is conceivable that
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The residues mutated in this study are shown as sticks. Color coding of mutated residues is the same as in Fig. 1. The β2-strand of each chain is highlighted in magenta. The pseudo 2-fold axis is indicated by a black line, but the dimeric arrangement is not really C2 symmetric; a displacement of roughly one amino acid along the β-sheet can be observed.

MET dimerization is achieved through the cooperative effect of several weak interactions (Fig. 7).

The crystal structure of the InlB B-repeat actually revealed a potential homodimer (17). The asymmetric unit contains four monomers that are arranged into two structurally equivalent pairs (chains A + B and chains C + D). The recurrence of packing contacts in crystals suggests that they may be biologically relevant (53). According to the PISA server (54), these homodimeric quaternary structures fall into a gray region of complex formation criteria, so that the complex may or may not be stable in solution. In solution, the B-repeat is definitely monomeric. Nevertheless, the interface between the protomers in the potential B-repeat homodimer may represent an evolved, biologically relevant dimerization site.

In our previous analysis, we had considered this potential homodimerization to be a crystal packing artifact rather than biologically relevant (17). This assessment was based on biochemical data, results from the PISA server, and the fact that the potential B-repeat homodimer is asymmetric (Fig. 8). Most biological homodimers have C2 point group symmetry (55, 56). In contrast, chains A and B (or C and D) of the InlB B-repeat are related by a rotation of roughly 176.5° (instead of 180° for a C2 dimer) and a translational component (a shift of roughly 3 Å along the rotation axis) introducing considerable asymmetry (Fig. 8). However, asymmetric dimers may be of physiological relevance as exemplified by the tyrosine kinase domain of EGFR (57, 58). Asymmetric tyrosine kinase domain dimers coupled to C2-symmetric extracellular domain dimers have also been described recently in electron microscopic reconstructions of the complete ligand-bound KIT and PDGF receptor (47, 48). Thus, it appears conceivable that an asymmetric B-repeat dimer could be linked to a C2-symmetric dimer of the InlB internalin domain bound to the MET ectodomain. The connection between the InlB internalin domain and the B-repeat is very flexible (14, 17) so that the linker between the two domains should allow for the small translational offset of the two protomers in the asymmetric B-repeat dimer.

The interface of the potential B-repeat homodimer is formed by strand β2 and the loop connecting β2 and β3. Interestingly, the amino acids that impair MET activation when mutated have the largest buried surface-accessible area among the interfacing residues (Fig. 8 and Table 2). Our data suggest that we identified a low affinity dimerization interface in the B-repeat with our mutations, similar to the interface on the convex side of the LRRs (11). In this case, the mutations of the small, uncharged residues to glutamate or lysine would turn a rather small but overall stabilizing contribution of the B-repeat into a repelling disturbance. A model in which the signaling active InlB-MET complex is stabilized cooperatively by several weak homophilic interactions from various domains of both the receptor and the ligand could explain many of the observed effects. The intrinsic dimerization propensity of MET would be enhanced upon binding of InlB, assembling a signaling active complex that is built around the weak InlB homodimer at its center (59). InlB321, the shortest InlB construct that we tested, has the ability to dimerize MET via its LRR dimerization interface (11, 51). InlB392, the dimerization interface of the wild type B-repeat would contribute an additional stabilizing interaction, whereas the mutation of amino acids in the dimerization interface would completely prevent formation of a signaling competent complex because of steric conflicts and charge repulsion. In the full-length protein, mutations in the dimerization interface of the B-repeat would result in a reduction but not in a complete loss of activity, because the clustering medi-

### TABLE 2

| Residue | ASA C | BSA C | % BSA C | ASA D | BSA D | % BSA D | ASA A–D Mean | BSA A–D Mean |
|---------|--------|--------|---------|--------|--------|---------|-------------|-------------|
| Tyr372  | 45.5   | 0.0    | 0.0     | 45.5   | 0.0    | 0.0     | 45.5        | 0.0         |
| Thr374  | 15.6   | 0.0    | 0.0     | 15.6   | 0.0    | 0.0     | 15.6        | 0.0         |
| Thr376  | 14.6   | 0.0    | 0.0     | 14.6   | 0.0    | 0.0     | 14.6        | 0.0         |
| Thr378  | 14.6   | 0.0    | 0.0     | 14.6   | 0.0    | 0.0     | 14.6        | 0.0         |
| Thr380  | 14.6   | 0.0    | 0.0     | 14.6   | 0.0    | 0.0     | 14.6        | 0.0         |
| Thr382  | 14.6   | 0.0    | 0.0     | 14.6   | 0.0    | 0.0     | 14.6        | 0.0         |
| Thr384  | 14.6   | 0.0    | 0.0     | 14.6   | 0.0    | 0.0     | 14.6        | 0.0         |
| Thr386  | 14.6   | 0.0    | 0.0     | 14.6   | 0.0    | 0.0     | 14.6        | 0.0         |
| Thr388  | 14.6   | 0.0    | 0.0     | 14.6   | 0.0    | 0.0     | 14.6        | 0.0         |
| Thr390  | 14.6   | 0.0    | 0.0     | 14.6   | 0.0    | 0.0     | 14.6        | 0.0         |
| Thr392  | 14.6   | 0.0    | 0.0     | 14.6   | 0.0    | 0.0     | 14.6        | 0.0         |
| Thr394  | 14.6   | 0.0    | 0.0     | 14.6   | 0.0    | 0.0     | 14.6        | 0.0         |
| Thr396  | 14.6   | 0.0    | 0.0     | 14.6   | 0.0    | 0.0     | 14.6        | 0.0         |
| Thr398  | 14.6   | 0.0    | 0.0     | 14.6   | 0.0    | 0.0     | 14.6        | 0.0         |
| Thr400  | 14.6   | 0.0    | 0.0     | 14.6   | 0.0    | 0.0     | 14.6        | 0.0         |
| Thr402  | 14.6   | 0.0    | 0.0     | 14.6   | 0.0    | 0.0     | 14.6        | 0.0         |
ated by the GW domains and their interaction with heparan sulfate proteoglycans (10, 14–16) can partially compensate the repulsive forces in the mutated B-repeat. In essence one could envisage InlB to consist of an essential MET-binding module (the internalin domain) and two partially redundant modules (B-repeat and GW domains) that enhance signaling by dimerization or clustering, where one domain can compensate the lack of the other to a certain degree.

**Experimental Procedures**

Cloning, Protein Expression, and Purification—Expression vectors coding for the different InlB variants were created by site-directed mutagenesis or round the horn cloning of the published expression vector of InlB<sub>392</sub> (17), the InlB B-repeat (17), InlB<sub>B</sub>, and full-length InlB (10). The sequence was verified by the in house sequencing service at Bielefeld University. All proteins were expressed as GST fusion protein in *Escherichia coli* BL21 CodonPlus-RIL (Invitrogen). Protein expression was induced at an A<sub>600</sub> of 0.6–0.8 in LB or TB medium at 20 °C overnight by the addition of 1 mM isopropyl-β-D-thiogalactoside. The cells were pelleted by centrifugation and lysed (cell homogenizer; Stansted Fluid Power) in ice-cold PBS containing complete protease inhibitors (Roche). The GST fusion protein was captured on a glutathione affinity matrix, and the GST tag was removed by cleavage with tobacco etch virus protease. Cleaved protein was eluted using an appropriate buffer and further purified by either ion exchange chromatography (Source S or Source Q; GE Healthcare) or size exclusion chromatography (Superdex 75 or Superdex 200; GE Healthcare) depending on estimated protein purity on SDS-polyacrylamide gels.

**CD Spectroscopy**—Proteins were dialyzed twice against a 5000-fold excess of 5 mM sodium phosphate, pH 7.4, 5 mM NaCl buffer and diluted to a final concentration of 0.1 mg/ml in the same buffer. Measurements were performed on a Jasco J810 CD spectropolarimeter (Jasco Inc.) in a wavelength range between 190 and 270 nm with a step width of 0.1 nm and a scanning speed of 50 nm/min. Each spectrum represents the average of three single spectra of the same sample. Secondary structure estimation was performed with the web interface based on the algorithm released by Micsonai et al. (32).

**Differential Scanning Fluorimetry**—DSF experiments were essentially carried out as described (33, 60). The samples were prepared with a final protein concentration of 0.5 mg/ml and a final SYPRO® Orange (Thermo Scientific) concentration of 1× in PBS. Melting curves were recorded in a temperature range from 25 to 95 °C in 1 °C increments on a StepOnePlus™ Thermocycler (Applied Biosystems). The data were evaluated using an Excel chart provided by the structural genomics consortium (33) with Excel (Microsoft) and Prism (GraphPad).

**Cell Lines and Media**—All media were supplemented with 2 mM glutamine (Lonza), 100 IU/ml penicillin, and 100 μg/ml streptomycin (Lonza) and different concentrations of FCS (PAN Biotech). MDCK cells were maintained in DMEM high glucose (GE Healthcare) with 5% FCS. HT-29 (human colon adenocarcinoma) cells were grown in Hyclone DMEM/F12 1:1 (GE Healthcare) with 10% FCS. A549 (human adenocarcinoma alveolar basal epithelial) and Vero (African green monkey kidney) cells were grown in DMEM high glucose (GE Healthcare) with 10% FCS. T47D (human ductal breast epithelial tumor) cells were cultivated in RPMI 1640 medium (Lonza) with 10% FCS.

**MDCK Cell Colony Scatter Assay**—For scattering assays, the cells were seeded in 24-well plates with a density of 1×10<sup>4</sup> cells/well. After incubation overnight, the medium was exchanged, and ligands were added in the stated final concentrations. Scattering was scored blindly after 24 h by three people. The experiments were performed at least three times.

**HT-29 Cell Colony Scatter Assay**—HT-29 cells were seeded with a density of 5×10<sup>3</sup> cells/well in 24-well plates and grown until well defined colonies were visible. The cells were washed twice with warm PBS, serum-starved overnight, and incubated with the stated ligands diluted in serum-free medium for at least 16 h. Scattering of the cells was scored blindly by three people before images were taken. The experiments were performed at least three times.

**Phospho Western Blots**—Vero cells were plated in 6-well plates and grown to confluence. After starvation in serum-free medium overnight, ligands diluted in serum-free medium were added for 7 min. The medium was removed, and the cells were immediately lysed in ice-cold RIPA buffer containing phosphatase and protease inhibitors (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 μg/ml PMSF) for 30 min on ice. Afterward the lysates were boiled in SDS sample buffer, separated by SDS-PAGE and analyzed by Western blot using specific antibodies (phospho-MET (Tyr<sup>1254</sup>/1255)) D26 XP® rabbit mAb, Met (pan) 25H2 mouse mAb, Akt (pan) 11E7 rabbit mAb, phospho-Akt (Ser<sup>473</sup>) D9E XP® rabbit mAb, phospho-ERK p44/42 (Thr<sup>202</sup>/Tyr<sup>204</sup>) D13.14.4E XP® rabbit mAb, and ERK (pan) 137F5 rabbit mAb). All primary antibodies were acquired from Cell Signaling Technology, and secondary antibodies were from Jackson ImmunoResearch. Phospho-MET blots were densitometrically quantified using ImageJ (61). To account for loading and transfer errors, the intensities of bands of interest were normalized against cross-reactive protein band with a molecular mass of 90 kDa. For each sample value, the negative control was subtracted, and the value was normalized to the value of 500 pm InlB full on the same blot that served as internal reference.

**FACS Analysis**—The cells were detached with 5 mM EDTA in PBS by incubating at 37 °C for 30 min and resuspended in PBS at a density of 2–3×10<sup>6</sup> cells/ml. To hinder unspecific antibody binding, 1 ml of cell suspension/sample was incubated with 10 μl of Ig receptor blocking reagent (Miltenyi Biotec) dissolved in PBS containing 1% BSA and 0.1% NaN<sub>3</sub>. Unlabeled reference cells were not treated further. The other samples were then incubated with ligand (500 nM HA-tagged InlB<sub>321</sub>, InlB<sub>392</sub>, InlB<sub>B</sub>F104S, and B-repeat) diluted in PBS with 1% BSA and 0.1% NaN<sub>3</sub> for 1 h on a rotating wheel followed by incubation with phycoerythrin-coupled anti-HA antibody (Columbia Biosciences) under the same conditions. The samples used as negative control were not incubated with ligand but only with the antibody after the Fc receptor blocking step. All steps were carried out in 1.5-ml reaction tubes on ice or at 4 °C, and centrifugation was conducted at 4 °C at 200 g for 5 min.
MET-activating Residues in the InlB B-repeat

In between incubation steps, the cells were washed twice with 1 ml of PBS. Finally, samples were prepared for analysis by filtration through a CellTrics® filter (Ø 30 μm; Partec) followed by vortexing. The cell suspension was directly probed in a CyFlow® space flow cytometer (Partec).

Author Contributions—W. M. B. and H. H. N. designed the study and wrote the paper. W. M. B. performed most experiments except FACS experiments. N. L. performed the FACS experiments and cloned the required proteins. M. E. designed and cloned the initial double and triple mutant vectors, purified the proteins, and performed some of the MDCK colony scattering assays. D. M. cloned the single-point mutations in InlB393 and prepared the proteins. C. G. purified full-length InlB variants and conducted the ERK and AKT Western blots. All authors analyzed the results and approved the final version of the manuscript.

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