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Ligand-Mediated Dimerization of the Met Receptor Tyrosine Kinase by the Bacterial Invasion Protein InlB

Davide M. Ferraris¹, Ermanno Gherardi², Ying Di², Dirk W. Heinz^{1*} and Hartmut H. Niemann^{3*}

¹Division of Structural Biology, Helmholtz Centre for Infection Research (HZI), Inhoffenstraße 7, 38124 Braunschweig, Germany

²MRC Centre, Hills Road, CB2 2QH, Cambridge, United Kingdom

³Department of Chemistry, Bielefeld University, Postfach 100131, 33501 Bielefeld, Germany

* *Corresponding authors.* E-mail addresses: Hartmut.Niemann@uni-bielefeld.de
Dirk.Heinz@helmholtz-hzi.de

Present address: D.M. Ferraris, DiSCAFF-Department of Chemical, Food, Pharmaceutical and Pharmacological Sciences, University of Piemonte Orientale A. Avogadro, I-28100 Novara, Italy.

Abbreviations used:

CD, crystal dimer; HGF/SF, hepatocyte growth factor/scatter factor; HSPG, heparan sulfate proteoglycan; IR, inter-repeat; LRR, leucine rich repeat; MDCK, Madin-Darby canine kidney; RTK, receptor tyrosine kinase;

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The *Listeria monocytogenes* surface protein InlB mediates bacterial invasion into host cells by activating the human receptor tyrosine kinase Met. So far it is unknown how InlB or the physiological Met ligand HGF/SF cause Met dimerization, which is considered a prerequisite for receptor activation. We determined two new structures of InlB revealing a recurring, antiparallel, dimeric arrangement, in which the two protomers interact through the convex face of the leucine rich repeat domain. The same contact is found in one structure of the InlB-Met complex. Mutations disrupting the inter-protomeric contact of InlB reduced its ability to activate Met and downstream signaling. Conversely, stabilization of this crystal contact by two intermolecular disulfide bonds generates a constitutively dimeric InlB variant with exceptionally high signaling activity, which is able to stimulate cell motility and cell division. These data demonstrate that the signaling-competent InlB-Met complex assembles with 2:2 stoichiometry around a back-to-back InlB dimer, enabling the direct contact between the stalk region of two Met molecules.

Introduction

Growth factor receptors belonging to the receptor tyrosine kinase (RTK) family are allosteric enzymes important in the control of cell growth, proliferation, differentiation and migration.¹ RTKs are transmembrane proteins that are typically activated by ligand-induced dimerization (or oligomerization).^{2; 3; 4; 5} This enables them to efficiently cross-phosphorylate their adjacent cytoplasmic tyrosine kinase domains and provides docking sites for a large variety of phosphotyrosine-binding proteins involved in signal transduction. Overactivation and/or impaired deactivation of RTK signaling pathways is strongly associated with carcinogenesis, making RTKs important targets for anti-cancer therapy.⁶ The RTK and proto-oncogene Met⁷ which is specifically activated by the hepatocyte growth factor/scatter factor (HGF/SF)^{8; 9; 10; 11} mediates cell proliferation, motility and morphogenesis. While normal Met-signaling is required during embryonic development^{12; 13}, liver regeneration^{14; 15} and wound healing¹⁶, abnormal signaling has been strongly implicated in the development of invasive and metastatic tumor phenotypes.⁷

The ectodomain of Met is composed of six individual domains, the N-terminal Sema domain, a 7-bladed β -propeller, followed by a cysteine-rich PSI domain and four immunoglobulin-like domains (Ig1-Ig4). While the β -chain of HGF/SF specifically interacts with the Sema domain¹⁷, binding sites on Met for the α -chain of HGF/SF have been identified both on the Sema domain¹⁸ and Ig3 and Ig4.¹⁹ Met is also the receptor for the invasion protein InlB from the food-borne human pathogenic bacterium *Listeria monocytogenes*.²⁰ InlB is a cell surface protein belonging to the leucine-rich repeat (LRR) family of internalins²¹ that promotes bacterial uptake by a variety of human host cells.^{22; 23; 24} Most interestingly InlB-mediated activation of Met leads to cellular phenotypes similar to those caused by HGF/SF, i.e. cell migration, DNA synthesis and tubulogenesis, making InlB a functional mimic of the structurally unrelated human growth factor.^{20; 25; 26} The N-terminal solenoid-like leucine

rich repeat (LRR) domain of InlB^{27; 28} is responsible for specific binding to the Met receptor.^{20; 29} The recently determined crystal structure of Met in complex with InlB²⁵ unexpectedly revealed that the LRR-domain of InlB interacts with the first Ig-like domain of the Met stalk. A second, less pronounced interface is formed between the inter-repeat (IR) region of InlB, an immunoglobulin-like domain adjacent to the LRR-domain, and the Sema domain of Met, causing stiffening of the otherwise flexible Met ectodomain. Despite the available structural information on Met in complex with fragments of HGF/SF¹⁷ and InlB²⁵ the precise mode of Met activation remains elusive. Here we aimed at defining the structural determinants of InlB-induced Met dimerization. We show that disruption of a dimer contact recurrent in different InlB crystal forms inhibits Met activation. Conversely, stabilization of this crystal contact by means of two intermolecular disulfide bonds generates a potent Met agonist. These data strongly suggest that InlB-mediated Met activation proceeds through the formation of discrete 2:2 complexes that are stabilized by several weak contacts.

Results

Two new crystal forms of InlB and the structure of the InlB-Met complex reveal a recurrent InlB-InlB dimer

Efforts to crystallize the InlB-Met complex resulted in two novel crystal forms of the InlB internalin domain (InlB₃₂₁, amino acids 36-321). This fragment, which comprises the cap, the LRR and the IR region, is capable of inducing Met phosphorylation but not cellular responses, such as colony scattering and cell division.²⁵ In the new triclinic and rhombohedral crystal forms (Table 1), two InlB₃₂₁ molecules, pack as an antiparallel dimer with two-fold symmetry. The dimer interface is formed by the LRR convex face and centered on the 3₁₀-helices of LRRs 6 and 7 (Fig. 1a), leaving the Met binding site on the concave face accessible. This “crystal dimer” occurs four times in these crystal forms, suggesting it to be a favorable, low-energy arrangement. Subsequent inspection of the published low resolution crystal form II of the InlB-Met complex (PDB code 2UZY)²⁵ revealed the presence of an identical crystal dimer contact (Fig. 1b). This crystal form contains two 1:1 InlB-Met complexes in the crystallographic asymmetric unit. Each of these complexes forms the InlB crystal dimer contact with the second complex from a neighboring asymmetric unit, so that the two InlB molecules are related by a non-crystallographic two-fold rotation axis. By choosing a different origin, the 2:2 complex assembled around the InlB crystal dimer can also be placed in the asymmetric unit. In this structure the Met molecules are related by a 2-fold axis and the C-termini point in the same direction. The distance between the C-termini of the Ig2-domains, the most C-terminal domains present in this structure, is approximately 30 Å (Fig. 1c). Actually, the Ig2 domains form a second contact mainly through residues from strands F and G, but the contact is structurally not well defined due to the low resolution of crystal form II. The contributions of individual side chains and details of the contact like salt-bridges or hydrogen bonds are thus not resolved. Sterically the arrangement of

Met in this 2:2 complex is most plausible in a physiological setting where the cytoplasmic tyrosine kinase domains need to approach each other closely for efficient cross-phosphorylation and downstream signaling.

Disruption of the InlB-InlB crystal dimer interface impairs Met activation

The frequent occurrence of the crystal dimer contact in complexed and uncomplexed crystal forms of InlB prompted us to study its physiological relevance. Analysis of the interface revealed a total buried surface area of about 1400 Å². The interface area is characterized by surface exposed residue pairs Ala205/Gly206 and Ala227/Gly228 allowing for a close approach of two InlB monomers. In addition, hydrogen bonds occurring between the carbonyl oxygen of Asp263 and the side chain of Ser183 and salt bridges between Asp200/Arg224-pairs contribute to the specificity of the interaction (Fig. 2a). The interface size is typical for a crystal contact with two-fold symmetry (crystal dimer) but too small for a homodimer that forms in solution.³⁰ To test the hypothesis of physiological relevance of InlB dimerization we introduced mutations aimed at disrupting the dimer interface.

The interface is small and very open and modelling at the graphics workstation showed that for any single mutation, at least one frequently populated rotamer exists, for which no clashes would occur. Only combining several mutations ensured the occurrence of clashes that prevent formation of the crystal dimer contact. We mutated Gly206 and Ala227 to destroy the flat, hydrophobic surface, and Asp200 to disrupt the salt bridges. All three residues were mutated to arginine, additionally introducing strong electrostatic repulsion between approaching InlB protomers (Fig. 2b). A fourth mutation (S199R) was fortuitously introduced by the mutagenesis reaction. The resulting InlB variant was termed InlB_{321_4R}. The crystal structure of InlB_{321_4R} shows no significant differences to wild-type (Table 1 and Fig. S1, Supplementary Information) confirming the structural integrity of the

quadruple mutant. On gel filtration it behaved like wild type InlB₃₂₁ (Fig. S2b, Supplementary Information). As expected, InlB₃₂₁_4R bound Met with the same affinity as wild type InlB₃₂₁ (Fig. 2c). The ability of InlB₃₂₁_4R to activate Met and Met-dependent downstream targets, such as Erk1/2 and Akt, was tested. After stimulation with InlB₃₂₁_4R, Met, Erk1/2 and Akt phosphorylation were severely reduced compared to wild-type InlB₃₂₁ (Fig. 2d and Fig. S3a, Supplementary Information). Because InlB₃₂₁ does not elicit cellular responses like cell colony scattering, the D200R, G206R and A227R mutations were also introduced into full-length InlB (InlBfl), and the resulting protein was termed InlBfl_3R. This variant was assayed for its ability to stimulate Met, Erk1/2 and Akt phosphorylation and cell scattering. The latter was tested in the standard Madin-Darby canine kidney (MDCK) cell scattering assay¹⁰ using the physiological Met ligand HGF/SF and InlBfl as references. InlBfl_3R showed reduced Met, Erk1/2 and Akt phosphorylation (Fig. 3a and Fig. S3b, Supplementary Information) and a decrease in scattering potency of two orders of magnitude compared to wild type InlBfl (Fig. 3b,c). The residual activity of InlBfl_3R may be attributed to unspecific clustering of 1:1 InlB-Met complexes by host cell heparan sulfate proteoglycans (HSPGs).²⁵ This clustering is mediated by the InlB GW domains, three C-terminal domains named for a conserved Gly-Trp motif that are positively charged and bind HSPGs with high affinity.^{31; 32} These results clearly establish that an intact InlB-InlB dimer interface is a prerequisite for efficient activation of Met and downstream key Met effectors, such as Erk1/2 and Akt.

Stabilization of the InlB-InlB crystal dimer interface by two intermolecular disulfide bonds generates a potent Met agonist

Having demonstrated the essential role of the InlB dimer interface in the assembly of the ligand receptor complex and in Met phosphorylation and signaling, we next hypothesized that stabilization of

this interface would enhance both Met phosphorylation and signaling potency. We designed a constitutively dimeric variant termed InlB₃₂₁ crystal dimer (InlB₃₂₁_CD) in which the crystal dimer geometry is fixed by two intermolecular disulfide bonds across the crystal dimer interface. To this end, two cysteine mutations were introduced on the 3₁₀-helices of LRR6 (Gly206) and LRR7 (Ala227) (Fig. 4). The purity and the oligomeric state of the dimeric variant were assessed by SDS-PAGE and gel filtration chromatography (Fig. S2, Supplementary Information). The success of the design was further confirmed by determining the crystal structure of InlB₃₂₁_CD. The protein crystallized isomorphously under conditions similar to the rhombohedral crystal form of monomeric InlB₃₂₁ (Table 1). The electron density of the intermolecular disulfides is well defined and the two InlB protomers bear the expected crystal dimer arrangement (Fig. 4) with an r.m.s.d. of 1.2 Å for the Cα-positions when the InlB₃₂₁_CD dimer is compared to the symmetric arrangement of two monomers in the triclinic or rhombohedral crystal forms.

It is noteworthy that Met activation by InlB₂₄₈, a frequently used InlB construct truncated at amino acid 248, can be attributed to fortuitous dimerization *via* a disulfide bond, as the single cysteine 242 in the InlB internalin domain, which is otherwise buried, becomes accessible upon truncation.³³ Our rationally designed InlB₃₂₁_CD differs from this accidentally formed dimer in two respects. First, InlB₂₄₈ lacks the IR region. Second, this shorter dimer is linked by only one disulfide, allowing for rotational freedom around this single bond. Hence, no defined orientation of the two monomers with respect to one another can be expected for InlB₂₄₈, whereas InlB₃₂₁_CD specifically fixes the orientation of the two monomers from the biologically active InlB-Met complex.

We tested the activity of InlB₃₂₁_CD in Met, Erk1/2 and Akt phosphorylation and in MDCK cell scattering assays. In the phosphorylation assay, InlB₃₂₁_CD was some two orders of magnitude more potent than monomeric InlB₃₂₁ (Fig. 5a, 2d and Fig. S3a, c, Supplementary Information) and it rivaled

the activity of HGF/SF (Fig. 5a). In cell scattering assays, monomeric InlB₃₂₁ was inactive, whereas InlB₃₂₁_CD showed activity at concentrations below 10⁻¹¹ M. Thus, InlB₃₂₁_CD was more potent than InlBfl and it matched the activity of HGF/SF (Fig. 5b,c). In addition to the remarkable mitogenic response, InlB₃₂₁_CD also caused a pronounced mitogenic effect as assayed by increased DNA synthesis (Fig. 5d). Again, monomeric InlB₃₂₁ was inactive in this assay, whereas InlB₃₂₁_CD exceeded the activity of InlBfl, and approached that of HGF/SF.

In order to investigate the role of the IR domain in Met activation and signaling, a second InlB variant bearing the crystal dimer arrangement stabilized by two disulfide bonds was generated. This variant comprises the Cap and LRR but lacks the IR region and is termed InlB₂₄₁_CD (residues 36-241 of InlB harboring the G206C and A227C mutations). Again, purity and the oligomeric state were checked (Fig. S2, Supplementary Information). The biological activity of InlB₂₄₁_CD was tested in MDCK cell colony scattering assays. This shorter dimer stimulated cell scattering down to concentrations below 10⁻⁹ M (Fig. 5b,c). Thus it was ~ 100-fold less active than InlB₃₂₁_CD although the InlB₂₄₁_CD dimer in turn was still ~ 100 fold more active in MDCK colony scattering assays than the previously described construct dimerized by a single disulfide involving Cys242³³ (our unpublished data).

Discussion

InlB dimerizes upon binding to Met

The data presented here reveal the structure of the signaling active 2:2 InlB-Met complex, representing the first crystal structure of a dimeric complex between Met and a ligand, the physiological relevance of which could be proven by mutagenesis. Mutations disrupting the contact site of the crystal dimer abolish Met activation by monomeric InlB₃₂₁ and strongly reduce the activity of InlBfl without effects on Met binding, while stabilization of the crystal contact by two disulfide bonds dramatically enhances signaling potency. Our initial model of InlB-mediated Met activation considered the formation of 1:1 complexes and subsequent receptor clustering by heparan sulfate proteoglycans²⁵. This can now be refined to include discrete 2:2 complexes as the basic signaling unit, clustered in the case of InlBfl by heparan sulfate (Fig. 6).

InlB exists as a monomer in solution.³³ Analytical ultracentrifugation and small angle X-ray scattering analysis have not revealed dimeric species of InlB or the InlB₃₂₁/Met complex in solution,^{25; 34} suggesting low binding affinities between the single InlB-Met units. Nevertheless, the recurrence of the crystal dimer in several crystal forms of InlB alone and in complex with Met suggests that it is an energetically favorable arrangement. Indeed, further analysis of available structures of InlB uncovered that this contact is present in two further crystal forms of InlB, namely that of the full-length InlB protein³² and that of an InlB₃₂₁ variant with an additional LRR (Niemann and Heinz, unpublished). In solution this contact also seems to be favored, as we observed only a dimeric species for the cross-linked InlB₃₂₁ G206C/A227C variant (Fig. S2, Supplementary Information), although the formation of higher oligomers would theoretically be possible due to the presence of two accessible cysteines per molecule. This assumption is also supported by the structure of the cross-linked InlB₃₂₁_CD that was crystallized only after dimers had formed in solution. Although too weak to cause stable dimerization

of purified proteins *in vitro*, the crystal dimer contact of InlB promotes Met dimerization on cells as shown by receptor phosphorylation and cell scattering assays. InlB₃₂₁_CD potently activates Met because it stabilizes an otherwise weak InlB-InlB dimer interface, locking the InlB-Met complex into a stable signaling competent conformation, thus replicating the core of the genuine 2:2 InlB/Met complex as it is formed on cells.

Our model, in which a monomeric ligand can dimerize only once it is bound to the receptor, can be rationalized because the receptors are anchored in the membrane. This will pre-orient the ligand correctly and limit diffusion from three to two dimensions, both of which will favor dimerization. In addition, the transmembrane domain itself was shown to contribute to the dimerization of several receptors^{35; 36} and this may be true for Met as well. Hence, in the context of membrane embedded receptors, the weak contact between the convex sides of the InlB LRR is obviously sufficient to induce dimerization of InlB-Met complexes. A similar phenomenon has been described for the receptor tyrosine kinase Axl and its ligand Gas6.³⁷ The soluble Axl ectodomain and the ligand Gas6 form a 1:1 complex in solution, as determined by analytical ultracentrifugation. The crystal structure of the Gas6-Axl complex revealed a potential 2:2 complex with two distinct ligand/receptor contacts, a major contact burying 2366Å² and a minor contact burying 765Å² of solvent-accessible surface area. Mutations of Axl or Gas6 at the minor contact did not appreciably affect their binding affinity but resulted in a marked reduction of Axl activation. Thus, Axl and Gas6 provide another example of a receptor tyrosine kinase and its ligand, for which a 2:2 complex found in the crystalline state was shown to be physiologically relevant by cellular assays with mutants although it could not be observed by biophysical methods applied to purified proteins in solution.

Follicle stimulating hormone receptor (FSHR) in complex with the follicle stimulating hormone (FSH)³⁸ sets another precedent for the physiological significance in receptor activation of a small

contact mediated by the convex side of an LRR. FSHR is a transmembrane protein with an extracellular LRR domain that dimerizes upon binding of its ligand FSH. In the crystal structure of the complex, the FSHR LRR domain dimerizes *via* the convex side. The arrangement is analogous to the crystal dimer arrangement of InlB, but the solvent accessible surface buried in this contact is even smaller (470 Å² per monomer).

The crystal dimer arrangement of InlB places Met in such a way that, in addition to the contact at the concave side of the InlB LRR, there is a second interaction between the Ig2 domains of the two Met molecules. This interaction between Ig domains within the Met stalk may well be physiologically relevant. In Kit, another RTK, a similar interaction was found.³⁹ A dimeric contact between receptor Ig domains is likewise critical for activation of the RTK MuSK⁴⁰. Whether the membrane proximal Ig domains Ig3 and Ig4 of Met engage in comparable interactions can only be clarified by further structural work.

The IR domain contributes to the potency of InlB

For monomeric forms of InlB, the IR region is crucial for receptor activation. Although InlB₂₄₁ binds Met with the same affinity as InlB₃₂₁,²⁵ only the latter can induce phosphorylation of Met and its downstream targets like Erk1/2.³³ We have previously proposed that InlB₃₂₁ induces a conformational change in the Met ectodomain through the two-sites interaction of the InlB LRR region with Met Ig1 and the InlB IR region with the Met Sema domain. InlB₃₂₁ would thus work as a “molecular clamp” that locks the otherwise flexible receptor ectodomain into a signaling competent conformation.²⁵ The present data support this concept. Dimeric InlB₂₄₁_CD conceivably forces two Met molecules to arrange according to the crystal dimer geometry, which alone is sufficient to trigger Met activity. However, InlB₃₂₁_CD that additionally contains the IR region is ~ 100-fold more active. This suggests

that, in addition to dimerization, the proposed stiffening of the Met ectodomain contributes to receptor activation.

Dimeric InlB variants as highly potent Met agonists

InlB₃₂₁_CD rivals HGF/SF in activity and is definitely more active than InlBfl. It had been noted already that InlB₂₄₈, which is inactive in its monomeric form, can activate Met and Erk when it is dimerized fortuitously by a disulfide bond involving Cys242.^{33; 41} Recently it has been shown that this InlB₂₄₈ dimer inhibits Met down regulation and promotes recycling of the receptor to the cell surface following clathrin-mediated endocytosis.²⁶ A similar mechanism may operate for InlB₃₂₁_CD leading to a prolonged half-life of Met after receptor activation. In all aspects tested, including motogenesis, mitogenesis and activation of Akt, the key effector of Met-dependent stimulation of cell survival,^{42; 43} InlB₃₂₁_CD behaved as a full Met agonist. This is of particular interest, as some dimeric Met ligands like the monoclonal antibody DN-30 act as partial agonists that only stimulate motility.⁴⁴ It is also interesting to note that InlB₃₂₁_CD acts independently of and does not bind to HSPGs, whereas both HGF/SF and InlBfl bind HSPGs with high affinity. The facile production and the extraordinary stability of InlB₃₂₁_CD make it a valuable tool that is likely to find applications in the study of Met activation and signaling. Due to its advantages over HGF/SF InlB₃₂₁_CD may moreover lend itself to the development of a protein therapeutic for tissue engineering and regeneration.

Materials and Methods

Protein expression and purification

The idea to reinforce a crystal contact by introducing inter-molecular disulfide bonds is based on earlier work.⁴⁵ In order to prevent formation of undesired disulfide bonds during folding, Cys242 of InlB₃₂₁ was mutated into Ala in InlB₃₂₁_CD. All mutations were performed using the Quikchange mutagenesis kit (Invitrogen). N-terminally GST tagged InlB₂₄₁ and InlB₃₂₁(residues 36-241 and 36-321, respectively) and their mutants were expressed from vector pETM30 in *E. coli* BL21-CodonPlus (DE3) and purified by affinity chromatography. The GST-tag was then removed by addition of TEV protease followed by ion exchange chromatography (MonoQ for wt; MonoS for InlB₃₂₁_4R). Formation of InlB₂₄₁_CD and InlB₃₂₁_CD dimers was enhanced by addition of 1 mM H₂O₂ in dialysis buffer prior to size exclusion chromatography. Purified dimeric species were analysed by SDS-PAGE and their oligomeric state assessed by analytical gel filtration on a Superdex 200 10/300 column equilibrated in PBS (Fig. S2b, Supplementary Information). InlBfl (residues 36-630) and InlBfl_3R were expressed from vector pGEX-6P-1 in *E. coli* BL21-CodonPlus (DE3). Purification was as described above except for the use of PreScission protease for tag removal. Untagged protein was then loaded on a MonoS column (GE Healthcare) and eluted with a linear salt gradient. HGF/SF was expressed and purified as described.⁴⁶

Protein crystallization

The triclinic crystal of InlB₃₂₁ grew in 200 nl of the InlB₃₂₁-Met₇₄₁ complex at 5 mg/ml (corresponding to 1.4 mg/ml of InlB₃₂₁) mixed with 100 nl reservoir solution (0.1 M Li₂SO₄, 0.1 M Na-citrate, pH 5.6, 30% PEG 400) in a sitting drop at 20° C. Only one crystal measuring about

50x50x15 μm was found 11 months after set-up, cryo-protected in reservoir solution containing 15% glycerol and flash frozen in liquid nitrogen. InlB₃₂₁ crystals of space group H32 grew from a complex of deglycosylated Met₇₄₁ with InlB₃₂₁ that had been purified by gel filtration. Drops were set up with 200 nl protein (2.5 – 5 mg/ml of complex, corresponding to 0.7 – 1.4 mg of InlB₃₂₁) + 100 nl reservoir (0.2 M NaCl, 0.1 M CHES, pH 9.5 and 40% PEG 300). Crystals of about 40x20x10 μm appeared within weeks to months and were flash frozen in liquid nitrogen without further cryo-protection. InlB₃₂₁_4R was crystallized by vapor diffusion in sitting drops at 20° C with 1 μl protein (3.8 mg/ml in 50 mM Hepes, pH 7.0, 100 mM NaCl) and 0.5 μl reservoir solution (17% PEG 1000 MME, 90 mM Na-citrate). Crystals were cryo-protected in 23% PEG 1000 MME, 0.1 M Na-citrate, 20% glycerol and flash frozen in liquid nitrogen. Diffraction-quality crystals of InlB₃₂₁_CD were obtained by mixing protein (6.7 mg/ml) in a 2:1 ratio with reservoir solution containing 44-52% PEG 200, 0.1M CHES, pH 9.5, 0.2 M NaCl. Fully grown crystals were flash frozen without further cryo-protectants.

Data collection, processing and structure determination

X-ray diffraction data for the InlB₃₂₁ monomers were collected on ESRF beam line ID23-2. Data for InlB₃₂₁_4R were collected on a Rigaku MicroMax-007 HF microfocus rotating anode generator operated at 40 kV, 30 mA equipped with VariMax HF Confocal Optical System and a Saturn 944+ CCD detector. Data were indexed, integrated and scaled with the XDS package.⁴⁷ Data for InlB₃₂₁_CD were collected on ESRF beam line ID14-4 and processed with MOSFLM and SCALA⁴⁸ from the CCP4 package.⁴⁹ All structures were solved by molecular replacement using the program Phaser.⁵⁰ Model building was performed using Coot.⁵¹ The structures were refined using Refmac5⁵² and validated with Molprobit.⁵³ TLS groups were determined with the TLSMD server.⁵⁴ Statistics of data collection and refinement are reported in Table 1. Interface analysis and calculation of buried

surface areas were carried out using the PISA server.⁵⁵ The electrostatic potential was calculated using APBS.⁵⁶ Figures were prepared with PyMol.⁵⁷

Western blots

The activity of the InlB mutants InlB₃₂₁_4R, InlB₃₂₁_CD and InlBfl_3R on Met, Erk1/2 and Akt phosphorylation was tested on Vero cells and compared with those of InlB₃₂₁, InlBfl and HGF/SF. For this, stock cultures of Vero cells were grown to 80-90% confluence in 10% FCS in DMEM and then starved for 24 hours. Test proteins were added in serum-free DMEM at suitable concentrations and incubated at 37 °C for 5 min. Phosphorylated and total Met were detected using rabbit anti-phospho Met Tyr1234/1235 (Cell Signaling 3126) and mouse anti-Met 25H2 (Cell Signaling 3127) antibodies, respectively. Phosphorylated and total Erk1/2 were detected using a mouse monoclonal anti-diphosphorylated Erk1/2 (Sigma-Aldrich M8159) and a rabbit polyclonal (Promega 9PIV114) antibody, respectively. Phosphorylated (Ser473) and total Akt were detected using rabbit polyclonal antibodies 9271 and 9272, respectively, from Cell Signaling Technology. Secondary antibodies were goat anti-rabbit (Dako P0448) and rabbit anti-mouse (Dako P0260) HRP-conjugated.

Colony scatter assay

For measuring the ability of InlB proteins to stimulate cell motility (colony scattering), the Madin-Darby canine kidney (MDCK) cell line was used essentially as described.¹⁰ Briefly, sub-confluent MDCK cells were seeded in 96-well plates at 5000 cells per well in 5% FCS in DMEM in the presence of semilog dilutions of test proteins and incubated overnight in 5% FCS in DMEM at 37 °C / 5% CO₂. Cultures were fixed for 5 minutes in 10% formaldehyde in PBS, stained with Coomassie

Blue and scored for activity. Two independent experiments were performed assaying each ligand in triplicate. At least two different batches of protein were assayed in both experiments for InlBfl, InlBfl_3R and InlB₃₂₁_CD. The mean and standard deviation of the minimal active concentration shown in the bar graphs were calculated from all available (at least six) data points for each ligand. For photography, MDCK cells were seeded in 6-well tissue culture plates (Beckton Dickinson) at a density of 2500 cells per well in 5% FCS in DMEM at 37 °C / 5% CO₂ for 48 hours before overnight incubation with semilog dilutions of test proteins. Live colonies were photographed using a Hamamatsu camera connected to a Leica inverted microscope with a phase contrast lens at 100 times magnification. A comprehensive collection of photographs of representative colonies from one titration is shown in Fig. S4, Supplementary Information.

DNA synthesis

DNA synthesis assays were carried out as described.¹⁸ Briefly, mouse keratinocyte (MK) cells were grown to confluence and then treated with the various ligands in the presence of [³H]-methylthymidine. Cells were lysed and mixed with scintillant to measure levels of incorporation of the radioactive isotope into DNA.

Binding assay

Nunc MaxiSorp plates were coated with 80 nM of the complete Met ectodomain. After blocking, wells were incubated with a concentration series of wild type GST-InlB₃₂₁ or GST-InlB₃₂₁_4R fusion protein. HRP-coupled anti-GST antibody was used for detection with an ABTS staining reaction.

Accession numbers

Atomic coordinates and structure factors have been deposited in the PDB with accession codes 2WQU (monomer, triclinic crystal form), 2WQV (monomer, rhombohedral crystal form), 2WQX (InlB₃₂₁_4R) and 2WQW (disulfide-linked InlB₃₂₁_CD).

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Figure Legends

Fig. 1. Two-fold symmetric crystal dimer arrangement of InlB. (a) Arrangement of InlB₃₂₁ in the rhombohedral crystal form. The view is along the two-fold axis relating the two protomers. (b) View along the non-crystallographic two-fold axis similarly relating two molecules of InlB in the low resolution crystal structure of the InlB-Met complex along (PDB: 2UZY²⁵). (c) Side view of the 2:2 InlB-Met complex. Color code in panels b and c: InlB: green, cap; blue, LRR; red, IR. Met: yellow, SEMA; orange, PSI; light grey, Ig-like domain 1; dark grey, Ig-like domain 2.

Fig. 2. Disruption of the InlB crystal dimer contact does not alter Met binding but impairs Met activation. (a) Interactions at the InlB-InlB dimer interface in the rhombohedral crystal form. Hydrogen bonds form between Ser 183 and Asp 263, and salt bridges between Asp 200 and Arg 224. (b) The surface patch that is neutral in InlB₃₂₁ shows a strongly positive electrostatic potential in InlB₃₂₁_4R. Red: negative potential; blue: positive potential. (c) Results of solid phase binding assays. InlB₃₂₁_4R binds Met like wt InlB₃₂₁. The quadruple mutation on the convex face of the InlB LRR does not impair Met binding. (d) Met, Erk1/2 and Akt phosphorylation in Vero cells upon incubation with InlB₃₂₁ and InlB₃₂₁_4R.

Fig. 3. Disruption of the InlB crystal dimer contact reduces the potency of full-length InlB. (a) Met, Erk1/2 and Akt phosphorylation in Vero cells upon incubation with InlBfl and InlBfl_3R. (b) MDCK cell colony scattering following incubation with InlBfl and InlBfl_3R. (c) Bar graphs of colony scattering assays. Bars indicate the lowest concentration at which colony dispersion was observed.

Molar concentrations of the ligands are reported in a negative logarithmic scale. Statistical significance of the difference was determined with a *t*-test ($p < 0.001$).

Fig. 4. The crystal dimer arrangement can be fixed covalently through two inter-molecular disulfide bonds. Crystal structure of the InlB₃₂₁ crystal dimer viewed along the two-fold axis. Arrows in the left inset indicate residues Gly206 and Ala227 of the wild type, which were chosen for mutation to cysteine. The right inset shows a magnification of the disulfide bonds between Cys206 and Cys227. The electron density of the intermolecular disulfide bonds is contoured at 1.8 σ .

Fig. 5. The covalently fixed crystal dimer is a highly potent Met agonist. (a) Met, Erk1/2 and Akt phosphorylation in Vero cells upon incubation with HGF/SF and InlB₃₂₁_CD. (b) Representative photographs of MDCK cell colony scattering in response to HGF/SF, InlBfl, InlB₂₄₁_CD and InlB₃₂₁_CD. (c) Bar graphs of MDCK colony scattering assays. Bars indicate the lowest concentration at which colony dispersion was observed. Molar concentrations of the ligands are reported in a negative logarithmic scale. Statistical significance of the difference between InlB₂₄₁_CD and InlB₃₂₁_CD was determined with a *t*-test ($p < 0.001$). (d) Mitogenic effect of InlB₃₂₁_CD. Stimulation of cell division was measured as [³H]-methylthymidine incorporation into DNA in MK cells upon addition of InlB₃₂₁, InlB₃₂₁_CD, HGF/SF and InlBfl.

Fig. 6. Model of Met activation by InlB. The structure of InlBfl (PDB 1M9S ³²) was overlaid on the InlB₃₂₁-Met₇₄₁ structure to derive the position of the GW domains. A discrete, signaling active 2:2 complex is shown on the left. The GW domains of soluble InlBfl induce clustering of several discrete 2:2 InlB-Met complexes via interaction with heparan sulfate proteoglycans (HSPGs) present on the host cell (cartoon on the right). Color coding for InlB: green, cap; dark blue, LRR; red, IR; light blue, B-repeat (represented as oval); grey, GW domains. Color coding for Met: yellow, SEMA domain; orange, PSI (omitted on the right); dark grey and light grey, Ig-like domains; violet, tyrosine kinase domains.

Table 1 Data collection and refinement statistics

	InIB ₃₂₁ monomer triclinic	InIB ₃₂₁ monomer rhombohedral	InIB ₃₂₁ _4R	InIB ₃₂₁ _CD
Data collection statistics				
Beamline	ESRF ID23-2	ESRF ID23-2	Rotating anode	ESRF ID14-4
Space group	P1	H32	P1	H32
Unit cell dimensions [Å], [°]	a = 59.66 b = 70.65 c = 124.66 α = 74.47 β = 83.13 γ = 85.65	a = b = 185.9 c = 115.07 α = β = 90.0 γ = 120.0	a = 38.70 b = 57.45 c = 64.30 α = 102.99 β = 90.21 γ = 93.56	a = b = 187.0 c = 115.1 α = β = 90.0 γ = 120.0
Resolution [Å] (highest resolution shell)	20-2.6 (2.67-2.60)	20-2.8 (2.87-2.80)	20-2.03 (2.09-2.03)	50-2.24 (2.36-2.24)
Completeness [%]	92.1 (74.8)	99.3 (100)	96.8 (68.5)	99.9 (99.7)
Redundancy	5.0 (3.4)	7.0 (7.1)	5.1 (3.7)	7.3 (6.9)
Observations (highest resolution shell)	273360 (11267)	130614 (9549)	172109 (6417)	271785 (36562)
Unique reflections (highest resolution shell)	55139 (3291)	18763 (1384)	33525 (1726)	37017 (5335)
I/ σ (I)	10.1 (2.6)	11.0 (2.6)	12.7 (4.0)	10.5 (2.0)
R-meas [%]	13.8 (70.2)	16.3 (91.1)	12.5 (40.7)	12.7 (95.7)
Molecules per asymm. unit	6	2	2	2
Solvent content [%]	52.7	58.6	42.6	59.1
Wilson B factor (Å ²)	41	45	20.1	37.8
Refinement statistics				
R / R _{free} ° [%]	19.1 / 24.9	20.3 / 23.8	19.4 / 25.7	19.9 / 23.9
Number of Atoms total	13405	4539	4959	4717
Protein / Solvent / others	13241 / 61 / 103	4442 / 31 / 66	4590 / 369 / -	4512 / 142 / 63
R.m.s. deviation				
Bonds [Å] /Angles [°]	0.006 /1.0	0.008 / 1.1	0.022 / 1.8	0.024 / 1.9
Ramachandran Plot				
Residues in favored regions [%]	96.3	94.8	96.7	95.4
Residues in disallowed regions [%]	0.1	0.2	0.0	0.0