Structure of Shigella IpgB2 in Complex with Human RhoA

IMPlications FOR THE MECHANISM OF BACTERIAL GUANINE NUCLEOTIDE EXCHANGE FACTOR MIMICry

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A common theme in bacterial pathogenesis is the manipulation of eukaryotic cells by targeting the cytoskeleton. This is in most cases achieved either by modifying actin, or indirectly via activation of key regulators controlling actin dynamics such as Rho-GTPases. A novel group of bacterial virulence factors termed the WXXXE family has emerged as guanine nucleotide exchange factors (GEFs) for these GTPases. The precise mechanism of nucleotide exchange, however, has remained unclear. Here we report the structure of the WXXXE-protein IpgB2 from Shigella flexneri and its complex with human RhoA. We unambiguously identify IpgB2 as a bacterial RhoA-GEF and dissect the molecular mechanism of GDP release, an essential prerequisite for GTP binding. Our observations uncover that IpgB2 induces conformational changes on RhoA mimicking DbI- but not DOCK family GEFs. We also show that dissociation of the GDP-Mg2+ complex is preceded by the displacement of the metal ion to the α-phosphate of the nucleotide, diminishing its affinity to the GTPase. These data refine our understanding of the mode of action not only of WXXXE GEFs but also of mammalian GEFs of the DH/PH family.

Remodeling of the actin cytoskeleton is essential for eukaryotic life, taking place in processes as diverse as division, motility, or cell-cell communication. The actin cytoskeleton is composed of actin monomers and helical filaments constantly assembling and disassembling at their ends, which is tightly regulated by multiple signaling pathways employing hundreds of actin-binding proteins. Most if not all signaling pathways converge on small GTPases of the Rho family, which are long considered as key signaling switches inducing different types of actin reorganization. The activation rate (1–3). Activation of the name-giving family member RhoA induces cell contraction. This drives the formation of stress fiber bundles and focal adhesions in fibroblasts or endothelial cells, exerting force onto the substratum or neighboring cells (5). In neurons for instance, RhoA activation induces growth cone retraction (6). Other Rho family members trigger the formation of cell protrusions antagonistic to RhoA, with Rac1 and Cdc42 inducing lamellipodia and filopodia, respectively (7, 8).

Given their importance in cell physiology, Rho-GTPases have also emerged as key targets of pathogens modifying cell signaling or actin remodeling for their own needs (9). For instance, multiple bacterial toxins inhibiting RhoA, such as C3 exoenzyme of Clostridium botulinum, have served as invaluable tools to study RhoA function in vivo (10, 11). As an alternative to inhibition, Rho-GTPase signaling pathways driving different types of actin reorganization are also frequently bypassed or activated by pathogens, at least transiently, allowing them to induce their tight adherence to or entry into eukaryotic cells (12–14). Pathogens often modulate host protein functions by virulence factors acting as perfect mimics of mammalian proteins. For example, the Salmonella toxin SopE can activate Cdc42 in a similar fashion as Dbl-like mammalian GEFs (15). Interestingly, SopE does not display any significant structural or sequence similarity to the mimicked mammalian protein.

An intriguing novel family of bacterial effectors shares a WXXXE sequence motif, the members of which were originally classified as GTPase mimics, despite the lack of any sequence homology or guanine nucleotide binding (16, 17). Prototypic members of the family include IpgB1 and IpgB2 from Shigella flexneri and Map from pathogenic Escherichia coli, which are all translocated into the host by type III secretion. Interestingly, these proteins were reported to induce filopodia (for Map) or lamellipodia and stress fiber formation, indicative of Rac1 and RhoA activity in the case of IpgB1 and IpgB2, respectively (16, 18). In another study, IpgB1 was described to drive Rac1 and

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‡ The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–4.

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Cdc42 activation (19). In addition, more recently identified family members such as EspM and EspT have been interpreted to activate rather than mimic RhoA and Rac1/Cdc42, respectively (20, 21), which was recently verified in the case of EspM2 (22). The finding that the WXXXY protein SiIA displays a fold similar to the bacterial GEF SopE (15, 23) provided the first structural evidence for WXXXY proteins acting as GEFs. Finally, Map has recently been defined as Cdc42-GEF (24), refuting the mimicry hypothesis and questioning the relevance of IpgB2 binding to downstream effectors of RhoA (16).

Here we provide crystal structures of IpgB2 both in its free form and in complex with human RhoA. Our data reveal the mechanism and structurally decipher the sequence of events inducing GDP release catalyzed by this novel family of bacterial GEFs, constituting yet another remarkable example of convergent evolution, a common theme in bacterial pathogenesis (12, 25, 26).

EXPERIMENTAL PROCEDURES

Cloning, Expression, and Purification—IpgB2 (residues 1–188, NCBI gene ID 56404000) was cloned into the expression vector pET-M41 (kindly provided by Gunter Stier, EMBL, Heidelberg). This pET-derived vector allows expressing the protein of interest as a fusion to the C terminus of His-tagged maltose binding protein (MBP) with a linker sensitive to tobacco etch virus protease. RhoA (residues 2–181, NCBI gene ID 10835049) and ROCK I were cloned into a modified pET-tobacco etch virus protease vector pET-M41 (kindly provided by Gunter Stier, EMBL, Heidelberg). This pET-derived vector allows expressing the protein of interest as a fusion to the N terminus of tobacco etch virus protease fused to the N terminus. The original ROCK I construct was purchased from GENEART and was optimized for protein production in E. coli. It encoded for amino acid residues 947–1015 according to NCBI gene ID 4885583. IpgB2 and RhoA were produced in E. coli Tuner (DE3) cells in LB medium containing additional 2 g/liter glucose after induction with 1 mM isopropyl 1-thio-β-D-galactopyranoside. The expression temperature was 15 °C for IpgB2 and 27 °C for RhoA. ROCK I was produced in E. coli Tuner (DE3) cells at 22 °C in 2×YT medium after induction with 0.8 mM isopropyl 1-thio-β-D-galactopyranoside. Selenomethionine-substituted IpgB2 was produced in minimal medium (1 g/liter NH4Cl, 3 g/liter KH2PO4, 6 g/liter Na2HPO4·7H2O) mixed 10:1 with supplement medium (21.82 g/liter d-glucose monohydrate, 0.45 g MgSO4·7H2O, 10.45 mg/liter Fe3(SO4)2·H2O, 10 mg/liter thiamine) and 50 mg/liter 1-selenomethionine, with the pH adjusted to pH 7.4. Harvested cells containing IpgB2 were resuspended in buffer (50 mM HEPES, pH 7.5, 250 mM NaCl, 8.7% (v/v) glycerol, 7.5 mM imidazole, 250 mM trisodium citrate, and 10 mM β-mercaptoethanol). In the case of RhoA, the buffer composition was 30 mM Tris/HCl, pH 7.5, 50 mM NaCl, 5 mM MgCl2, 5% (v/v) glycerol, 10 mM β-mercaptoethanol, and 0.05 mM GDP, and in the case of ROCK I the buffer contained 50 mM Tris/HCl, pH 8.5, 10% (v/v) glycerol, 20% (w/v) sucrose, 0.2 mM Na2S2O4, 2 mM MgCl2, and 2 mM dithiothreitol. To all resuspension buffers, a temperature was 15 °C for IpgB2 and 27 °C for RhoA. ROCK I was first purified by gel-filtration chromatography on a Superdex 75 column (Amersham Biosciences). During gel-filtration chromatography, the buffer was exchanged against 200 mM trisodium citrate/citric acid, pH 6.0, and 10 mM dithiothreitol in the case of IpgB2 and against 30 mM Tris/HCl, pH 7.5, 50 mM NaCl, 5 mM MgCl2, 5% (v/v) glycerol, 5 mM dithiothreitol in the case of RhoA.

Nucleotide Exchange and GTPase and GEF Assays—GTPase and GEF assays were performed according to methods described by Eberth and Ahmadian (27). RhoA loaded with different nucleotides was prepared by a modified method similar as described by Hutchinson and Ecclestone (28), utilizing the enhanced nucleotide dissociation rate of RhoA in buffers lacking Mg2+ (29).

Recombinant Protein Pulldown Experiments—His-MBP-fused IpgB2 was incubated with 5 µl of nickel-nitritolriatic acid-agarose (Qiagen) per milligram of fusion protein for 12 h at 4 °C in buffer containing 50 mM HEPES, pH 7.5, 250 mM NaCl, 8.7% (v/v) glycerol, 250 mM trisodium citrate, 10 mM β-mercaptoethanol. The resin was washed in a gravity flow column with 5 column volumes of buffer to remove unbound protein. Aliquots of 35 µl of packed resin mixed 1:1 (v/v) with glycerol were used in a typical binding assay with a 1–ml total reaction volume or stored at −70 °C until usage. The resin complexed with His-MBP-IpgB2 was incubated with recombinant tagless test protein (RhoA, ROCK 1 (Rho-binding domain (RBD))) for 10 min. The agarose was pelleted at 1000 x g, the supernatant was removed, and the agarose was washed three times to remove unbound test protein. Proteins were eluted with buffer additionally containing 250 mM imidazole, separated by SDS-PAGE, and visualized by Coomassie staining. To exclude unspecific binding of the test protein to the resin, all experiments were repeated with resin without complexed His-MBP-IpgB2.

Preparation of Soluble Bacterial WXXXYE Effector Proteins—As untruncated wild-type IpgB2 was not soluble in standard buffers after cleavage of the MBP fusion tag, buffer conditions were optimized to obtain soluble, untagged IpgB2. 96 conditions from a standard crystallization screen (classics suite, Qiagen) were tested for optimization. The MBP-tagged IpgB2 was concentrated to ~10 mg/ml, and 1.2 µl of protein solution was mixed with 1.2 µl of H2O, 0.3 µl of tobacco etch virus Protease (~2.5 mg/ml), and 1.2 µl of reservoir solution from the crystallization screen in batch experiments. After incubation at 4 °C
for 3 days, the solution was mixed with 10 µl of \( H_2O \), and insoluble particles were pelleted by centrifugation. 10 µl of supernatant of each experiment was separated by SDS-PAGE and visualized by Coomassie staining. In almost all tested conditions, complete cleavage of the MBP tag was observed, and approximately one-third of all conditions contained varying quantities of soluble IpgB2. Buffer components that repetitively were present in conditions leading to soluble IpgB2 were individually tested in further cleavage experiments. Citrate proved to be the most successful buffer component at concentrations of 100–200 mM, pH 5–7. Fresh IpgB2 was produced as described above using buffer systems based on this finding, which yielded >100 mg of soluble MBP-fused IpgB2 per liter bacterial culture and >10 mg of untagged IpgB2 per liter after purification to apparent homogeneity. Purified, untagged IpgB2 can also be handled in buffers without citrate (e.g. 10 mM Tris/HCl, pH 7.0) at 4 °C as long as no mechanical or oxidizing stress is applied to the protein. The proper folding state of purified IpgB2 was tested by \(^{15}N\), \(^{13}C\)-NMR spectroscopy on appropriately labeled IpgB2 (data not shown), indicating that the protein accommodates a compact, folded state in solution.

The buffer systems found for IpgB2 also allowed purification of the homologous WXXXE protein Map in full length, and allowed production of MBP-tagged IpgB1 (data not shown). IpgB1, however, slowly precipitates upon cleavage of the fusion tag and might require further buffer optimization.

**Cell Assays**—cDNA of the constitutively active variant Q14V of human RhoA was a kind gift from Laura Machesky (Glasgow, UK). cDNA of IpgB2 was amplified by PCR from genomic DNA of *S. flexneri* (serotype 5), and the mutants of IpgB2 were custom-synthesized (Genescript, Piscataway, NJ). All cDNAs were subcloned into pEGFP-C1 (Clontech, Mountain View, CA). The fidelity of all constructs was verified by sequencing. For transfections and fluorescence microscopy, SV40 large T-antigen-immortalized mouse embryonic fibroblasts were grown in Dulbecco's modified Eagle's medium, 4.5 g/liter glucose containing micro crystals. Crystals of IpgB2 in complex with RhoA were obtained from a (stoichiometric) mixture of 25 µl of RhoA (19.3 mg/ml) with 5.7 µl of IpgB2 (89.6 mg/ml). 1 µl of this protein solution was mixed with 1 µl of reservoir solution containing 100 mM Hepes, pH 7.5, and 20% (w/v) polyethylene glycol 3350 for crystallization of complex A. To obtain complex B, excess Mg\(^{2+}\) was removed from the mixture prior to crystallization by five cycles of 20-fold dilution with buffer containing 30 mM Tris/HCl, pH 7.5, 50 mM NaCl, 5% (v/v) glycerol, 20 mM EDTA, 0.05 mM GDP, and 5 mM dithiothreitol following concentration to the original volume using a Vivaspin concentrator unit (Sartorius, 5-kDa cutoff). The final concentration of the IpgB2-RhoA complex was 20.9 mg/ml. Crystals of complex A and B were cryoprotected in reservoir buffer with an additional 20% (v/v) glycerol and frozen in liquid nitrogen. Complex C was obtained by treatment of complex B crystals with a buffer containing 100 mM LiSO\(_4\), 100 mM HEPES, pH 7.5, 30 mM Tris, pH 7.0, 22.5% (w/w) polyethylene glycol 3350, 100 mM NaCl, 12% (v/v) glycerol, and 40 mM EDTA. Complex C crystals were directly frozen from this solution after 2–5 min of incubation time (30).

Free IpgB2 crystallized as cuboids with dimensions of up to 0.2 × 0.2 × 0.2 mm\(^3\) belonging to space group P42.2. IpgB2 in complex with RhoA crystallized as rhomboids with dimensions of ~0.2 × 0.2 × 0.08 mm\(^3\). Complex A crystals belonged to space group P2\(_1\). Despite similar crystal morphology, complex B crystals belonged to space group P2\(_2\), 2\(_2\). Complex C crystals had a gel-like consistency and melted within ~10 min after preparation. When frozen prior to complete disintegration, they showed an improved diffraction behavior compared with complex B. Changes in the crystal lattice led to the new space group P2\(_2\), 2\(_2\).

**Data Collection, Structure Determination, and Refinement**—X-ray diffraction data were collected at the beamlines ID23-1 and ID23-2 of the European Synchrotron Radiation Facility and by using a Rigaku MicroMax 007HF (home-source) x-ray generator equipped with a Saturn 944+ charge-coupled device detector. Data were processed and scaled using the XDS program package (31, 32). SHELXC, -D, and -E (33, 34) were used for structure solution and phasing of free selenomethionine-substituted IpgB2, and an initial model was built with AutoBuild of PHENIX (35). The structures of IpgB2 in complex with RhoA were solved by molecular replacement with MOLREP (36) using the partially refined structure of free IpgB2 and the structure of RhoA (37) as search models. The MOLREP solution was automatically corrected using ARP/wARP (38), manually completed in COOT (39), and refined with REFMAC5 (40). The figures were prepared using PyMOL (41). Data statistics are summarized in Table 1. Similarities between dif-
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### RESULTS

IpgB2 Is a GEF for Human RhoA—Consistent with the proposed function of IpgB2 acting as a GEF for RhoA, a weak but detectable interaction was found with GDP-bound RhoA in pulldown assays. Furthermore, a complex containing nucleotide-free RhoA in approximately stoichiometric ratio could be purified using gel-filtration chromatography buffer (30 mM Tris/HCl pH 7.5, 50 mM NaCl, 5 mM MgCl₂, 5 mM dithiothreitol), and the exchange was started by addition of 200 μM GDP and the indicated concentrations of (untagged) IpgB2 in a stopped flow apparatus.

### TABLE 1

| Protein Data Bank Accession Numbers—The atomic coordinates and structure factors (code 3LYQ for free IpgB2, 3LW8 for IpgB2-RhoA complex A, 3LWN for complex B, and 3LXR for complex C) have been deposited in the RCSB Protein Data Bank. |

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**TABLE 1**

| Crystallographic data and refinement statistics | Selenomethionine-labeled free IpgB2 | free IpgB2 | IpgB2/RhoA complex A | IpgB2/RhoA complex B | IpgB2/RhoA complex C |
|---|---|---|---|---|---|
| Data collection statistics | Beamlne | ESRF (ID23-1) | ESRF (ID23-1) | Rigaku MicroMax 007HF | ESRF (ID23-1) |
| Space group | P42,2 | P42,2 | P2₁ | P2₁,2,2,2,2 |
| Unit cell dimensions | 116.2 | 116.2 | 90.8 | 90.90, 90.90, 90.90 |
| α, β, γ (°) | 90, 90, 90 | 90.90, 90.90, 90.90 | 90.90, 90.90, 90.90 | 90, 90, 90 |
| Resolution (Å) | 48.9-2.9 (3.0-2.9) | 22.9-2.3 (2.36-2.3) | 48.2-1.85 (1.9-1.85) | 37.8-2.28 (2.34-2.28) | 42.5-1.68 (1.72-1.68) |
| Unique reflections (merge) | 26125 (1950) | 25363 (1205) | 133487 (9777) | 34100 (2183) | 45588 (3248) |
| Multiplicity | 6.3 (6.4) | 18.2 (6.4) | 3.5 (2.6) | 5.3 (3.5) | 4.7 (4.0) |
| Completeness (%) | 98.9 (99.9) | 95.4 (63) | 98.4 (97.8) | 98.7 (86.3) | 98.6 (96.9) |
| I/σ(I) | 26.2 (3.35) | 33.35 (2.56) | 15.11 (2.49) | 9.81 (2.06) | 23.76 (2.64) |
| Wilson B-factor (Å²) | 4.5 (55.7) | 5.6 (67.5) | 5.2 (49.2) | 13.4 (65.4) | 4.1 (58.8) |
| Solvent content (%) | 64.9 | 63.8 | 48.0 | 44.5 | 47.4 |

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**FIGURE 1.** GEF activity of IpgB2 for RhoA. A, His₆-MBP-IpgB2 binds RhoA but not ROCK I (RBD) in vitro. Lanes 1–5, SDS-PAGE analysis of pulldown experiments using immobilized His₆-MBP-IpgB2 incubated with RhoA-GDP or ROCK I (RBD); lanes 6–9, SDS-PAGE showing His₆-MBP-IpgB2 in complex with nucleotide-free RhoA after purification by gel-permeation chromatography. B, time course of the dissociation of the RhoA-mantGDP complex, as followed by the decrease in mantGDP fluorescence after excitation at 335 nm. The reaction mixture contained 0.25 μM RhoA in gel-filtration chromatography buffer (30 mM Tris/HCl pH 7.5, 50 mM NaCl, 5 mM MgCl₂, 5 mM dithiothreitol), and the exchange was started by addition of 200 μM GDP and the indicated concentrations of (untagged) IpgB2 in a stopped flow apparatus.

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**a** Values in parentheses refer to statistics in the highest resolution shell.

**b** Rmerge = Σ|Iobs - <Iobs>|/ΣIobs.

**c** Rfree = Σ|Fo - Fc|/ΣFo × 100, where Fo and Fc are the observed and calculated structure-factor amplitudes, respectively.

**d** Rfree was computed using 5% of the data assigned randomly.

**r.m.s.d., root mean square deviation.**
0.005 s\(^{-1}\) was measured at the highest molar excess of IpgB2 over RhoA (128-fold), corresponding to a ~10\(^4\)-fold acceleration of nucleotide exchange by IpgB2 compared with the intrinsic rate of 3.8 ± 0.2 × 10\(^{-5}\) s\(^{-1}\). This activity is well in the range observed for the bacterial GEF SopE (15, 43) and more than an order of magnitude higher when compared with mammalian GEFs like p115RhoGEF and p190RhoGEF (44).

**Structure of IpgB2—IpgB2 consists of two distinct domains:** an extended N-terminal antiparallel \(\beta\)-sheet domain comprising the first 39 residues, followed by a C-terminal \(\alpha\)-helical domain (residues 40–188) divided into a four-helix bundle and a three-helix bundle. The \(\alpha\)-terminal domain does not interact with its own C-terminal domain but is instead engaged via domain-swapping in interactions with the C-terminal domain of a neighboring IpgB2 molecule in the crystal, leading to a Z-shaped IpgB2 dimer (Fig. 2A). The absence of an IpgB2 dimer in solution (data not shown) and in the complex with RhoA (see below) indicates that the domain-swapping most likely represents a crystallographic feature. This is further supported by a disrupted hydrogen bonding pattern of the formed intermolecular antiparallel \(\beta\)-sheet at residues 31–33 (Fig. 2A), which is unlikely to occur in a physiologically relevant dimer.

The rather loosely packed \(\alpha\)-helical domain is further stabilized by a loop linking helices \(\alpha3\) and \(\alpha4\) that we refer to as the “catalytic loop” (residues Ile\(^{108}\)–Ser\(^{118}\)) herein, in line with previous nomenclature (15, 24). The loop winds around helix \(\alpha2\) and is oriented toward the N terminus of this helix. The conserved WXXXE motif is located close to the N-terminal end of helix \(\alpha2\). Trp\(^{62}\) and Glu\(^{66}\) of this motif form hydrogen bonds with Ser\(^{118}\) and Ser\(^{117}\) in the catalytic loop, respectively.

Both IpgB2 monomers in the asymmetric unit are structurally similar, but show a marked difference at the interface of GTPTase targets. One, representing the initial step of the nucleotide exchange reaction, contains RhoA GTPase with Mg\(^{2+}\)-bound GDP (termed complex A). In the second complex structure (complex B), the Mg\(^{2+}\) is displaced from its original \(\beta\)-phosphate position of the nucleotide to a secondary binding site, thus representing the next step of the exchange reaction. In a third structure (complex C), the Mg\(^{2+}\) is completely removed from the complex.

The obtained IpgB2-RhoA complexes crystallized in different space groups, with four, two, and one heterodimers per asymmetric unit for complex A, B, and C, respectively. All complex structures show similar overall folds. The interaction surface between IpgB2 and RhoA buries roughly 1220 Å\(^2\). It is formed by extensive interactions between the helical bundles of IpgB2 and the flexible switch regions I and II, as well as a region involving residues of sheets \(\beta1\), \(\beta2\), and \(\beta3\) of RhoA (in the following called the "\(\beta123\) region") (Fig. 2B). Although the overall fold of RhoA and the conformation of its bound GDP ligand remained essentially unchanged when compared with the free enzyme structure (46), the N-terminal domain of IpgB2 now packs against the four-helix bundle of the helical domain of the same protomer, without interacting with RhoA. This is in line with the observation that the first 19 residues of IpgB2 are dispensable for exerting GEF activity (24). The surface of this \(\beta\)-domain shows a pronounced positive charge, indicating potential interactions with lipids or other proteins.

**Complex A—** Upon complex formation, the catalytic loop of IpgB2 reorients along helix \(\alpha2\) toward its C terminus (8.4-Å movement) and is now cradled between the RhoA switch regions I and II. In complex A, it interacts with the Mg\(^{2+}\) hydration shell via Gln\(^{116}\) (Fig. 3A). Interestingly, replacement of this residue by alanine (Q116A) partially preserved stress fiber induction in cells upon expression of the IpgB2 mutant when
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FIGURE 3. Nucleotide binding site and catalytic loop region in IpqB2–RhoA structures. IpqB2 is shown as a blue schematic, with the catalytic loop marked in red. GDP is shown as orange balls and sticks, and Mg$^{2+}$ and water molecules are shown as yellow and red spheres, respectively. A, GDP-binding pocket and interaction of IpqB2 residue Gln$^{116}$ in complex A. The ($F_o$ - $F_c$) electron density map contoured at 1 $\sigma$ for GDP, Mg$^{2+}$, the Mg$^{2+}$ coordination sphere, and Gln$^{116}$ is colored as blue mesh. B and C, similar representation as in A for complexes B and C, respectively. For A–C, RhoA is shown as a surface representation, with switch I, switch II, and other regions of RhoA as yellow, green, and brown surfaces, respectively (compare also supplemental Fig. 2 for a more detailed view in stereo representation). D, helix $\alpha_2$ in complex A, showing a conformation that is disrupted by two water molecules. Such a disrupted helix conformation is not observed in crystals of complex B (E) or of complex C (not shown). RhoA residues are shown as brown balls and sticks in D and E.

compared with the respective positive and negative controls (Fig. 4, A–C and F, and supplemental Fig. 1). In contrast, mutation of Gln$^{116}$ to glutamate (Q116E) completely abolished IpqB2-mediated stress fiber formation (Fig. 4G). This suggests that Gln$^{116}$ is not essential for complex formation, but instead might have a supportive role for the nucleotide release induced by IpqB2. Substitution by glutamate may abolish this function due to electrostatic repulsions with Asp$^{59}$ in RhoA, whereas mutation to alanine only results in a lowered catalytic efficiency of IpqB2.

Switch I is partially reoriented upon IpqB2 binding. Thr$^{37}$, one of two threonine residues coordinating Mg$^{2+}$ in free RhoA (46), is no longer involved in forming the octahedral Mg$^{2+}$ coordination sphere. Instead, Asp$^{80}$ of IpqB2 pulls switch I toward IpqB2 by forming hydrogen bonds to Tyr$^{34}$, Thr$^{37}$, and Val$^{38}$. Additional hydrogen bonds are formed between IpqB2 residues Arg$^{73}$, Gln$^{77}$, and Asn$^{154}$ and Glu$^{40}$ of RhoA (Fig. 5A). In contrast to switch I, the conformation of switch II remained essentially unaffected upon formation of complex A. Minor rearrangements within switch II allow a closer approach of the catalytic loop of IpqB2 to the active site of RhoA (Fig. 5B). RhoA residues Arg$^{5}$, Asp$^{35}$, and Glu$^{54}$ of the $\beta_{123}$ region are the main determinants of specificity of RhoA–GEFs (47) and are involved in extensive interactions with complementary charged IpqB2 residues (Fig. 5C).

Complex B—After binding to a GTPase, GEFs destabilize the GDP-bound state to allow GDP dissociation and binding of GTP for activation. This is thought to be facilitated by a stepwise ligand release: First, Mg$^{2+}$ is released, followed by dissociation of GDP (48–50). To obtain a structure of the RhoA–IpqB2 complex in the magnesium-free state, the metal ion was sequestered by repeated cycles of EDTA treatment prior to complex formation. Upon magnesium ion depletion, RhoA in the structure of complex B undergoes changes in switch II that are virtually identical to those described in the structure of magnesium-free RhoA (49). A flip of switch II residue Ala$^{61}$ toward the GDP-binding site occludes the primary Mg$^{2+}$ binding site at the $\beta$-phosphate. At the same time, Glu$^{64}$ is repositioned and forms a hydrogen bond with Lys$^{18}$ from the phosphate-binding loop (P-loop), thus lowering the affinity to the nucleotide (supplemental Fig. 2). However, while the Mg$^{2+}$ is completely absent in free RhoA in complex with GDP (49), the complex with IpqB2 strikingly reveals a new, secondary Mg$^{2+}$ binding site at the $\alpha$-phosphate group of the nucleotide (Fig. 3B). Although the metal ion is well defined in the secondary binding site, it is no longer coordinated by protein atoms, but instead by five water molecules and an $\alpha$-phosphate oxygen. Indirect interactions of this Mg$^{2+}$ (\(\alpha\)) with RhoA residues are mediated by two magnesium-coordinating water molecules to the side chain of Thr$^{19}$ and to the carbonyl oxygens of Pro$^{36}$ and Thr$^{37}$ of switch I, respectively (supplemental Fig. 3).

Complex C—Shimizu and coworkers (49) observed that high concentrations of LiSO$_4$ are required to obtain the GDP-loaded, magnesium-free state of RhoA. In the current study, a milder method of Mg$^{2+}$ depletion by EDTA treatment was utilized, which allowed remaining Mg$^{2+}$ to bind to the $\alpha$-phosphate. Indeed, LiSO$_4$ can be used to completely remove the Mg$^{2+}$ ion from the active site: treatment of complex B crystals with a buffer containing LiSO$_4$ leads to significant rearrangements of the crystal lattice, an improved diffraction behavior, and quantitative Mg$^{2+}$ elimination from the newly observed secondary binding site. The protein fold in this complex (com-
plex C) essentially remains identical, showing virtually no conformational changes in the environment of the depleted site (Fig. 3C and supplemental Fig. 2). Switch I in complexes B and C is similar to the conformation observed in complex A, whereas in the free RhoA_{H18528}GDP structure (49) it is moved away from the (magnesium-free) GDP binding pocket.

In addition to the conformational changes in RhoA, differences were also observed in helix H_9251 of IpgB2, which contacts the switch II region. In complex A, helix H_9251 preferentially adopts a conformation with two water molecules disrupting the helix between residues Lys_67/Lys_68 and Ser_72/Val_71 (Fig. 3D). In contrast, it adopts a well defined continuous -helical arrangement in all IpgB2 molecules in complexes B and C (Fig. 3E). This suggests that the conformational rearrangements in switch II at low Mg^{2+} concentrations favor the undisrupted helix conformation.

**DISCUSSION**

**Different Bacterial GEFs Induce GDP Release in a Similar Manner**

IpgB2 shows a close structural homology to Map, the C-terminal region of SifA, and a less pronounced homology to SopE (r.m.s.d. of 2.6 Å, 3.5 Å, or 4.3 Å for 150, 158, or 140 common Cα positions, respectively). All four proteins share a V-shaped helical arrangement and a catalytically important loop connecting both lobes of the V. This loop winds around a helix that contains the conserved WXXXE motif in IpgB2, Map, and SifA.

Map in complex with the small GTPase Cdc42 (24) is the only other WXXXE family member co-crystallized with its cognate GTPase, allowing for a close comparison with the present IpgB2-RhoA complex. Both complexes share a number of common features. Structurally, the V-shaped C-terminal helical domain of Map (residues 51–203) and IpgB2 is basically conserved (Fig. 5B and E). The interactions of IpgB2 and Map with switch I of their cognate GTPases are also remarkably similar (Fig. 5A and D). As expected, the region involved in binding to the B123 region displays strong differences between IpgB2 and Map. Although the GTPase interaction surface of Map is predominantly apolar, IpgB2 interacts with RhoA via charged or polar interactions (Fig. 5C and F). Both Map and IpgB2 contain the stabilizing double serine motif (Ser_117 and Ser_118 in IpgB2). The two adjacent serines form hydrogen bonds with the con-
served residues of the WXXE motif (Fig. 3, D and E) and are part of a sequence motif AQ(S/T)S(I/V) in the catalytic loop that is conserved throughout most currently known members of the WXXE family. Substitution of both serines by alanine completely abolished induction of stress fiber formation by IpgB2 in vivo (Fig. 4H), consistent with the abrogation of Cdc42 binding observed for the S130H mutant of Map (24). This shows that the serines are crucial for biological activity and underscores their indirect function in catalyzing nucleotide exchange. Because the catalytic loop is directly involved in GTase interactions whereas the WXXE motif is not, mutations of the critical tryptophan in the WXXE motif were tested for biological activity. A substitution of Trp62 for alanine led to a complete loss of cellular response (Fig. 4D), as described (16). However, a conservative change to tyrosine partially preserved IpgB2 activity (Fig. 4E), demonstrating that the tryptophan itself is not strictly required for biological function. This is also in line with the data on the novel family member EspM3 expressed in certain pathogenic E. coli strains, because the corresponding mutant W66Y also induced stress fibers when translocated into host cells (51).

The structure of the Map-Cdc42 complex represents a rather late state of the nucleotide exchange reaction where GDP is already released. Switch II of Cdc42 shows a conformation that is also observed in the complex with SopE, another bacterial GEF lacking a WXXE motif (15). This switch II conformation is highly similar to the conformation of RhoA in IpgB2-RhoA complex B, but not in complex A (Fig. 5, B and E). Similarly, the contact surfaces of Map with switch II show a continuous conformation of helix α2, as seen in complex B. This demonstrates that Map, SopE, and IpgB2 induce very similar changes in their “chaise longue” shape, with all the long helices packed into the “seat” and the short helices forming the “seatback” (52, 54). Interactions with the β123 region of RhoA are the main determinants for specificity of RhoA-GEFs (47). IpgB2 and DH/PH interact with this region in a remarkably similar fashion. Asp65 and Glu84 in RhoA form salt bridges with Arg142 of IpgB2 and Arg868 of DH/PH, while Arg3 forms hydrogen bonds with IpgB2-Asp134 and DH/PH-Asp873, respectively (Fig. 5, C and I). In both the (DH/PH)-RhoA and the IpgB2-RhoA complexes, switch I adopts a similar conformation. Asp60 of IpgB2 and Glu741 of DH/PH occupy similar positions in the RhoA interface and participate in essentially the same interactions with the switch I residues Tyr34, Thr37, and Val38 (Fig. 5, A and G). In addition, RhoA residue Glu40 forms hydrogen bonds with Asn154 and Gln77 of IpgB2 and with Ser746 of DH/PH (Fig. 5, A and G). The switch II conformation in (DH/PH)-RhoA resembles the state in complexes B and C of IpgB2-RhoA. Gln80 of RhoA is repositioned by >10 Å compared with complex A (Fig. 5, B and H) and now forms a water-mediated interaction with DH/PH residue Gln880 or a hydrogen bond to the main-chain carbonyl of IpgB2-Gln116 (supplemental Fig. 2), respectively. Additional hydrogen bonds with both GEFs are formed by RhoA residues Tyr66 and Arg68, although they occur with different binding modes (Fig. 5, B and H).

This structural comparison suggests that bacterial WXXE GEFs and mammalian GEFs of the DbI family are the result of convergent evolution. In addition it ranks complexes B and C at a late stage of the nucleotide exchange reaction, strongly resembling the state in (DH/PH)-RhoA in which the GDP nucleotide is already released.
Role of the WXXXE Motif in Bacterial GEFs

The crystal structures of SifA and a Map-Cdc42 complex already revealed that WXXXE proteins are GEFs instead of GTPase mimics (23, 24, 30). Information on mechanistic details and GTPase selectivity of WXXXE proteins, however, is still limited. The structures of free and RhoA-complexed IpgB2 presented in this work together with the structures of SifA and Map-Cdc42 now provide a framework for the mode of action of bacterial WXXXE proteins. Although the WXXXE motif is only indirectly required for catalytic activity, residues of the catalytic loop form direct interactions with the cognate GTPase. For this, one important function of the WXXXE motif is to stabilize the catalytic loop by forming interactions with the double serine motif (Ser117/Ser118). This allows productive interactions between the catalytic loop by forming interactions with the double serine motif (Ser117/Ser118). This allows productive interactions with the cognate GTPase, e.g. of the catalytically important catalytic loop residue Gln116 with the Mg2+ hydration shell. The double serine motif is strictly required for activity, and interactions involving the WXXXE motif are strictly conserved in IpgB2 and Map. SifA most likely utilizes a similar stabilization, despite harboring a more extended catalytic loop that is stabilized by several proline residues, leading to a different overall loop conformation (supplemental Fig. 4A). His255 of the catalytic loop in SifA is oriented toward Trp197 and Glu201 of the WXXXE motif, suggesting that it substitutes the hydrogen bonding potential of the two serine residues present in IpgB2, Map, and numerous other WXXXE proteins (supplemental Fig. 4B). However, the question arises whether the significant structural differences in the catalytic loop of SifA compared with IpgB2 suggest that RhoA might not be the main target of SifA, as recently proposed by Ohlsen et al. (23). An overlay with IpgB2-RhoA reveals highly unfavorable interactions between SifA and RhoA, particularly within the specificity determining regions (supplemental Fig. 4C). Interestingly, phenotypes induced by SifA in host cells as the formation of Salmonella-induced filaments (Sifs) do not typically coincide with RhoA activation but could derive, rather, from interference with Rab-GTPase signaling.

We conclude that the WXXXE motif in all family members forms an essential part of a conserved structural motif, which is complemented by the catalytic loop motif AQ(S/T)S(L/V) or related residues in the distinct sequence of SifA. Interactions between both motifs promote a functionally competent conformation of the catalytic loop that is required for productive interactions with the cognate GTPase. The role of the different catalytic loop in SifA still requires further analysis and might reveal other GTPases as targets of SifA which might not belong to the Rho family.

GEF Mechanism of Bacterial WXXXE Proteins

Various families of mammalian Rho-GTPase specific GEFs exist that activate their cognate GTPases by different mechanisms. The largest of these is the Dbl family of GEFs containing a DH/PH domain. In contrast to other Rho-specific GEFs like proteins from the DOCK family (55), Dbl proteins do not directly insert residues into the active site of their cognate GTPase (4). Bacterial WXXXE GEFs utilize a similar mechanism for GTPase activation, despite having a different overall fold. By combining structural information about free and RhoA-complexed IpgB2 with knowledge about RhoA-GDP (with and without Mg2+ (46, 49)), and the structurally related complex of nucleotide-free Map-Cdc42 (24), it is now possible to outline the sequence of events finally leading to nucleotide release, the essential prerequisite of GTP loading (45). At least four distinct states, States 1−4 as follows, can now be discriminated (Fig. 6).

State 1: Uncomplexed Proteins—The structures of free IpgB2 and RhoA-GDP-Mg2+, respectively, represent the state before an interaction occurs (Fig. 6A). The catalytic loop of IpgB2 is oriented toward the N terminus of helix α2. The octahedral Mg2+ coordination sphere in RhoA includes switch I residue Thr37 (46).

State 2: Complex Formation—In a first step, IpgB2 binds to GDP-bound RhoA (Fig. 6B). Upon complex formation, the catalytic loop reorients along helix α2 toward its C terminus. In contrast to later states, the α-helical hydrogen-bonding pattern in helix α2 is disrupted by two water molecules. This way, a helical tilt is induced that bends the N-terminal residues of IpgB2 helix α2 toward switch II. While switch II stays mostly unchanged at this stage and IpgB2 reorients to form favorable interactions, the situation is opposite for switch I. Here, the contacting regions of IpgB2 remain similar to the uncomplexed protein, with switch I of RhoA undergoing a large reorientation. It adopts a conformation that is common to other GEF/GTPase structures, including Map-Cdc42 (24) and (DH/PH)-RhoA (52). Upon complex formation, Thr37 is removed from the octahedral coordination sphere of Mg2+ and is replaced by a fourth Mg2+-coordinating water molecule. This way, the GDP binding pocket becomes more solvent-exposed to initiate dissociation of GDP and Mg2+.

State 3: Change of Switch II and Release of Mg2+ from the β-Phosphate of GDP—In a second step, conformational changes are induced within switch II that displace Mg2+ and prepare the dissociation of GDP (Fig. 6C). This coincides with a conformation of helix α2 being no longer disrupted by water molecules, indicating that IpgB2 utilizes changes in this helix to adopt to different switch II conformations. Consistent with a subsequent release of Mg(β) and GDP, the Mg2+ ion is removed from its primary binding site at the β-phosphate. Such a stepwise mechanism has already been proposed based on a structure of GDP-loaded, magnesium-free RhoA in the absence of a GEF (49). It was discussed that the depletion of the Mg(β) ion reduces the affinity of GDP to RhoA to facilitate GDP release (49, 50), but the question remained as to whether a magnesium-free state would be stable under physiological conditions. The structure of complex B provides an intriguing answer to this question: the negative charge at the nucleotide is compensated by Mg2+ at a secondary, previously not described α-phosphate binding site. This secondary site can be depleted by soaking complex B crystals in buffers containing LiSO4 (Fig. 3C), which was also used to obtain the magnesium-free state of RhoA (49). This demonstrates that Mg2+ can bind to the α-phosphate of GTP without inducing any notable conformational rearrangements of the protein. The switch II conformation in complexes B and C is similar to free RhoA-GDP (49) and to nucleotide-free (DH/PH)-RhoA (52). Remarkably, even
though an magnesium-bound α-phosphate binding site has never been described before, all analyzed structures of GEF-GTPase complexes in the presence as well as in the absence of GDP (15, 24, 50, 52, 54, 55) contain a cavity that can fit Mg$^{2+}$ in a similar binding mode as observed in the IpgB2-RhoA complex B. This might indicate a broader relevance of this secondary site also for other GEF families such as mammalian Dbl-like GEFs. Because high concentrations of Mg$^{2+}$ are present in vivo, complex B might represent a relevant intermediate in GTPase activation that was not structurally characterized before. The secondary Mg$^{2+}$ binding site can be rationalized by the requirement to compensate the phosphate charge without interference with a switch II conformation blocking the primary Mg$^{2+}$ binding site at the β-phosphate. This switch II reorientation is an essential step in the GEF-catalyzed nucleotide release and is required for a lowered GDP affinity. By binding to the α-phosphate, Mg$^{2+}$ is able to compensate the phosphate charge without formation of additional interactions that could stabilize the binding of GDP to RhoA.

**State 4: Release of GDP**—In a final step, GDP and Mg(α) are released from the complex (Fig. 6D). The remarkable similarity of complex B with the GDP-free Map-Cdc42 complex (24) indicates that the latter can serve as a good model for a nucleotide-free IpgB2-RhoA complex, which is the final state in the nucleotide exchange reaction. As indicated by the comparison of both complexes, the dissociation of GDP does not require significant conformational changes. Under physiological conditions, a simultaneous release of Mg(α) and GDP cannot be excluded, which would place the magnesium-depleted complex C crystals as an additional intermediate between states 3 and 4.

The presented scheme provides the first example of a fully deduced mechanism of a bacterial GEF in action. The conformational changes induced in the GTPase are comparable to those observed upon interaction with mammalian GEFs containing a DH/PH domain (e.g. Ref. 52), despite completely distinct GEF-folds. Based on structural comparisons of the PRONE domain of RopGEF8 with Rho of plants (Rop), Thomas and colleagues (50) concluded that mammalian and plant GEFs utilize common...
intermediate states in small G protein/GEF reactions. Our work shows that this notion can now be extended to bacterial WXXE GEFs.

Conclusions

We report the first crystal structure of a free WXXE family member. The structure of IpgB2 from S. flexneri combined with its complexes with RhoA allows a structural description of the sequence of events accompanying GDP release induced by this bacterial GEF, which is an essential prerequisite for GTP loading. A function as a GTPase mimic—release induced by this bacterial GEF, which is an essential prerequisite for GTP loading. A function as a GTPase mimic during data collection.

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