Structural basis for the reversible activation of a Rho protein by the bacterial toxin SopE

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The bacterial enteropathogen *Salmonella typhimurium* employs a type III secretion system to inject bacterial toxins into the host cell cytosol. These toxins transiently activate Rho family GTP-binding protein-dependent signaling cascades to induce cytoskeletal rearrangements. One of these translocated *Salmonella* toxins, SopE, can activate Cdc42 in a Dbl-like fashion despite its lack of sequence similarity to Dbl-like proteins, the Rho-specific eukaryotic guanine nucleotide exchange factors. To elucidate the mechanism of SopE-mediated guanine nucleotide exchange, we have analyzed the structure of the complex between a catalytic fragment of SopE and Cdc42. SopE binds to and locks the switch I and switch II regions of Cdc42 in a conformation that promotes guanine nucleotide release. This conformation is strikingly similar to that of Rac1 in complex with the eukaryotic Dbl-like exchange factor Tiam1. However, the catalytic domain of SopE has an entirely different architecture from that of Tiam1 and interacts with the switch regions via different amino acids. Therefore, SopE represents the first example of a non-Dbl-like protein capable of inducing guanine nucleotide exchange in Rho family proteins.

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Introduction

Ras-related GTP-binding proteins of the Rho family (e.g. RhoA/B, Cdc42 and Rac1/2) are key regulators of vital cellular functions including cytoskeletal rearrangements, cell shape, motility, cytokinesis, gene expression, cell aggregation and cell–cell adhesion (Hall, 1998). They exert their function by cycling between a biologically inactive GDP-bound and an active GTP-bound conformation (Bourne *et al.*, 1990, 1991). As spontaneous guanine nucleotide dissociation is very slow, activation is depend-

ent on guanine nucleotide exchange factors (GEFs), which enhance nucleotide exchange rates by inducing structural changes in the nucleotide-binding site (Vetter and Wittinghofer, 2001). All cellular Rho family-specific GEFs identified so far share a common module, the Dbl homology (DH) domain. This domain forms the catalytically active part of these molecules responsible for binding to the switch I and switch II regions of the Rho component (Cerione and Zheng, 1996; Cherfils and Chardin, 1999). These regions in GTP-binding proteins define major conformational differences between the active and inactive states (Milburn *et al.*, 1990).

The bacterial enteropathogen *Salmonella typhimurium* is one of the leading causes of gastroenteritis in developed countries. During its evolution as a pathogen, *S. typhimurium* has acquired a variety of mechanisms to colonize, replicate and survive within its animal hosts (Groisman and Ochman, 1996). Essential for *S. typhimurium* virulence is a specialized protein secretion and delivery system termed type III that is encoded within the SPI-1 pathogenicity island of this bacterium (Galán and Curtiss, 1989; Watson *et al.*, 1998; reviewed by Wallis and Galyov, 2000). This system mediates the transfer of several bacterial proteins that have the capacity to modulate signal transduction into the host cell cytosol. The translocated toxins trigger responses including cell death in macrophages, nuclear responses resulting in pro-inflammatory cytokine production, and actin cytoskeletal rearrangements leading to bacterial internalization by normally non-phagocytic cells such as epithelial cells (reviewed by Galán, 1999). In fact, *S. typhimurium* serves as an important model to study the cell biology of bacterial host cell invasion (Galán and Zhou, 2000). Recent work has demonstrated that *S. typhimurium* invasion of host cells is triggered mainly by three translocated effector proteins (Mirolid *et al.*, 2001; Zhou *et al.*, 2001): SopE, SopE2 (see below) and SopB, a protein with inositol phosphatase activity (Hong and Miller, 1998; Norris *et al.*, 1998).

Cdc42 is an essential component of the host cellular signaling cascades triggered by the translocated *S. typhimurium* effector proteins during invasion (Chen *et al.*, 1996). The effector proteins SopE and SopE2 (70% amino acid sequence identity; Wood *et al.*, 1996; Hardt *et al.*, 1998b; Bakshi *et al.*, 2000; Stender *et al.*, 2000) bind directly to and activate host cellular Cdc42 by catalyzing guanine nucleotide exchange (Hardt *et al.*, 1998a; Rudolph *et al.*, 1999a; Stender *et al.*, 2000). In fact, transient expression of SopE or SopE2 in cultured cells is sufficient to activate Cdc42, induce actin cytoskeletal reorganization and mediate internalization of non-invasive *S. typhimurium* mutants (Hardt *et al.*, 1998b; Stender *et al.*, 2000). Biochemical analyses have demonstrated that, unlike many other bacterial toxins, SopE and SopE2 activate host cellular Rho family proteins by transient

interaction and not by chemical modification. Even though SopE and SopE2 do not share any recognizable sequence similarity to any known proteins from bacteria or eukaryotes, particularly the DH family proteins, they act as genuine GEFs (Rudolph *et al.*, 1999a; Stender *et al.*, 2000). Their ability to form nucleotide-free binary complexes with Rho proteins and to stimulate the rate of nucleotide exchange by several orders of magnitude are strikingly similar to those of genuine eukaryotic GEFs such as RCC1, a GEF for Ran (Klebe *et al.*, 1995), or Cdc25, a GEF for Ras (Lenzen *et al.*, 1998). Preliminary experiments indicate that its catalytic activity is much higher than that of genuine eukaryotic Rho-GEFs of the DH family (A. Wittinghofer, unpublished). For this reason, it was of great interest to study the structural basis of SopE-mediated guanine nucleotide exchange.

Here we have analyzed by X-ray crystallography at 2.3 Å resolution the interaction between the catalytic fragment of SopE and its host cellular target, Cdc42. We found that the structure of SopE is entirely different from that of Dbl-like RhoGEFs. Yet, the interaction of SopE locks the switch I and switch II regions of Cdc42 in the same conformation as that observed in the equivalent regions of Rac1 when bound to its cellular GEF Tiam1 (Worthylake *et al.*, 2000), or in the Cdc42–Dbs complex (Rossman *et al.*, 2002). These data provide the first insight into how proteins lacking any similarity to the Dbl-like protein family can act as efficient GEFs for Rho family GTP-binding proteins.

Results and discussion

Structure determination and overview

For crystallographic analysis, we used C-terminally truncated Cdc42 (amino acids 1–178) and the catalytic domain of SopE (SopE_C, residues 78–240) (SopE from now onwards). It was shown previously that C-terminal truncation of Cdc42 does not interfere with SopE-mediated GEF activity (Rudolph *et al.*, 1999a). The components of the complex were expressed in *Escherichia coli* and purified from cell lysates according to standard protocols. After mixing the constituents with a 2-fold molar excess of Cdc42, the SopE–Cdc42 complex was purified by size exclusion chromatography, crystallized, and the structure determined as described in Materials and methods. The current structural model of the asymmetric unit consists of two SopE–Cdc42 complexes, with most of the structure being well defined in its electron density. Altogether, six sulfate ions and 160 solvent molecules were added on the basis of $F_o - F_c$ electron density maps. A summary of the crystallographic data analysis is given in Table I. Figure 1A shows a representative segment of the experimental electron density map.

SopE

SopE is composed of six α -helices arranged in two three-helix bundles. The two bundles measure ~40 Å in length and 30 Å in width and are arranged in a V-shaped fashion (Figure 1B). We observed only one small two-stranded β -sheet, which is followed by a peptide segment (residues 165–172) connecting the two sides of the 'V'. This segment harbors a ¹⁶⁶GAGA¹⁶⁹ motif, which is proposed to represent the catalytic core of SopE (see Figure 1C).

Analysis of the surface charge distribution with the program GRASP (Nicholls *et al.*, 1991) did not show preferentially acidic or basic character.

A homology search with the program DALI (Holm and Sander, 1993) did not identify similarity with the Rho family-specific DH domains from the eukaryotic RhoGEFs Sos, Trio, β PIX or Tiam1 (Aghazadeh *et al.*, 1998; Liu *et al.*, 1998; Soisson *et al.*, 1998; Worthylake *et al.*, 2000). The DH domain is also an α -helical protein, but the location and topological arrangement of helices are quite different, as shown in Figure 1D. SopE also has no similarity to other nucleotide exchange factors for Ras-related GTP-binding proteins (Boriack-Sjodin *et al.*, 1998; Cherfils *et al.*, 1998; Mossessova *et al.*, 1998; Renault *et al.*, 1998). Structural relationships were found with proteins containing three-helix bundle 'motifs', with ribosome recycling factor (Selmer *et al.*, 1999; Kim *et al.*, 2000) and the helical segment of human guanylate-binding protein showing the highest scores of 5.8 and 5.7, respectively (Prakash *et al.*, 2000). These similarities do not reflect common functional aspects, but rather confirm the three-helix bundle as a structural element of a large number of proteins.

The complex with Cdc42

The overall structure of Cdc42 is similar to that found in crystal structures of other Rho family proteins (e.g. Hirshberg *et al.*, 1997; Wei *et al.*, 1997). Conformational changes are restricted to the switch regions of Cdc42 that interact directly with SopE (discussed below). The switch I and switch II regions of Ras-related GTP-binding proteins are involved in nucleotide binding and define the major conformational differences between GTP- and GDP-bound forms (Milburn *et al.*, 1990) (see below). Presumably as a consequence of the crystallization conditions (see Materials and methods), sulfate ions are found in various favorable binding sites, the functionally most important of which is the phosphate-binding (P) loop (Saraste *et al.*, 1990). This sulfate ion is located in the position which would be occupied by the β -phosphate moiety when nucleotide is present. Like the β -phosphate, it is stabilized by interactions with main chain amide groups derived from the P-loop motif (GxxxxKS/T) and the side chain amino group of Lys16. A similar situation is found in the complex between Rac1 and the RhoGEF Tiam1 (Worthylake *et al.*, 2000) (see below), in the Ran–RCC1 complex (Renault *et al.*, 2001) or in guanosine-bound Ras (Scheffzek *et al.*, 1994).

The complex interface

The complex interface is formed primarily by helices α 2, α 4 and α 5 from SopE, including flanking regions that communicate mainly with the switch regions of Cdc42 (Figure 2A and B). The interface buries ~2800 Å² of accessible surface area, which is similar to that of the Rac1–Tiam1 (Worthylake *et al.*, 2000) and Dbs–Cdc42 (Rossman *et al.*, 2002) complexes and somewhat higher than those of other Rho regulatory complexes (Rittinger *et al.*, 1997; Hoffman *et al.*, 2000; Scheffzek *et al.*, 2000). As in other G-protein–GEF complexes (Kawashima *et al.*, 1996; Wang *et al.*, 1997; Boriack-Sjodin *et al.*, 1998; Goldberg, 1998), the base-binding pocket is not occluded by the GEF component and is accessible from the solvent.

Table I. Summary of crystallographic analysis

	Native	CH ₃ HgCl	K ₃ UO ₂ F ₅
Data collection ^a			
Resolution (Å)	20–2.3	25–2.3	20–2.5
Highest shell (Å)	2.4–2.3	2.4–2.3	2.6–2.5
Unique reflections	40 507 (4737)	76 154 (9239)	59 109 (6611)
Redundancy	8.7 (7.6)	2.6 (2.6)	1.9 (1.9)
<i>I</i> / σ	15.2 (5.5)	12.48 (5.18)	7.49 (2.37)
<i>R</i> _{sym} (%) ^b	11.2 (32.8)	5.6 (20.2)	7.2 (29.4)
<i>R</i> _F (%) ^c	–	28.9	13.8
Completeness (%)	99.9 (100.0)	97.5 (98.6)	99.3 (99.8)
MIRAS analysis			
Resolution (Å)	–	20–2.6	20–2.6
Soaking			
Concentration/time		0.1 mM/13 h	5 mM/108 h
No. of sites		4	3
<i>R</i> _{cullis} ^d		0.75	0.86
Anomalous <i>R</i> _{cullis}		0.89	0.99
Phasing power ^e		1.29	0.78
Anomalous phasing power		7.15	1.85
Overall figure of merit	0.52		
Refinement			
Resolution (Å)	20–2.3		
Reflections	40 297		
<i>R</i> _{work} (%) ^f	22.7		
<i>R</i> _{free} (%) ^g	25.8		
No. of atoms			
Protein	5308		
Sulfate	6		
Solvent	160		
R.m.s. deviations			
Bonds (Å)	0.006		
Angles (°)	1.25		

^aNumbers in parentheses give values for the highest resolution bin.

^b $R_{\text{sym}} = \sum |I_{\text{hi}} - \bar{I}_{\text{h}}| / \sum I_{\text{hi}}$, where I_{hi} is the scaled intensity of the *i*th symmetry-related observation of reflection *h* and $\bar{I}_{\text{h}}$ is the mean value.

^c $R_{\text{F}} = 2 \sum |F_{\text{PH}} - F_{\text{P}}| / \sum |F_{\text{PH}} + F_{\text{P}}|$, where F_{PH} and F_{P} are the derivative and native structure amplitudes.

^dGeneralized *R*_{cullis} as implemented in CNS (Brünger *et al.*, 1998).

^eAs defined in CNS (Brünger *et al.*, 1998).

^f $R_{\text{work}} = \sum |F_{\text{o}} - F_{\text{c}}| / \sum F_{\text{o}}$, where F_{o} and F_{c} are the observed and calculated structure factor amplitudes for reflection *h*, respectively.

^gValue of *R*_{free} calculated for 10% randomly chosen reflections not included in the refinement.

SopE does not interact with P-loop residues, which is consistent with the observation that Cdc42_{G12V} is an efficient substrate (Rudolph *et al.*, 1999a).

In order to explore the catalytic mechanism of SopE-mediated guanine nucleotide exchange, we have superimposed the structure of the nucleotide-free SopE–Cdc42 complex (Figure 2A) with the structure of GDP-loaded Cdc42 (Rudolph *et al.*, 1999b). The core of the G-domains is virtually identical in both structures, but major conformational changes are observed in the switch I/II regions of Cdc42 (Figure 2C). The most notable feature of SopE contributing to these conformational changes is the catalytic ¹⁶⁶GAGA¹⁶⁹-loop of SopE, which is inserted between the switch regions of Cdc42 and appears to exert a push-and-pull-type movement whereby switch I is pushed aside and switch II is pulled towards the loop (Figure 2C). Insertion of the ¹⁶⁶GAGA¹⁶⁹-loop of SopE induces two consecutive peptide flips in the C-terminal half of switch I, rotating Phe37_{cdc42} by ~90° and Tyr40_{cdc42} by almost 180° (Figure 2D). The resulting conformation is stabilized by a salt bridge between Asp38_{cdc42} and Lys198_{sopE} along with a hydrogen bond between the hydroxyl group of Tyr40_{cdc42} and the main chain carbonyl group of Gln194_{sopE}. In addition, the hydrophobic core burying the aromatic side chains of Phe37_{cdc42} and Tyr40_{cdc42}

appears to contribute to stabilization (Figure 2). The major contribution of the catalytic ¹⁶⁶GAGA¹⁶⁹-loop to the proposed mechanism of SopE-mediated nucleotide release is supported by site-directed mutagenesis of Gly168_{sopE} to alanine. The mutant protein, under the conditions employed, does not stimulate nucleotide exchange on Cdc42 as measured by a fluorescence assay where Cdc42-bound fluorescent mant-nucleotide is exchanged against a large excess of unlabeled nucleotide (Figure 2E).

The displacement of switch I seems to be stabilized by interactions of Asp124_{sopE} with the hydroxyl group of Tyr32_{cdc42}, as well as with the main chain amide groups of Thr35_{cdc42} and Val36_{cdc42}. The side chains of these residues also participate in the interface. Presumably as a consequence of the observed interactions, the N-terminal portion of switch I becomes disordered (residues 26–31) and extruded from the nucleotide-binding pocket (Figure 2B and C). Switch II is displaced towards the catalytic loop and its C-terminal end is involved in interactions with the area around helices $\alpha 2$ and $\alpha 4$ of SopE, forming an extensive interface (Figure 2B and C). Hydrophobic contributions come from Tyr64_{cdc42}, and a number of polar contacts involving Tyr64_{cdc42}, Asp65_{cdc42}, Arg66_{cdc42} and Ser71_{cdc42} apparently stabilize the interface in this area (Figure 2B and C).

Fig. 1. The GEF domain of SopE. (A) Segment of the experimental electron density map, contoured at 1.5σ showing a section of the complex interface. (B) Diagram of the structure of SopE, with helices as cylinders and the catalytic loop highlighted in red. (C) Sequences of the GEF domains of SopE (*S. typhimurium*, accession No. O52623; *S. dublin*, accession No. O06949; *S. typhi*, accession No. Q9RPM6) and SopE2 (accession No. Q9KIZ2), with the assignment of secondary structure elements according to the program DSSP (Kabsch and Sander, 1993). Selected residues in the interface (see Figure 2) are marked with filled circles. The catalytic loop involved in close contact with Cdc42 is highlighted. (D) Diagram of the structure of the DH domain of Tiam1, with helices as cylinders, as seen in the Rac1-Tiam1 complex (Worthylake *et al.*, 2000), in the same orientation relative to the GTP-binding protein as SopE in (B). The functionally important lysine residue (see Figure 3) is included.

The most extensive contacts are formed between the catalytic loop of SopE and the N-terminal part of switch II, involving residues Ala59_{Cdc42}, Gly60_{Cdc42} and Gln61_{Cdc42}. They form a network of polar main chain and van der Waals interactions with Ser165_{SopE}, Gly166_{SopE}, Gly168_{SopE} and Ala169_{SopE}. The most dramatic effect on the conformation of switch II is a peptide flip involving Ala59_{Cdc42}, whereby its methyl group points into and thus blocks the Mg²⁺-binding site (Figure 2C). Strikingly, a similar conformation of Ala59_{Cdc42} has been observed in GDP-bound RhoA in the absence of Mg²⁺ (Shimizu *et al.*, 2000) and also in other G-protein–GEF complexes (Boriack-Sjodin *et al.*, 1998; Worthylake *et al.*, 2000). The conformation of Ala59_{Cdc42} is stabilized by a main chain–main chain interaction with Ala169_{SopE}.

Implications for nucleotide release

We have shown earlier that SopE increases the guanine nucleotide exchange on Rho proteins 10⁵-fold above the intrinsic reaction rate (Rudolph *et al.*, 1999a), which is similar to the catalytic rates of GEFs for Ras-related proteins (Klebe *et al.*, 1995; Lenzen *et al.*, 1998; Hutchinson and Eccleston, 2000). The structure of the nucleotide-free SopE–Cdc42 complex provides important

insights to explain the accelerated guanine nucleotide release stimulated by the bacterial exchange factor.

Reorientation of switch I causes extrusion of residues potentially important for binding of the nucleotide, especially of the guanine base. The rearrangements in switch I include the disordering of its N-terminal segment, as inferred from ill-defined electron density in this region (Figure 2C). This leads to extrusion of Phe28_{Cdc42} from the nucleotide-binding pocket, where it usually provides a major contribution to the binding of GDP/GTP. Similarly to Ras, the mutation of this amino acid to leucine leads to 140-fold increased nucleotide dissociation rates (Reinstein *et al.*, 1991; Lin *et al.*, 1997), highlighting the importance of this hydrophobic interaction for nucleotide affinity.

In the nucleotide-free Ras–Sos complex, the switch I region of Ras is flipped out of its position close to the nucleotide by a long helical extension on Sos, and it has been argued that this is the key feature driving guanine nucleotide release (Boriack-Sjodin *et al.*, 1998). Here we also find that most of the switch I region is pushed aside due to the action of the catalytic loop of SopE, arguing that reorientation of residues such as Glu31_{Cdc42} in addition to Phe28_{Cdc42} might contribute to accelerated nucleotide release. In the Rac1–GppNHp, but not in the Cdc42–GDP complex, Glu31_{Rac1} is coordinated to the 3'-oxygen of the

Fig. 2. The Cdc42–SopE complex. (A) Ribbon representation of the complex with SopE in blue and Cdc42 in yellow. The switch regions are shown in green. The catalytic loop inserted between the switch regions is highlighted in red. (B) Schematic representation of the Cdc42–SopE interaction with important interactions indicated. Hydrophobic contacts are in green, polar interactions in red. Arrowheads mark side chain and straight ends indicate main chain contributions. (C) Close up view of the interface region with the switch regions of GDP-bound Cdc42 (in light gray) included. Side chains of residues considered important for the interaction are shown. Dashed segments indicate disordered regions, modeled stereochemically. The gray ball near Ala59 is the Mg²⁺ ion from the Cdc42–GDP structure. (D) Stereo view of the nucleotide-binding region, showing the specific rearrangements of the switch regions associated with SopE–Cdc42 interaction, as discussed in the text. (E) Multiple turnover kinetics of nucleotide exchange on Cdc42-mantGDP mediated by GST–SopE_{G168A}. Release of mantGDP was followed by fluorescence spectrometry in buffer S (50 mM NaCl, 50 mM Tris–HCl pH 7.6, 5 mM MgCl₂) in the presence of 1 mM GDP. The assays were performed with 1 μM Cdc42-mantGDP and 250 nM GST, 10 mM EDTA, 25 nM GST–SopE_{G168A} (gray line) or 25 nM GST–SopE (excitation wavelength 366 nm, emission wavelength 440 nm, step size 1 nm, band-pass 4 nm).

C

D

E

ribose and this residue is conserved in Rho family proteins. Therefore, we speculate that the specific reorientation of Phe28_{cdc42} and Glu31_{cdc42} and/or the general rearrangement of the effector loop, which runs alongside the nucleotide and partially shields it from the solvent, might both contribute to the accelerated nucleotide release.

In switch II, a peptide flip positions the methyl group of Ala59_{cdc42} within 2 Å of the Mg²⁺-binding site (Figure 2C and D). Since Mg²⁺ is important for high-affinity binding of GDP/GTP from Ras-like GTP-binding proteins (Vetter and Wittinghofer, 2001) and excess Mg²⁺ decreases dissociation of GDP from Cdc42 by 140-fold (Zhang *et al.*, 2000), the SopE-induced displacement of Mg²⁺ might be an important step in guanine nucleotide release. The conformation of switch II in SopE-bound Cdc42 is strikingly similar to that of switch II regions in Mg²⁺-free GDP-bound RhoA (Shimizu *et al.*, 2000) and Tiam1-bound Rac1 (Worthylake *et al.*, 2000), with Ala59_{rac1} pointing into the Mg²⁺-binding site. It is possible that part of the switch II region is stabilized in a Mg²⁺-free conformation and that the GEF activity of SopE is due at least partially to its effect on removal of the metal ion. The role of the methyl group of Ala59 in the GEF reaction has been investigated for the Ras–Sos system by site-directed mutagenesis (Hall *et al.*, 2001). These studies showed that this residue might not be important for nucleotide release in the Ras–Sos reaction. In order to assess the role of the almost invariant Ala59 for the RhoGEF mechanism in more detail, however, site-directed mutagenesis combined with fast kinetic analysis would be necessary. Reorientation of Thr35 in switch I, in turn, is unlikely to have an effect on nucleotide affinity since it is coordinated to Mg²⁺ only in the triphosphate form, and its mutation to alanine in Ras reduces Mg²⁺ affinity in that complex only 4-fold (Spoerner *et al.*, 2001).

Studies on Ras have shown that the β-phosphate contributes significantly to binding affinity of the nucleotide, since GMP and guanosine bind with five orders of magnitude weaker affinity than GTP or GDP (John *et al.*, 1990). Since removal of Mg²⁺ or Phe28_{cdc42} would only partially account for the 10⁵-fold stimulation of nucleotide release from Cdc42 by SopE, it is possible that SopE, like other GEFs, interferes with binding of the phosphate moiety of the nucleotide. Structures of Ras, Arf and EF-Tu complexed with their cognate GEFs indeed show GEF side chains pointing into phosphate-binding regions (Kawashima *et al.*, 1996; Wang *et al.*, 1997; Boriack-Sjodin *et al.*, 1998; Goldberg, 1998). However, no such residues were found in our structure or in other GEF–G-protein complexes where a sulfate ion is bound in the P-loop (Worthylake *et al.*, 2000; Renault *et al.*, 2001). On the basis of the Ran–RCC1 complex (Renault *et al.*, 2001), it has been suggested that the sulfate-bound structure in the crystal represents an intermediate along the kinetic pathway of nucleotide exchange, and that rearrangement of the nucleotide-binding site after release of the anion may produce conformational changes that lead to the nucleotide-free structures observed in Ras–Sos, Arf–Sec7 and EF-Tu–EF-Ts (Kawashima *et al.*, 1996; Boriack-Sjodin *et al.*, 1998; Goldberg, 1998).

The SopE-induced conformational rearrangements of switch I and switch II shown here disrupt several favorable interactions necessary for tight guanine nucleotide binding

and induce nucleotide release. Since the nucleotide-binding pocket is almost freely accessible from the solvent, unrestricted rebinding of nucleotide and formation of a low-affinity trimeric complex would be possible. In the course of the complete GEF reaction, concomitant restructuring of the switch regions would not be compatible with the SopE binding and would thus lead to dissociation of SopE and formation of a high-affinity nucleotide complex.

Comparison with the eukaryotic Rac1–Tiam1 complex and evolutionary relationship

In order to compare the catalytic mechanism of SopE-mediated guanine nucleotide exchange with that of eukaryotic RhoGEFs, we have superimposed the structure of the Cdc42–SopE complex with that of the Rac1–Tiam1 complex (Worthylake *et al.*, 2000). Alignment of both complexes on the G-domain cores revealed that the switch regions of the Rho proteins have a remarkably similar local arrangement (Figure 3) such as the flip of Phe28_{cdc42/rac1} and Ala59_{cdc42/rac1} as well as displacement of Phe37_{cdc42/rac1}. Only the local arrangement around Tyr40_{cdc42/rac1} differs significantly between the two structures (Figure 3). In the Rac1–Tiam1 complex, the side chain of Tyr40_{rac1} is rotated by 180° relative to its orientation in the Cdc42–SopE complex. Nevertheless, SopE and the eukaryotic GEF Tiam1 induce very similar overall rearrangements in the switch I and switch II regions of the Rho component to accelerate nucleotide release.

In sharp contrast, however, the GEF components of both protein complexes are completely different (Figure 1B and D). While both GEFs have a high α-helical content, they have different tertiary folds and both use different amino acids to interact with their Rho target (Figure 3). Even the catalytic cores of both GEFs differ markedly: to distort switch I and II, SopE employs main chain contacts of the ¹⁶⁶GAGA¹⁶⁹-loop, which is composed entirely of small side chains, while Tiam1 uses helix α8 and particularly the large side chain of Lys1195 (Figure 3). Mutations of Lys1195 in Tiam1 or of the analogous amino acid in the DH domain of Trio have been reported to decrease the GEF activity by at least 80%, supporting its importance for the catalytic mechanism (Liu *et al.*, 1998; Worthylake *et al.*, 2000). The only similarity between both GEFs is that Asp124_{sopE} and Glu1047_{tiam} engage in analogous contacts with the side chain of Tyr32_{cdc42/rac1} and the main chain amides of Thr35_{cdc42/rac1} and Val36_{cdc42/rac1} (see Figure 5 in Worthylake *et al.*, 2000). Based on mutational analyses of DH proteins, substitution of the homologous glutamate by alanine in the exchange factor Trio reduces its activity to 50% of the wild-type rate (Liu *et al.*, 1998). Comparing the complexes leads us to conclude that SopE is not just mimicking Rho family-specific eukaryotic GEFs of the DH family, but that it represents an entirely novel class of RhoGEFs. Why SopE appears to be a more efficient guanine nucleotide exchange catalyst is presently unclear and has been investigated in more detail, using as a conceptual framework the structural information from the SopE and DH complexes.

Bacterial pathogens have evolved a variety of strategies to manipulate signaling inside animal cells. Many bacterial toxins modulate Rho signaling pathways by covalently

Fig. 3. Similar GEF mechanisms by SopE and Tiam1 using distinct structural elements. The Cdc42–SopE and Rac1–Tiam1 complexes have been aligned on the G-domain shown in gray. The P loop and switch I/II regions are shown for the individual molecules. Helices $\alpha 2$ and $\alpha 4$ from SopE are in blue, and helix $\alpha 8$ from Tiam1 in red. The view shows the similar location of the catalytic loop from SopE and the important Lys1195 from Tiam1.

modifying Rho proteins (Aktories *et al.*, 2000). These modifications either activate or inactivate Rho proteins. The translocated toxins SopE, SopE2 and SptP from *S.typhimurium* and ExoS from *Pseudomonas aeruginosa* belong to a new group of toxins interfering with Rho signaling by non-covalent interaction with host cell GTP-binding proteins of the Rho subfamily. These toxins allow *S.typhimurium* to modulate reversibly the activity of Rho proteins. Therefore, they can be viewed as functional analogs of regulators of their host cells. Here we have demonstrated that SopE very closely copies the functional mechanism of GEFs from eukaryotic cells, but uses a different fold and residues for the task.

The structural and functional properties of SopE are in sharp contrast to those of SptP and other similar toxins from *Yersinia* spp. and *Pseudomonas* spp. These proteins, which act as transient inactivators of Rho family members by increasing the rate of GTP hydrolysis, use the same catalytic motif (the ‘arginine finger’; Scheffzek *et al.*, 1998) as eukaryotic GTPase-activating proteins (GAPs) and therefore constitute examples of molecular mimicry (Stebbins and Galán, 2000; Würtele *et al.*, 2001).

Considering the long standing co-evolution between *Salmonella* spp. and their mammalian hosts, it is intriguing to speculate that hosts have little chance to evolve variants of Rho resistant to modulation. Since SopE (and SptP) binds to virtually identical elements of the Rho target as the eukaryotic GEFs (and GAPs), any mutation interfering with the SopE interaction would almost certainly also negatively affect interaction with the host cellular GEFs (and GAPs). In addition, SopE recognizes several different members of the Rho family. Therefore, multiple mutations in different genes would be necessary to evolve host resistance.

Materials and methods

Protein expression and purification

Salmonella typhimurium SopE (residues 78–240) and human Cdc42 (residues 1–178) were expressed in *E.coli* BL21 as recombinant GST fusion proteins. Cells grown to an OD₆₀₀ of 0.8 in LB medium with 100 µg/ml ampicillin were induced at 30°C with 0.1 mM isopropyl- β -D-thiogalactopyranoside (IPTG) and cultivated for 7–10 h. After homogenizing the cells with a fluidizer (Microfluidics, Newton, MA), the suspension was centrifuged at 30 000 g for 30 min. GST fusion proteins of SopE and Cdc42 were purified on glutathione–Sephadex (Pharmacia). After application, the column was washed extensively with buffer A [50 mM Tris–HCl pH 7.5, 100 mM NaCl, 5 mM dithiothreitol (DTT)], followed by buffer B (50 mM Tris–HCl pH 7.5, 500 mM NaCl, 5 mM DTT) and again with buffer A. After cleavage with thrombin overnight on the column, the proteins were eluted with buffer A and thrombin was removed by chromatography on benzamidine–Sephadex 6B (Pharmacia). Further purification by gel filtration chromatography (Superdex 75, Pharmacia) resulted in the SopE construct (residues 78–240), which contains two additional amino acids (Gly76–Ser77), and the Cdc42 construct that also included glycine and serine at the N-terminus. We obtained ~1 mg of protein per gram of cells in both cases.

The Cdc42–SopE complex was formed by incubating the two purified proteins at 4°C in buffer [20 mM Tris–HCl pH 7.5, 150 mM NaCl, 5 mM MgCl₂, 200 mM (NH₄)₂SO₄, 0.1 mM ZnCl₂ and 5 U of alkaline phosphatase/mg Cdc42] using an ~2-fold molar excess of Cdc42. Alkaline phosphatase and ZnCl₂ were used in order to remove the nucleotide. The complex solution was purified through size exclusion chromatography using a Superdex 75 gel filtration column (Pharmacia, 16/60) and a running buffer with 20 mM Tris–HCl pH 7.5, 30 mM NaCl, 1 mM (NH₄)₂SO₄, 2 mM EDTA and 5 mM DTT. Fractions containing the complex were pooled and concentrated to 44 mg/ml.

Crystallization

Crystals were grown at room temperature by vapor diffusion from hanging drops formed by equal volumes (1 + 1 µl) of protein (44 mg/ml) and crystallization buffer [1.9 M ammonium sulfate, 0.1 M sodium citrate pH 5.6, 2% polyethylene glycol (PEG)], suspended over a reservoir of 500 µl. Small crystalline needles (average size 100 × 10 × 10 µm³) appeared after 1–2 days. They were improved by addition of 0.05 M betaine and using microseeding protocols, and reached a typical size of

$250 \times 40 \times 20 \mu\text{m}^3$. Crystals belong to the trigonal space group $P3_121$ with cell dimensions $a = b = 87.5 \text{ \AA}$, $c = 200.5 \text{ \AA}$, $\alpha = \beta = 90^\circ$, $\gamma = 120^\circ$, and contain two Cdc42–SopE complexes in the asymmetric unit with a solvent content of 58%.

Data collection

Initial data sets of tiny needles, diffracting to a resolution of $\sim 2.5 \text{ \AA}$, were collected at the microfocus beamline (ID13) of the European Synchrotron Radiation Facility (ESRF, Grenoble, France). Improved crystals were used for routine data collection on beamline ID14-1 at ESRF.

Crystals were prepared for flash cooling by transfer to a stabilization buffer (2.0 M ammonium sulfate, 0.1 M sodium citrate pH 5.6, 2% PEG, 0.05 M betaine) containing 15% (v/v) glycerol as cryoprotectant. Data sets of typically $2.3 \text{ \AA}$ in resolution were collected from cryofrozen crystals at a temperature of 100 K using the rotation method (crystal to detector distance, 150 cm; oscillation angle, 1° ; wavelength, $0.934 \text{ \AA}$; detector MAR 165 mm CCD). Data were processed and scaled with the XDS package (Kabsch, 1993) (see Table I).

Structure determination and refinement

The structure was determined by multiple isomorphous replacement anomalous scattering (MIRAS), combined with molecular replacement. Heavy atom soaks were performed in stabilization buffer (see above) containing 0.1–5 mM heavy metals of seven tested compounds. CH_3HgCl - and $\text{K}_3\text{UO}_2\text{F}_5$ -soaked crystals produced derivatives of sufficient quality to be used for phasing. All MIRAS analysis (see Table I) was carried out with the program CNS (Brünger *et al.*, 1998). Initial heavy atom positions in the mercury derivative were determined using difference Patterson methods and used to obtain the heavy atom coordinates in the uranyl derivative by difference Fourier methods. Phases were calculated, and improved using ‘solvent flipping’ as implemented in CNS. The resulting electron density map was of good quality and was used to position the Cdc42 model (Rudolph *et al.*, 1999b) in the unit cell, followed by tracing the SopE portion of the asymmetric unit. After several cycles of interactive model building with O (Jones *et al.*, 1991) and refinement with CNS, the model was completed finally to two Cdc42–SopE complexes, 160 water molecules and six sulfate ions. Inspection of the model with PROCHECK (Laskowski *et al.*, 1993) did not reveal unfavorable Φ, Ψ combinations. Visualization of structural models was done with the programs MOLSCRIPT (Kraulis, 1991) and RASTER3D (Merritt and Bacon, 1997).

Fluorescence spectrometry

The Cdc42-mantGDP complex was prepared as published recently (Friebe *et al.*, 2001). Fluorescence measurements were performed as multiple turnover kinetics in an Aminco-Bowman Series 2 Luminescence spectrometer. The excitation wavelength was 366 nm, and emission was recorded at 440 nm. Release of mantGDP was followed in buffer S (40 mM HEPES pH 7.4, 100 mM NaCl, 5 mM MgCl_2) in the presence of 1 mM GDP. The assays contained 1 μM Cdc42-mantGDP and 250 nM GST, 10 mM EDTA, 25 nM GST–SopE_{G168A} or 25 nM GST–SopE.

Coordinates

Coordinates have been deposited with the Protein Data Bank under the ID code 1gzs.

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