Crystal structure of the C3bot–RalA complex reveals a novel type of action of a bacterial exoenzyme

Alexander Pautsch^{1,4}, Martin Vogelsgesang^{2,4}, Jens Tränkle³, Christian Herrmann³ and Klaus Aktories^{2,*}

¹Structural Research, Department of Integrated Lead Discovery, Boehringer Ingelheim Pharma GmbH & Co. KG, Biberach an der Riss, Germany, ²Institut für Experimentelle und Klinische Pharmakologie und Toxikologie der Universität Freiburg, Otto-Krayer-Haus, Freiburg, Germany and ³Physikalische Chemie I, Ruhr-Universität Bochum, Bochum, Germany

C3 exoenzymes from bacterial pathogens ADP-ribosylate and inactivate low-molecular-mass GTPases of the Rho subfamily. Ral, a Ras subfamily GTPase, binds the C3 exoenzymes from *Clostridium botulinum* and *C. limosum* with high affinity without being a substrate for ADP ribosylation. In the complex, the ADP-ribosyltransferase activity of C3 is blocked, while binding of NAD and NAD-glycohydrolase activity remain. Here we report the crystal structure of C3 from *C. botulinum* in a complex with GDP-bound RalA at 1.8 Å resolution. C3 binds RalA with a helix–loop–helix motif that is adjacent to the active site. A quaternary complex with NAD suggests a mode for ADP-ribosyltransferase inhibition. Interaction of C3 with RalA occurs at a unique interface formed by the switch-II region, helix α 3 and the P loop of the GTPase. C3-binding stabilizes the GDP-bound conformation of RalA and blocks nucleotide release. Our data indicate that *C. botulinum* exoenzyme C3 is a single-domain toxin with bifunctional properties targeting Rho GTPases by ADP ribosylation and Ral by a guanine nucleotide dissociation inhibitor-like effect, which blocks nucleotide exchange.

The EMBO Journal (2005) 24, 3670–3680. doi:10.1038/sj.emboj.7600813; Published online 22 September 2005

Subject Categories: structural biology; microbiology & pathogens

Keywords: bacterial ADP-ribosyltransferase; exoenzyme C3; GDP-binding; Ral; Rho

Introduction

Clostridium botulinum C3 exoenzyme (C3bot) is the prototype of a family of ADP-ribosyltransferases, which modify eukaryotic low-molecular-mass GTPases of the Rho family (Aktories *et al.*, 1987, 1989; Rubin *et al.*, 1988; Chardin *et al.*,

1989). Other members of this family of bacterial exoenzymes are C3lim from *Clostridium limosum*, C3cer from *Bacillus cereus* and three isoforms of C3stau (also called EDIN) from *Staphylococcus aureus*, which exhibit about 35–70% sequence identity between each other (Aktories *et al.*, 2004). C3 transferases are ~25 kDa proteins, which harbor the enzyme activity, but apparently miss any specific cell binding and transportation unit. Therefore, these exoenzymes differ from typical AB toxins, which contain in addition to the enzyme domain a unit for cell binding and transportation (Aktories *et al.*, 2004).

Crystal structures have shown that C3bot (Han *et al.*, 2001) and C3stau2 (Evans *et al.*, 2003) share the canonical mixed α/β -fold of ADP-ribosylating enzymes. An extended central cleft functions as the binding site for the cosubstrate NAD (Ménétrey *et al.*, 2002; Evans *et al.*, 2003). A structural motif, termed ‘ADP-ribosylating-toxin-turn-turn-motif’ (ARTT motif) was first identified in C3bot (Han *et al.*, 2001). The ARTT motif consists of two short amino-acid stretches (C3bot²⁰⁷–C3bot²¹⁰ and C3bot²¹¹–C3bot²¹⁴), which are suggested to be responsible for substrate recognition and important for catalytic activity. Amino-acid exchanges in this region caused major reduction in ADP ribosylation of Rho GTPases and decreased interaction of C3 with Rho GTPases in precipitation assays (Wilde *et al.*, 2002b). So far, no direct structural analysis of the interaction of C3 (or other toxins) with its protein substrates is available, but a recent report (Sun *et al.*, 2004) has delineated regions that are necessary and sufficient for substrate binding of the C3-related ADP-ribosyltransferases ExoS and ExoT from *Pseudomonas aeruginosa*. These transferases recognize their respective substrates via three regions: ARTT loop, phosphate-nicotinamide (PN) loop and ‘region B’. Whereas the PN loop links strands β 3– β 4 (C3bot¹⁷⁷–C3bot¹⁸⁷), region B corresponds to an α 2–loop– α 3 motif in C3-like toxins (C3bot⁷⁹–C3bot⁸⁸).

All C3-like ADP-ribosyltransferases modify RhoA, B and C at asparagine-41 (Sekine *et al.*, 1989). C3stau additionally modifies RhoE (Wilde *et al.*, 2001). Other Rho GTPases are poor (Rac) or not at all substrates for ADP ribosylation by C3-like exoenzymes (Just *et al.*, 1992). ADP ribosylation of Rho GTPases inhibits their cellular functions most likely by inhibition of activation by guanine nucleotide exchange factors (GEFs) (Sehr *et al.*, 1998) and/or by stabilizing a tight complex of ADP-ribosylated Rho with the Rho regulatory guanine nucleotide dissociation inhibitor (GDI) (Genth *et al.*, 2003).

Ral is a member of the Ras subfamily of small GTPases and exists in two isoforms, RalA and B, which share 82% amino-acid identity between each other and ~35% to RhoA (Chardin and Tavittian, 1986; Feig, 2003). Ral GTPases have been implicated in several cellular processes, including transcriptional activation (Hernandez-Munoz *et al.*, 2000),

*Corresponding author. Institut für Experimentelle und Klinische Pharmakologie und Toxikologie der Universität Freiburg, Otto-Krayer-Haus, Albertstrasse 25, 79104 Freiburg, Germany.

Tel.: +49 761 203 5301; Fax: +49 761 203 5311;

E-mail: Klaus.Aktories@pharmakol.uni-freiburg.de

⁴These authors contributed equally to this work

Ras-mediated cell transformation (Feig *et al*, 1996; Urano *et al*, 1996) and cytoskeleton rearrangement (Jullien-Flores *et al*, 1995; Park and Weinberg, 1995; Ohta *et al*, 1999). Ral activates phospholipase D1 (PLD1) (Jiang *et al*, 1995; Luo *et al*, 1998) and both RalA and PLD1 modulate receptor endocytosis (Jullien-Flores *et al*, 2000) and vesicle transport (Shen *et al*, 2001). RalA is involved in regulation of the mammalian exocyst complex (Moskalenko *et al*, 2002). In mammals, this complex is involved in transport of Golgi-derived vesicles to basolateral membranes. Activated Ral interacts directly with Sec5 (Moskalenko *et al*, 2002), an interaction which is responsible for secretory and cytoskeletal effects (Sugihara *et al*, 2002). The structural basis for Ral-dependent effector activation was delineated by a complex between the Ral-binding domain of Sec5 and RalA (Fukai *et al*, 2003).

Surprisingly, C3 exoenzymes from *C. botulinum* and *C. limosum* bind with high affinity to RalA (Wilde *et al*, 2002a) without modifying the GTPase by ADP ribosylation. Binding of RalA to C3bot blocks the ADP ribosylation of RhoA by the exoenzyme. Moreover, activation of the Ral-effector PLD1 was inhibited for the RalA-C3bot complex (Wilde *et al*, 2002a). During submission of our work, Holbourn *et al* (2005) presented the crystal structure analysis of a RalA-GDP-C3bot complex, where an astonishingly small interface involves helix $\alpha 4$ and strand $\beta 6$ of RalA and the ARTT loop of C3bot (for simplicity, this model will be referred to as ' $\alpha 4$ -ARTT model', hereafter).

Here, we solved the crystal structure of the RalA-GDP-C3bot complex and demonstrate that the biological unit is fundamentally different from that proposed in the $\alpha 4$ -ARTT model. Structural and biochemical data show that C3bot is a single-domain toxin with bifunctional properties targeting Rho GTPases by ADP ribosylation and Ral by a 'GDI-like effect', which blocks nucleotide exchange and effector interaction.

Results and discussion

Ral and C3bot form a tight complex

Recently, we reported that RalA inhibits the C3-catalysed ADP ribosylation of RhoA by formation of a tight RalA-C3 complex (Wilde *et al*, 2002a). A similar inhibition of ADP ribosylation of RhoA by C3bot was observed in the presence of RalB, but not with H-Ras (Figure 1A). Precipitation experiments with GST-RalA beads suggested that interaction of the GTPase with C3bot was slightly more stable than with C3lim. Similar data were obtained with GST-RalB (data not shown). By contrast, no stable complexes were precipitated with C3cer and C3stau2 (Figure 1B).

The affinity of the RalA-C3bot interaction was quantified by two independent methods. First we used protein fluorescence titration with a tryptophan-deficient C3bot mutant (see Materials and methods) and measured a K_D value of 35 ± 15 nM (Supplementary Figure 1). The affinity of the interaction was confirmed by isothermal titration calorimetry (ITC). A similar K_D value of 60 ± 20 nM was observed for the RalA-GDP-C3bot complex (Figure 1C and Table I). In contrast, binding of C3bot to the active RalA-GppNHp (a non-hydrolysable GTP analogue) could not be detected (Table I). In an earlier publication, Wilde *et al* (2002a) reported no significant difference between RalA-GDP and RalA-GTP γ S in

Figure 1 C3bot and Ral form a stable complex. (A) Influence of RalA, RalB and H-Ras on the ADP ribosylation of RhoA. Rat brain lysate was incubated with C3bot and [32 P]NAD in the presence or absence of the indicated GTPases. Samples were subjected to SDS-PAGE followed by autoradiography. For detection of the RhoA level after the ADP ribosylation, a second aliquot of each sample was applied to Western blot analysis. (B) Precipitation of C3 by GST-RalA. GST-RalA immobilized to glutathione-Sepharose beads was incubated with the indicated C3 proteins. Thereafter, beads were washed three times and subjected to SDS-PAGE. Coomassie Blue-stained gel is shown. (C) Calorimetric titration of C3bot with RalA-GDP. The upper panel shows the heating power with peakwise changes after each 5 μ l injection of RalA-GDP. These data are integrated and plotted versus the concentration ratio in the lower panel. The evaluation according to a single-site binding model results in a K_D value of 60 ± 20 nM.

Table I Calorimetric data for the RalA-C3bot interaction

Variant ^a	ΔH° (kcal/mol)	K_A (10^6 M ⁻¹)	K_D (μ M)
RalA-GDP/C3bot	-20 ± 2	16 ± 5	0.06 ± 0.02
RalA-GppNHp/C3bot	—	< 0.1	> 10
RalA-GDP/C3bot ^{G99D}	-25 ± 5	0.33 ± 0.15	3 ± 1
RalA-GDP/C3bot ^{E109A}	—	< 0.1	> 10

^aThe measurements were performed at 37°C in 20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 2 mM MgCl₂ and 2 mM DTE. Data were fit with a single-site binding model as described in Materials and methods, yielding the stoichiometric ratio of the complex N , the association enthalpy ΔH° and the affinity constant K_A . From the experiments with RalA-GppNHp and with the C3bot^{E109A} mutant, only estimates for the affinities could be obtained as indicated. The number of binding sites N was 1.4 ± 0.2 for RalA-GDP-C3bot and 0.8 ± 0.2 for RalA-GDP-C3bot^{G99D}.

complex formation. These data are presumably an artefact due to incomplete GTP γ S loading of RalA.

Due to its stability, we decided to study the interaction of C3bot and RalA in more detail by X-ray crystallography. The complex between C3bot and GDP-bound RalA could be purified using size-exclusion chromatography as the final purification step, and was stable for at least 1 week at 4°C against precipitation or dissociation. The apparent molecular mass of the eluted protein corresponded to a quantitative 1:1 complex (not shown).

Structure determination

The N- and C-termini of RalA were found disordered in crystal structures of RalA–GDP (Dr A Wittinghofer, personal communication) as well as in RalA–GppNHp complexed to Sec5 (Fukai *et al*, 2003). To improve the crystallization probability, we truncated the N-terminal 8 and C-terminal 23 amino acids of the 206-residue protein. This truncation did not affect C3bot binding and inhibition (data not shown). For clarity, the RalA(9–183) fragment will be referred to as RalA.

Crystals grew under several nonredundant conditions (not shown) with the best crystals obtained in space group P2₁ with one complex in the asymmetric unit. Models for the GppNHp-bound form of RalA and the free form of C3bot were used to identify a molecular replacement solution for the RalA–GDP–C3bot complex. Electron density maps, calculated using phases from the molecular replacement solution, showed strong electron density for the two RalA switch regions, which were not included in the search model. The model of the RalA–GDP–C3bot complex was refined to a resolution of 1.8 Å with an R_{free} value of 0.217, and consists of residues Ral¹³–Ral¹⁸² of RalA and C3⁴⁵–C3²⁵¹ of C3bot (Table II). A representative portion of the final electron density is shown in Supplementary Figure 2.

Structure of the RalA–C3bot interface

The RalA–GDP–C3bot complex has roughly a bilobal shape (Figure 2). An extensive interface forms a constriction between the two lobes. It is composed of a combination of polar and nonpolar interactions (Figure 3) and buries 1764 Å² of accessible surface area. Besides direct protein–protein interactions, additional hydrogen-bonding contacts are mediated by water molecules (Supplementary Table I). Overall, the structures of C3bot and GDP–Mg²⁺ bound RalA are remarkably similar to those of their isolated homologues. C3bot docks rigidly on RalA without major structural rearrangements. It can be superimposed onto the structure of free C3bot (PDB code 1G24) with a C $_{\alpha}$ atom root-mean-square deviation (r.m.s.d.) of 0.58 Å. The catalytic ARTT loop (C3bot²⁰⁷–C3bot²¹⁴) is not involved in Ral interaction. C3bot docks to RalA with a side, which is distal to the NAD-binding cleft (using the ARTT loop as a reference point) (Figures 2 and 5). Residues suggested to be involved in the catalytic activity of C3, such as the catalytic glutamine (C3bot^{Q214}) or phenylalanine (C3bot^{F209}), are ~25 Å apart from the Ral-binding site. The most prominent binding contribution is made from a stretch of 23 residues (C3bot⁸⁹–C3bot¹¹²), which build the tip of a well-ordered helix–loop–helix motif. The loop between helices α 3 and α 4 (C3bot⁹³–C3bot¹⁰¹) inserts into a groove of the RalA interface (Figure 3A). Additional contributions from C3bot are made

Table II Data collection and refinement

Data set ^a	RalA–GDP–C3bot	RalA–GDP–C3bot–NAD
<i>Space group</i>	P2 ₁	P2 ₁
<i>Cell dimensions</i>		
<i>a, b, c (Å)</i>	34.9, 112.8, 56.4	35.3, 113.9, 56.3
β (deg)	105.1	106.4
<i>Data collection</i>		
<i>Resolution (Å)</i>	20–1.80 (1.91–1.80)	20–1.73 (1.83–1.73)
<i>Observed reflections</i>	82091	114088
<i>Unique reflections</i>	35816	40078
<i>Completeness (%)</i>	91.8 (62.9)	90.2 (62.6)
R_{sym}^b (%)	5.9 (34.5)	4.9 (23.9)
$\langle I/\sigma(I) \rangle$	11.8 (2.3)	13.2 (3.6)
<i>Refinement</i>		
<i>Resolution (Å)</i>	20–1.81 (1.85–1.81)	20–1.73 (1.78–1.73)
$R_{\text{work}}^c/R_{\text{free}}^c$ (%)	18.0/0.21.7 (31.3/43.5)	18.6/22.5 (29.7/33.8)
<i>No. of non-H atoms</i>		
<i>Protein</i>	2995	2995
<i>Solvent</i>	330	210
<i>Mg²⁺–GDP</i>	29	29
<i>NAD</i>	—	44
<i>Average B-factor (Å²)</i>	17.4	21.0
<i>R.m.s.d.</i>		
<i>Bond length (Å)</i>	0.007	0.008
<i>Bond angles (Å)</i>	1.05	1.10
<i>Coordinate error index^d (Å)</i>	0.134	0.119

^aValues in parantheses are for the highest resolution shells.

^b $R_{\text{sym}} = \sum_{hkl} \sum_i |I_i - \langle I \rangle| / \sum_{hkl} \sum_i I_i$

^c $R_{\text{work}} = \sum_{hkl} |F_{\text{obs}} - k|F_{\text{calc}}| / \sum_{hkl} |F_{\text{obs}}|$, R_{free} was calculated using 5% of data excluded from refinement.

^dData precision indicator (DPI) as calculated by REFMAC (Murshudov *et al.*, 1997).

by a turn connecting β sheets β 6 and β 7 (C3bot²¹⁹–C3bot²²⁰) and by three C-terminal residues (C3bot²⁴⁷–C3bot²⁴⁹).

RalA contributes to the interface with its switch-II region (Ral⁷⁰–Ral⁷⁸), two adjoining residues from helix α 2 (Ral⁸³–Ral⁸⁴) and residues from one face of helix α 3. Additional contributions are made by Ral^{T46} from switch I and by three residues (Ral²¹–Ral²³) of the canonical P loop, which connect β -strand β 1 and helix α 1. The most remarkable feature of the RalA interface is a deep groove (Figure 3A) that is flanked by switch II and helix α 3 and harbors the α 3– α 4 loop of C3bot. Upon binding, the overall conformation of the G-domain is maintained and RalA of the RalA–C3bot complex can be superimposed onto the GDP-bound RalA (PDB code 1U8Z; Nicely *et al*, 2004) with a C $_{\alpha}$ atom r.m.s.d. of 0.48 Å for all atoms. Conformational changes upon complex formation are restricted to the switch-I (Ral⁴⁰–Ral⁴⁸) and switch-II regions (Ral⁷⁰–Ral⁷⁸), respectively, and are discussed below.

An alternative dimer interface?

Holbourn and co-workers have recently reported the structure of an RalA–GDP–C3bot complex in an orthorhombic crystal form (Supplementary Results and Supplementary Table II) and suggested the α 4–ARTT model for the dimer. This model places the main contact between C3bot and RalA on helices α 1 and α 2 and the ARTT loop of C3bot and helix α 4

and strand $\beta 6$ of the RalA molecule. Major interface residues are Asp204, Pro205 and Ser207 located in the ARTT loop, and residues Lys73 and Lys81 in helix $\alpha 2$ of C3bot and Glu140 and Lys143 of RalA. Thus, the $\alpha 4$ –ARTT model is fundamentally different from the RalA–C3bot complex presented in our analysis.

Although crystal packing is different, the RalA–C3bot interface presented in our analysis is conserved in both crystal forms, whereas the RalA–C3bot interface deduced from the $\alpha 4$ –ARTT model is restricted to the crystal form analysed by Holbourn *et al* (2005) (Supplementary Tables III and IV). Moreover, the RalA–C3bot interface presented in our analysis is significantly larger (1764 Å² of buried surface area) than any other crystal contact, including the $\alpha 4$ –ARTT interface, which only buries 642.3 Å² of accessible surface area. Based on crystallographic inference, we suggest that the $\alpha 4$ –ARTT interface represents a nonfunctional association induced by crystallization.

Mutational analysis of the dimer interface

To corroborate our model of the C3bot–RalA complex, an extensive mutational analysis was performed. Exchange of glycine-99 to aspartate of C3bot, which is located in the $\alpha 3$ – $\alpha 4$ loop (Figure 3B and C), significantly reduced precipitation by GST–RalA beads (Figure 4A). Accordingly, a drop in the affinity was also observed by ITC (K_D 3 μ M $\pm$ 1) (Table I). These findings corroborated our structural data: the acidic side chain of C3bot^{G99D} presumably results in electrostatic repulsion and steric clashes with RalA^{D106} and RalA^{F107}, respectively. In line with the precipitation experiment, ADP ribosylation of RhoA by C3bot^{G99D} was no longer inhibited by addition of RalA (Figure 4B), indicating that the interaction of C3bot and RalA was altered by the amino-acid exchange. Also, the glucosylation of RalA by *C. sordellii* lethal toxin, which is blocked in the RalA–C3bot complex (Wilde *et al*, 2002a), was observed in the presence of C3bot^{G99D} (Supplementary Figure 3A). Glucosylation of RalA by the clostridial toxin occurs at Thr46, which is located in the switch-I region of RalA and part of the C3bot–RalA interface in our model, but opposite to the contact site in the $\alpha 4$ –ARTT model of the Acharya group. Therefore, recovery of RalA glucosylation is also a strong argument for our model.

Exchange of glutamate-109 to alanine, located at helix $\alpha 4$ in C3bot, completely abolished the interaction with GST–RalA (Table I and Figure 4A). C3bot^{E109} contributes to binding by packing against the phenol group of RalA^{V75} and additionally interacts with the helix dipole of RalA $\alpha 2$. In contrast, exchange of C3bot^{E214} to alanine did not affect the interaction with RalA (Figure 4A). C3bot^{E214} is crucial for ADP-ribosyl-transferase activity in C3 exoenzymes, but is located in the ARTT loop, which is not part of the interface of our model.

Next we changed RalA^{A103}, which interacts with C3bot, to aspartate (Ras contains aspartate at the equivalent position) (Figure 3D). RalA^{A103} belongs to the 14 ‘tree-determinant’ residues, which are specific for the Ral subtype group (Bauer *et al*, 1999). This exchange diminished complex formation of RalA with C3bot, because first the precipitation of RalA^{A103D} by GST–C3bot (Supplementary Figure 4B) was significantly reduced and second the ADP ribosylation of RhoA by C3bot was not affected in the presence of this mutant (Supplementary Figure 5B). Finally, glucosylation of this mutation (RalA^{A103D}) was not affected by C3bot, indicating that the mutation blocked the interaction of RalA with C3bot (Figure 4C), presumably due to steric clashes with C3bot^{N93}. We changed alanine-102 of RalA to glutamate (Ras contains glutamate at the equivalent position). RalA^{A102} is involved in van-der-Waals interactions with C3bot^{Q92}; however, its side chain is directed away from the protein interface and exchange of alanine to glutamate has probably no consequence for the interaction of the proteins. Accordingly, RalA^{A102E} was not glucosylated in the presence of C3bot, indicating complex formation (Figure 4C). Taken together, the mutations studied support the model of the RalA–C3bot interface deduced from the crystal structure.

Next we changed residues, which are crucial in the interface of the $\alpha 4$ –ARTT model proposed by Holbourn and co-workers. Exchange of two critical lysines (C3bot^{K73L} and C3bot^{K81E}) of C3bot did not affect precipitation by GST–RalA (Supplementary Figure 4A) and inhibition of C3-induced ADP ribosylation of RhoA (Supplementary Figure 5A). The same was true for the exchange of Pro205, which is located in the ARTT loop of C3bot to alanine (Supplementary Figure 4A and 5A). Finally, glucosylation of RalA was not affected by all

Figure 2 Structure of the RalA–GDP–C3bot complex. Ribbon diagram of RalA (grey) and C3bot (cyan) as a stereo view. The RalA switch-I and -II regions are coloured blue and red, respectively. The P loop is shown in green. The bound GDP molecule is shown as a stick model coloured by atom type (C, yellow; O, red; N, blue; P, magenta) and the Mg²⁺ ion as a black sphere. Helices $\alpha 2$ and $\alpha 3$ of RalA are labelled as a2 and a3, respectively. The catalytic ARTT loop of C3bot is shown in yellow. The secondary structure elements $\alpha 3$, $\alpha 4$, $\beta 6$ and $\beta 7$ of C3bot are labelled a'3, a'4, b'6 and b'7, respectively.

three C3bot mutants, indicating complex formation with RalA (Supplementary Figure 3A). Similarly, exchange of two main interface residues of RalA (RalA^{E140A} and RalA^{K143E}) did not affect precipitation by GST-C3bot (Supplementary Figure 4B), inhibition of ADP ribosylation of RhoA (Supplementary Figure 5B) and inhibition of glucosylation of RalA by the lethal toxin (Supplementary Figure 3B). Taken

together, all mutations designed to disrupt the interface of the $\alpha 4$ -ARTT model had no effect on the dimer in solution.

Specificity

It was shown that RalA inhibits the RhoA ADP-ribosylating activity of the toxins C3lim and C3cer to a weaker extent (63% for C3lim, 24% for C3cer), whereas a fourth toxin,

C3stau2, could not be inhibited at all (Wilde *et al*, 2002a). The C3-docking region is not fully conserved in the three toxins (Figure 3C): seven (out of 17) interface residues are conserved in C3lim, five in C3cer and four in C3stau2, respectively. Differences in amino-acid composition of the interface could explain why the other toxins bind weaker or not at all to Ral. Exchange of C3bot^{G99} to a larger residue, for example aspartate, is not tolerated (Table I and Figure 4A and B). Accordingly, the equivalent residues C3cer^{S67} and

C3stau^{K56} could lead to collision with RalA. In contrast, the conservation of the critical C3bot^{G99} in C3lim could be one determinant for its retained binding to RalA. A superposition of the C3bot interface residues with the equivalent region in C3stau2 (C3stau⁴⁵–C3stau⁶⁶ and C3stau¹⁸⁵–C3stau¹⁸⁶; PDB code 1OJQ) indeed reveals several steric clashes. Unfavourable interactions are, for example, observed between C3stau^{D53} and Ral^{G71}, C3stau^{K56} and Ral^{F107} and C3stau^{Q62} and Ral^{R79}.

The conservation of interfacial residues is in good agreement with the available binding data of other GTPases (Figure 3D). Interacting residues are invariant in RalB (Figure 3D), which is in line with the interaction of RalB with C3bot (Figure 1A). On the other hand, Ras, which does not bind C3bot (Figure 1A), has several amino-acid exchanges in helix α_3 , which sterically interfere with the observed interface, as for example Ras^{D92} (equivalent to Ral^{A103}, Figure 4C). RhoA also lacks significant sequence conservation in the corresponding region and, therefore, it is unlikely that Rho proteins are recognized by C3 in a Ral-like manner.

Figure 4 Mutational analysis of the interface. (A) Precipitation of mutant C3bot proteins by GST–RalA. GST–RalA immobilized to glutathione–Sepharose beads was incubated with equimolar concentrations of the indicated C3bot proteins. Beads were washed three times and subjected to SDS–PAGE. The Coomassie Blue-stained gel is shown. (B) ADP ribosylation of RhoA by C3bot^{G99D}. Rat brain lysate was incubated with C3bot or C3bot^{G99D} in the absence or presence of RalA. Thereafter, samples were analysed by SDS–PAGE. The autoradiography is shown. (C) Glucosylation of mutant RalA proteins. RalA proteins were incubated with *C. sordellii* lethal toxin and UDP-[¹⁴C]glucose in the absence or presence of C3bot. Samples were subjected to SDS–PAGE followed by autoradiography.

NAD binding and implications for the ADP-ribosylating activity of C3bot

In a previous study, we had shown that the RalA–C3bot complex is still capable of NAD hydrolysis (Wilde *et al*, 2002a). Using a GST-pulldown assay, we found that addition of NAD does not compete with RalA binding (not shown), which prompted us to prepare a quaternary RalA–GDP–C3bot–NAD complex. In this complex, the NAD molecule is well ordered (Supplementary Figure 6) and we did not find any signs of NAD hydrolysis as previously reported for two of four molecules in the asymmetric unit of the binary C3bot–NAD complex (Ménétrey *et al*, 2002). Upon NAD binding, only localized conformational changes in the catalytic ARTT and PN loops are observed (not shown). Moreover, no significant structural changes are observed with respect to the binary C3bot–NAD complex (PDB code 1GZF, molecule A). The r.m.s.d. over all C α atoms of C3bot is 0.55 Å and the NAD positions are identical. Taken together, there is no clear evidence that RalA binding renders the structural properties of C3bot to block its Rho–ADP-ribosylating activity.

To answer if RalA could compete with Rho for a common binding epitope, we mapped the areas that are involved in substrate binding (ARTT loop, PN loop and region B) of the C3-related ADP-ribosyltransferases ExoS and ExoT onto the

Figure 3 The RalA–C3bot interface. (A) Interaction of C3bot with RalA–GDP as a stereo view. The interface region of C3bot is displayed as a cyan worm and interacting residues are shown in stick representation. For residues C3bot⁹²–C3bot¹⁰⁰, all backbone atoms are shown and the worm is omitted for clarity. Ral–GDP is shown as a surface representation with C3bot-interacting regions colour coded: switch I in blue, switch II in red, P loop in green and helix α_3 in yellow. The Mg²⁺ ion is presented as a yellow sphere, GDP is shown in white. Atoms other than carbon are coloured as in Figure 2. Hydrogen bonds are shown as dotted blue lines. (B) Schematic representation of polar (left) and hydrophobic (right) interactions between RalA and C3bot at the protein–protein interface. Hydrogen bonds are shown as dotted blue lines between the interacting atoms. Most weak hydrogen bonds are omitted for clarity. Residues that are not part of the interface but couple C3bot binding to GDP binding are shown in green. The Mg²⁺ ion and coordinating water molecules are shown as magenta and blue balls, respectively. Other water molecules are omitted for clarity. Hydrophobic interactions (cutoff level 4.0 Å) are shown as yellow dashed lines. The π -cation interaction of Arg91 (green) to NAD-adenine is shown as a green dashed line. Residues that can be mutated to eliminate or reduce binding are coloured magenta or orange, respectively. (C) Sequence alignment of the RalA-interacting regions of C3. The secondary structure is shown at the top. The numbering corresponds to C3bot. Invariant residues are highlighted in red, conserved residues are boxed and in red. The amino-acid sequences of C3bot, C3lim, C3cer and C3stau2 are shown. Residues contributing to the complex interface are marked with black dots. Note that the C3bot C-terminal region is not present in the other C3 exoenzymes and is therefore not shown. (D) Sequence alignment of the C3bot interacting regions of RalA. The numbering corresponds to RalA. Invariant and conserved residues are marked as in (C), residues in the interface and secondary structure are shown correspondingly. The amino-acid sequences of RalA, RalB, Ras- and RhoA are shown.

Figure 5 The binding site of RalA and the putative Rho-binding site of C3 proteins. C3bot is shown as a grey surface representation with three regions, which are important for substrate recognition in the related transferases ExoS and ExoT (see text) shown in cyan. RalA is shown as a ribbon representation in magenta. GDP and NAD are shown as stick models coloured by atom type as in Figure 2. The boxed region is shown as a close-up with RalA as a space-filling model. Labelled residues of C3bot are marked by a dash.

C3bot context (Figure 5). Although RalA does not directly overlap with any of the three regions, it is directly neighbouring region B (C3bot⁷⁹–C3bot⁸⁸). The C_α atoms of C3bot^{G88} and Ral^{E99}, for example, are only 10 Å apart and the adjacent C3bot^{K89} forms a salt bridge to Ral^{D106}. The full Rho-binding site might extend further into the distal region of the active site cleft, such that RalA would sterically interfere with Rho binding, but more experiments will be needed to confirm this hypothesis.

Consequences for nucleotide binding

In the following section, we will discuss the structural implications of C3bot binding to RalA for guanine nucleotide binding. A superposition of the RalA–C3bot complex onto the GDP-bound RalA is shown in Figure 6A. In the structures of RalA–GDP as well as RalA–GppNHp (PDB code 1U8Y), the solvent-exposed switch II is either disordered (RalA⁷²–RalA⁷⁴) or has high-temperature factors (Figure 6B), indicating a flexible backbone. In contrast, the switch-II region becomes well ordered in the complex due to numerous polar and hydrophobic interactions with C3bot (Figures 3B and 6). In the complex, C3bot neither contacts the nucleotide nor does it directly interact with residues that are involved in nucleotide binding (Figure 3B). However, C3bot binds to and stabilizes the P loop and switch-II region. These residues in turn can then stabilize adjacent residues, which contact the GDP β-phosphate (RalA^{G24}, RalA^{K27}) or form hydrogen bonds (RalA^{T69}, RalA^{A70}) to water molecules, coordinating the Mg²⁺ ion, which is involved in high-affinity binding of the β-phosphate of the guanine nucleotide (Figure 6C). It is suggested that this indirect interaction of C3bot with the P loop and switch II of RalA stabilizes the nucleotide binding. Since the P loop is also found well ordered in the structure of free RalA–GDP (Figure 6B), we assume that the stabilization of switch II makes the more important contribution. Interestingly, a similar mode of nucleotide stabilization has been observed previously for two GDIs of Rab family proteins (Rak *et al*, 2003, 2004), suggesting that C3bot could also act as a GDI on Ral (see the biochemical analysis below).

To understand how C3bot binding discriminates between different nucleotide-binding states of RalA (Table I), we compared the structure of RalA–GppNHp in complex with Sec5 (Fukai *et al*, 2003) with the RalA–GDP–C3bot complex

(Figure 6D). The observed conformational changes in the switch-I and -II regions of RalA are a typical response of Ras family proteins to changes in the nucleotide-binding status (Cherfils *et al*, 1997). C3bot and Sec5 bind to opposite sides of the RalA–effector recognition region without steric overlap. Sec5 recognition of RalA occurs mostly through an intermolecular β-sheet, which involves RalA⁴⁸–RalA⁵², but binding would be sterically precluded by the GDP-bound conformation of switch I (Fukai *et al*, 2003). On the other hand, the GppNHp-bound RalA conformation would not be compatible with the observed RalA–GDP–C3bot complex due to severe steric clashes of switch-II residues with C3bot (Figure 6D), for example between RalA^{Q72} and C3bot^{V96}, RalA^{E73} and C3bot^{I97}. Complex formation of GTP-bound RalA with C3bot would additionally require structural rearrangements in switch I to bring RalA^{T46} into a position for coordinating Mg²⁺, as observed in all structures of G-domains in the trisphosphate conformation. A direct consequence would be that C3bot binding to the RalA–GTP state is impaired or at least largely reduced. In conclusion, C3bot competes indirectly with endogenous RalA effectors (e.g., Sec5) by stabilizing the GDP-bound ‘off-state’ of RalA.

Biochemical studies on nucleotide binding

Biochemical studies corroborated that nucleotide binding of RalA is altered in the RalA–C3bot complex. We studied the nucleotide exchange by using fluorescently labelled mant-nucleotides. In line with the high K_D of the RalA–GppNHp–C3bot complex (Table I), the release of mant-GppNHp from RalA was not significantly affected by the presence of a 12-fold molar excess of C3bot (data not shown). The release of mant-GDP from RalA was studied at increasing concentrations of C3bot in the presence of an excess of GDP (Figure 7). Addition of C3bot resulted in inhibition of mant-GDP release in a concentration-dependent manner. Accordingly, C3bot, but not C3stau, inhibited the binding of mant-GDP to RalA (data not shown). This inhibition of GDP exchange is reminiscent to the interaction of Ras with the Ras-binding domain of Ras effectors (Herrmann *et al*, 1995). As it is also found with RhoGDI and Rho GTPases, the term GDI effect was introduced for the trapping of GDP in such a complex (Herrmann *et al*, 1995). Notably, the GDI effect of C3bot could not easily be rationalized with the α4-ARTT model,

Figure 6 Switch regions and structural basis of the GDI effect. (A) Superposition of GDP-bound RalA with the RalA–GDP–C3bot complex. GDP-bound RalA is shown in yellow, except for the switch-I (magenta) and switch-II (orange) regions. RalA in complex with C3bot is shown in grey, except for the switch-I (blue) and switch-II (red) regions. Residues 89–112 of the C3bot-interface region are displayed as a cyan worm. GDP and Mg^{2+} are shown in white (RalA–GDP–C3bot) and yellow (RalA–GDP), with atoms other than carbon and Mg^{2+} coloured as in Figure 2. Labelled residues of C3bot are marked by a dash, throughout the figure. (B) Comparison of the temperature factors (B-factors) for GDP-bound RalA (orange line) and RalA–GDP–C3bot (black line). The switch-I and -II regions are highlighted in grey. (C) Indirect stabilization of GDP by C3bot binding. C3bot is displayed as a cyan worm. RalA is shown in grey, except for the switch-I (blue) and switch-II (red) regions. Interacting residues of RalA–GDP and C3bot are shown in stick representation with atoms other than carbon coloured as in Figure 2. Water molecules are shown as red spheres. The Mg^{2+} ion is presented as a black sphere, hydrogen bonds are shown as yellow dotted lines. (D) Superposition of the Sec5 bound RalA–GppNHp with the RalA–GDP–C3bot complex. RalA–GppNHp is shown as a grey worm except for the switch-I (magenta) and switch-II (orange) regions. RalA–C3bot is shown colour coded as in (A). Mg^{2+} –GDP is shown in white and Mg^{2+} –GppNHp is shown in orange. Sec5 is shown as a brown worm. The Sec5-binding region is marked by a brown circle. Prominent residues of RalA and C3bot that would be involved in steric clashes are marked by a sphere at their C_{α} position.

Figure 7 Influence of C3bot on the nucleotide exchange of RalA. RalA was preloaded with mant-GDP. The release of mant-GDP from RalA (100 nM) was determined in the presence of the indicated C3bot concentrations by monitoring the decrease in fluorescence.

where the Ral interface lies on a surface that is opposite to the Ral effector-binding region.

Conclusions

Here we solved the structure of RalA in a complex with the Rho–ADP-ribosylating C3 exoenzyme from *C. botulinum*. During submission of the manuscript, a similar crystal structure was reported, which differed fundamentally in the interface of the protein complex (Holbourn *et al*, 2005). Crystallographic and biochemical arguments strongly disfavoured the $\alpha 4$ –ARTT model proposed by Holbourn and co-workers. Moreover, site-directed mutational analysis of residues proposed to be involved in the one or the other interfaces of both complexes confirms our model of C3bot–RalA interaction. This study shows a GTPase–protein complex in which the GDP- and not the GTP-bound form of a small GTPase (RalA) is the species, which selectively interacts with

a partner. This mode of interaction is usually observed with GDIs. Interestingly, there is no GDI known for Ral proteins. The interaction of RalA with C3bot occurs mainly in a pocket between the switch-II region and helix $\alpha 3$ of the GTPase. Interestingly, this region was recently predicted to be involved in RalA effector binding on the basis of the crystal structures of GDP- and GppNHP-loaded RalA (Nicely *et al*, 2004). By contrast, the RalA effectors Sec5 and C3bot bind on different sides of the GTPase effector recognition region. Notably, the affinity of Sec5 for RalA $K_D \sim 118$ nM is lower than the affinity of C3bot for RalA (K_D 35 ± 15 nM (fluorescence titration) and 60 ± 20 nM (ITC)). The high-affinity interaction of C3bot with RalA has major consequences for the guanine nucleotide binding of RalA: the RalA–C3bot complex causes trapping of GDP (GDI effect). It has to be studied which functional consequence this GDI-like effect has for Ral signalling. The interaction site of C3bot with RalA is surprising, because no specific function could be ascribed to this region of the transferase up to now. Although distal from the catalytic centre, the $\alpha 3$ -loop- $\alpha 4$ region appears to be involved in the regulation of the transferase activity via RalA binding. The observed complex offers an explanation how Ral is able to block the ADP ribosylation of RhoA by C3bot without serving as a substrate for the enzyme. Studies from recent years have shown that many bacterial protein toxins and effectors act in a multifunctional manner. For example, *P. aeruginosa* exoenzyme S possesses an ADP-ribosyltransferase activity and a GTPase-activating protein (GAP) function for Rho-GTPases (Barbieri and Frank, 2002). However, these different functions of the bacterial effectors depend on their bimodular structure. Our findings suggest that C3bot, which consists of one domain only, accomplishes different functions (e.g., ADP-ribosyltransferase activity and inhibition of RalA) by interacting with different targets (e.g., Rho and Ral). With respect to the high affinity of the RalA–C3bot complex and the functional consequences of the RalA–C3bot interaction, it is highly likely that C3bot affects target cells not only by ADP ribosylation of Rho but also by interfering with Ral signalling (e.g., via PLD regulation) (Wilde *et al*, 2002a).

Materials and methods

Cloning, expression and purification

The RalA gene was cut from pTacRalA kindly provided by P Chardin (Valbonne, France) (Frech *et al*, 1990) and cloned into the pGEX-2T vector. For crystallization, a truncated version RalA(9–183) was amplified by PCR and cloned into pGEX-2T with a glycine linker using BamHI and EcoRI restriction sites. The RalB expression plasmid was kindly provided by Dr A Wittinghofer (MPI, Dortmund). The recombinant GTPases RalA, RalB, Ras and recombinant exoenzymes C3bot, C3lim, C3cer and C3stau were expressed as GST-fusion proteins and purified as described (Selzer *et al*, 1996).

C. sordellii lethal toxin was prepared as reported (Just *et al*, 1997). RalA(9–183) and recombinant C3bot were expressed at 37°C for 10–15 h in *Escherichia coli* (BL21, Stratagene, La Jolla, CA) as GST-fusion proteins. The purified GST-fusion proteins were cleaved with thrombin and subsequently purified by using a Superdex 75 size exclusion column. To form the RalA(9–183)–C3bot complex, equimolar amounts of the purified proteins were incubated at 4°C overnight in a buffer, containing 20 mM Tris–HCl, 50 mM NaCl, 2 mM MgCl₂ and 5 mM β -mercaptoethanol. The RalA(9–183)–C3bot complex was isolated by size-exclusion chromatography (Superdex 75).

Site-directed mutagenesis

Mutant proteins of RalA or C3bot were constructed using the Quick-Change kit, according to the manufacturer's instructions (Stratagene). Correct nucleotide sequences were confirmed by sequencing.

Mant-nucleotide exchange

For analysis of the release of mant-GDP, RalA was loaded with mant-nucleotides as described (Ahmadian *et al*, 2002). C3bot was added at the indicated concentrations to RalA(mant-GDP) (100 nM) in a buffer, containing 10 mM Tris–HCl (pH 7.5), 150 mM NaCl and 2.5 mM MgCl₂. The nucleotide exchange was started by addition of GDP (200 μ M). A time-dependent decrease in fluorescence was determined, using excitation and emission wavelengths of 357 and 444 nm, respectively, with a Perkin-Elmer LSB 50 fluorimeter.

ITC and fluorescence titration

The affinity of the binding of RalA to C3 from *C. botulinum* was measured by ITC and by fluorescence titration according to Herrmann *et al* (1995), using the tryptophan fluorescence of RalA excited at 290 nm and monitored at 348 nm. The tryptophan-deficient mutant C3bot^{W18L} was used to minimize the background fluorescence (Wilde *et al*, 2002a). To account for the small background which increases with the titration, we performed a control titration experiment with C3bot^{W18L} alone. This linear increase in fluorescence was subtracted from the original data. A quadratic binding curve (equation (1)) was fit to the corrected data.

$$F = F_{\max} + (F_{\min} - F_{\max}) \times \frac{([A]_0 + [B]_0 + K_D) - \sqrt{([A]_0 + [B]_0 + K_D)^2 - 4[A]_0[B]_0}}{2[A]_0} \quad (1)$$

where F is the relative fluorescence; $F_{\max}$ and $F_{\min}$ maximal and minimal fluorescence, respectively; $[A]_0$, total concentration of RalA–GDP; $[B]_0$, total concentration of titrant C3bot^{W18L}; K_D , dissociation equilibrium constant.

ITC experiments were performed using a VP-ITC calorimeter (Microcal, LCC, Northampton, MA) thermostatted to 37°C. In brief, 1.4 ml of a solution containing 4 μ M of C3bot (or the mutants C3bot^{G99D} and C3bot^{E109A}, respectively) was loaded into the sample cell and titrated with 73 μ M of RalA–GDP or RalA–GppNHP by stepwise injections from the syringe. The sample was stirred at 420 r.p.m. An interval of 4 min between each injection was used to allow baseline recording after each peak. The changes in the heating power were integrated to yield the enthalpy change upon complex formation. After subtraction of the background value, these data were evaluated with a single-site binding model using the Origin software, provided by Microcal, LCC. All titration measurements were carried out in a buffer containing 20 mM Tris–HCl, pH 7.5, 50 mM NaCl, 2 mM MgCl₂ and 2 mM DTE. The nucleotide loading of RalA–GDP and RalA–GppNHP was confirmed by HPLC analysis.

Enzyme assays

For ADP ribosylation of Rho protein in rat brain lysate, 60 μ g of total protein was incubated with wild-type or mutant C3bot proteins (50 nM) in a solution containing 0.3 μ M [adenylate-³²P]NAD, 2 mM MgCl₂ and 50 mM HEPES (pH 7.5) for 30 min at 37°C. Indicated recombinant GTPases were added in a final concentration of 2 μ M (Wilde *et al*, 2002a). Proteins were subjected to SDS–PAGE and further analysed by phosphorimaging.

For glucosylation of RalA by *C. sordellii* lethal toxin, wild-type or mutant RalA proteins (2 μ M) were incubated with lethal toxin (100 ng) in 25 μ l of a solution containing 10 μ M UDP-[¹⁴C]glucose, 100 mM KCl, 200 μ M MnCl₂, 0.1 mg/ml BSA and 10 mM HEPES (pH 7.4) in the presence or absence of equal concentrations of C3bot for 20 min at 37°C (Just *et al*, 1997). Phosphorimager data of the SDS–PAGE are shown.

Western blot analysis

For Western blotting, samples were subjected to SDS–PAGE and transferred onto polyvinyl difluoride membrane. RhoA was detected with a polyclonal antibody (anti-RhoA, Santa Cruz Biotechnology, Santa Cruz, CA). Binding of the second horseradish peroxidase-coupled antibody was detected with enhanced chemiluminescent detection reagent (100 mM Tris–HCl, pH 8.0, 1 mM luminal, 0.2 mM *p*-coumaric acid and 3 mM H₂O₂).

Precipitation assays

GST–RalA or GST–C3bot immobilized to Sepharose beads (1 μ M) were incubated with the indicated proteins (1 μ M) in a buffer containing 2 mM MgCl₂, 50 mM HEPES (pH 7.5), 1 mM dithiothreitol, 1 mM EDTA, 1 mM PMSF and 0.5% NP40 (total volume 300 μ l) for 45 min at 4°C. Subsequently, beads were washed three times with the same buffer and subjected to SDS–PAGE.

Crystallization, data collection and processing

Initially, 480 conditions were screened in 96-well crystallization plates, using low-volume drops dispensed by a Cartesian robot (Genomics Solutions, UK). Small crystals could be obtained from many conditions. Optimal crystals were obtained at 20°C in 1 + 1 μ l sitting drops from a complex concentrated up to 10 mg/ml over a reservoir of 0.2 M ammonium sulfate, 0.1 M bis–tris, pH 5.5 and 25% w/v polyethylene glycol 3350. Crystals were flash frozen in a 100 K nitrogen stream, after briefly soaking in reservoir solution supplemented with 20% glycerol. The NAD complex was obtained through a soaking experiment to limit hydrolysis during crystal growth. We used a crystal grown as described above, but from a reservoir solution consisting of 0.2 M tri–ammonium citrate, pH 7.0, and 25% w/v polyethylene glycol 3350. A 1 μ l aliquot of mother liquor supplemented with 5 mM NAD was added to the drop. The crystal was soaked for 3 h and then briefly transferred to cryoprotectant (reservoir solution supplemented with 20% glycerol) before freezing in a 100 K nitrogen stream. Data sets were collected at 100 K on a Rigaku RU–300 generator equipped with a MAR345dtb image plate. The diffraction data were processed with XDS (Kabsch, 1993). Statistics of the data processing are shown in Table II.

Structure determination and refinement

The structure of RalA–GDP–C3bot was solved with the molecular replacement method. As search models we used the coordinates of unliganded C3bot and GppNHp-bound RalA with the switch-I and -II regions omitted from the calculation (PDB accession codes 1G24 and 1UAD, respectively). Calculations were performed with

program Molrep from the CCP4 suite (Collaborative Computational Project N.4, 1994) and resulted in unambiguous signals for both proteins. The model was improved by iterative rounds of manual rebuilding and refinement using the programs O (Jones *et al*, 1991) and Refmac (Murshudov *et al*, 1997). Water molecules were added using arp-warp (Perrakis *et al*, 1999) at the last stages of refinement. The final model has been completed to residues 13–182 of RalA, residues 45–251 of C3bot, 1 Mg²⁺ ion and 330 water molecules. As defined by PROCHECK, there are 91.8% of residues in the most favoured regions of the Ramachandran plot and 8.2% in additionally allowed regions. The structure of the isomorphous NAD complex was obtained by difference Fourier methods and refined as outlined above. The final statistics for the models are listed in Table II. Buried surface areas were calculated by program AREAINMOL from the CCP4 suite. Figures were prepared using MOLSCRIPT (Kraulis, 1991) InsightII (accelerys INC) and PyMOL (DeLano Scientific LLC).

Coordinates

The coordinates and structure factors have been deposited in the Protein Data Bank (PDB accession codes 2A78 (RalA–GDP–C3bot) and 2A9K (RalA–GDP–C3bot–NAD)).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

Acknowledgements

The study was financially supported by the Deutsche Forschungsgemeinschaft and the Fonds of the Chemical Industry. We thank Dr Alfred Wittinghofer (Max-Planck-Institut für Molekulare Physiologie, Dortmund, Germany) for providing the unpublished coordinates of the GDP-bound RalA and Dr Manuel Dietz (Institut für physikalische Chemie, Universität Freiburg, Germany) for supporting the collection and refinement of fluorescence data.

References

- Ahmadian MR, Wittinghofer A, Herrmann C (2002) Fluorescence methods in the study of small GTP-binding proteins. *Methods Mol Biol* **189**: 45–63
- Aktories K, Braun U, Rösener S, Just I, Hall A (1989) The rho gene product expressed in *E. coli* is a substrate of botulinum ADP-ribosyltransferase C3. *Biochem Biophys Res Commun* **158**: 209–213
- Aktories K, Weller U, Chhatwal GS (1987) *Clostridium botulinum* type C produces a novel ADP-ribosyltransferase distinct from botulinum C2 toxin. *FEBS Lett* **212**: 109–113
- Aktories K, Wilde C, Vogelsang M (2004) Rho-modifying C3-like ADP-ribosyltransferases. *Rev Physiol Biochem Pharmacol* **152**: 1–22
- Barbieri JT, Frank DW (2002) *Pseudomonas aeruginosa* exoenzyme S, a bifunctional cytotoxin secreted by a type-III pathway. In *Bacterial Protein Toxins*, Aktories K, Just I (eds), pp 235–251. Berlin: Springer
- Bauer B, Mirey G, Vetter IR, Garcia-Ranea JA, Valencia A, Wittinghofer A, Camonis JH, Cool RH (1999) Effector recognition by the small GTP-binding proteins Ras and Ral. *J Biol Chem* **274**: 17763–17770
- Chardin P, Boquet P, Madaule P, Popoff MR, Rubin EJ, Gill DM (1989) The mammalian G protein rho C is ADP-ribosylated by *Clostridium botulinum* exoenzyme C3 and affects actin microfilament in Vero cells. *EMBO J* **8**: 1087–1092
- Chardin P, Tavittian A (1986) The ral gene: a new ras related gene isolated by the use of a synthetic probe. *EMBO J* **5**: 2203–2208
- Cherfils J, Ménétrey J, Le Bras G, Janoueix-Lerosey I, De Gunzburg J, Garel J-R, Auzat I (1997) Crystal structures of the small G protein Rap2A in complex with its substrate GTP, with GDP and with GTPgammaS. *EMBO J* **16**: 5582–5591
- Collaborative Computational Project N.4 (1994) The CCP4 suite: programs for protein crystallography. *Acta Crystallogr D* **50**: 760–763
- Evans HR, Sutton JM, Holloway DE, Ayriss J, Shone CC, Acharya KR (2003) The crystal structure of C3stau2 from *Staphylococcus aureus* and its complex with NAD. *J Biol Chem* **278**: 45924–45930
- Feig LA (2003) Ral-GTPases: approaching their 15 minutes of fame. *Trends Cell Biol* **13**: 419–425
- Feig LA, Urano T, Cantor S (1996) Evidence for a Ras/Ral signaling cascade. *Trends Biochem Sci* **21**: 438–441
- Frech M, Schlichting I, Wittinghofer A, Chardin P (1990) Guanine nucleotide binding properties of the mammalian RalA protein produced in *Escherichia coli*. *J Biol Chem* **265**: 6353–6359
- Fukai S, Matern HT, Jagath JR, Scheller RH, Brunger AT (2003) Structural basis of the interaction between RalA and Sec5, a subunit of the sec6/8 complex. *EMBO J* **22**: 3267–3278
- Genth H, Gerhard R, Maeda A, Amano M, Kaibuchi K, Aktories K, Just I (2003) Entrapment of Rho ADP-ribosylated by *Clostridium botulinum* C3 exoenzyme in the Rho-guanine nucleotide dissociation inhibitor-1 complex. *J Biol Chem* **278**: 28523–28527
- Han S, Arvai AS, Clancy SB, Tainer JA (2001) Crystal structure and novel recognition motif of Rho ADP-ribosylating C3 exoenzyme from *Clostridium botulinum*: structural insights for recognition specificity and catalysis. *J Mol Biol* **305**: 95–107
- Hernandez-Munoz I, Malumbres M, Leonardi P, Pellicer A (2000) The Rgr oncogene (homologous to RalGDS) induces transformation and gene expression by activating Ras, Ral and Rho mediated pathways. *Oncogene* **19**: 2745–2757
- Herrmann C, Martin GA, Wittinghofer A (1995) Quantitative analysis of the complex between p21^{ras} and the Ras-binding domain of the human Raf-1 protein kinase. *J Biol Chem* **270**: 2901–2905
- Holbourn KP, Sutton JM, Evans HR, Shone CC, Acharya KR (2005) Molecular recognition of an ADP-ribosylating *Clostridium botulinum* C3 exoenzyme by RalA GTPase. *Proc Natl Acad Sci USA* **102**: 5357–5362
- Jiang H, Luo J-Q, Urano T, Frankel P, Lu Z, Foster DA, Feig LA (1995) Involvement of Ral GTPase in v-Src-induced phospholipase D activation. *Nature* **378**: 409–412

- Jones TA, Zou JY, Cowan SW, Kjeldgaard M (1991) Improved methods for building protein models in electron density maps and the location of errors in these models. *Acta Crystallogr A* **47**: 110–119
- Jullien-Flores V, Dorseuil O, Romero F, Letourneur F, Saragosti S, Berger R, Tavitian A, Gacon G, Camonis JH (1995) Bridging Ral GTPase to Rho pathways—RLIP76, a Ral effector with CDC42/Rac GTPase-activating protein activity. *J Biol Chem* **270**: 22473–22477
- Jullien-Flores V, Mahe Y, Mirey G, Leprince C, Meunier-Bisceuil B, Sorkin A, Camonis JH (2000) RLIP76, an effector of the GTPase Ral, interacts with the AP2 complex: involvement of the Ral pathway in receptor endocytosis. *J Cell Sci* **113**: 2837–2844
- Just I, Mohr C, Schallehn G, Menard L, Didsbury JR, Vandekerckhove J, van Damme J, Aktories K (1992) Purification and characterization of an ADP-ribosyltransferase produced by *Clostridium limosum*. *J Biol Chem* **267**: 10274–10280
- Just I, Selzer J, Hofmann F, Aktories K (1997) *Clostridium difficile* toxin B as a probe for Rho GTPases. In *Bacterial Toxins—Tools in Cell Biology and Pharmacology*, Aktories K (ed), pp 159–168. Weinheim: Chapman & Hall
- Kabsch W (1993) Automatic processing of rotation diffraction data from crystals of initially unknown symmetry and cell contents. *J Appl Crystallogr* **26**: 795–800
- Kraulis PJ (1991) Molscript—a program to produce both detailed and schematic plots of protein structures. *J Appl Crystallogr* **24**: 946–950
- Luo JQ, Liu X, Frankel P, Rotunda T, Ramos M, Flom J, Jiang H, Feig LA, Morris AJ, Kahn RA, Foster DA (1998) Functional association between Arf and RalA in active phospholipase D complex. *Proc Natl Acad Sci USA* **95**: 3632–3637
- Ménétrey J, Flatau G, Stura EA, Charbonnier J-B, Gas F, Teulon J-M, Le Du M-H, Boquet P, Ménez A (2002) NAD binding induces conformational changes in Rho ADP-ribosylating *Clostridium botulinum* C3 exoenzyme. *J Biol Chem* **277**: 30950–30957
- Moskalenko S, Henry DO, Rosse C, Mirey G, Camonis JH, White MA (2002) The exocyst is a Ral effector complex. *Nat Cell Biol* **4**: 66–72
- Murshudov GN, Vagin AA, Dodson EJ (1997) Refinement of macromolecular structures by the maximum-likelihood method. *Acta Crystallogr D* **53**: 240–255
- Nicely NI, Kosak J, de SV, Mattos C (2004) Crystal structures of Ral-GppNHp and Ral-GDP reveal two binding sites that are also present in Ras and Rap. *Structure (Cambridge)* **12**: 2025–2036
- Ohta Y, Suzuki N, Nakamura S, Hartwig JH, Stossel TP (1999) The small RalA targets filamin to induce filopodia. *Proc Natl Acad Sci USA* **96**: 2122–2128
- Park SH, Weinberg RA (1995) A putative effector of Ral has homology to Rho/Rac GTPase activating proteins. *Oncogene* **11**: 2349–2355
- Perrakis A, Morris R, Lamzin VS (1999) Automated protein model building combined with iterative structure refinement. *Nat Struct Biol* **6**: 458–463
- Rak A, Pylypenko O, Durek T, Watzke A, Kushnir S, Brunsvelde L, Waldmann H, Goody RS, Alexandrov K (2003) Structure of Rab GDP-dissociation inhibitor in complex with prenylated YPT1 GTPase. *Science* **302**: 646–650
- Rak A, Pylypenko O, Niculae A, Pyatkov K, Goody RS, Alexandrov K (2004) Structure of the Rab7:REP-1 complex: insights into the mechanism of Rab prenylation and choroideremia disease. *Cell* **117**: 749–760
- Rubin EJ, Gill DM, Boquet P, Popoff MR (1988) Functional modification of a 21-Kilodalton G protein when ADP-ribosylated by exoenzyme C3 of *Clostridium botulinum*. *Mol Cell Biol* **8**: 418–426
- Sehr P, Joseph G, Genth H, Just I, Pick E, Aktories K (1998) Glucosylation and ADP-ribosylation of Rho proteins—effects on nucleotide binding, GTPase activity, and effector-coupling. *Biochemistry* **37**: 5296–5304
- Sekine A, Fujiwara M, Narumiya S (1989) Asparagine residue in the rho gene product is the modification site for botulinum ADP-ribosyltransferase. *J Biol Chem* **264**: 8602–8605
- Selzer J, Hofmann F, Rex G, Wilm M, Mann M, Just I, Aktories K (1996) *Clostridium novyi* α -toxin-catalyzed incorporation of GlcNAc into Rho subfamily proteins. *J Biol Chem* **271**: 25173–25177
- Shen Y, Xu L, Foster DA (2001) Role for phospholipase D in receptor-mediated endocytosis. *Mol Cell Biol* **21**: 595–602
- Sugihara K, Asano S, Tanaka K, Iwamatsu A, Okawa K, Ohta Y (2002) The exocyst complex binds the small GTPase RalA to mediate filopodia formation. *Nat Cell Biol* **4**: 73–78
- Sun J, Maresso AW, Kim JJ, Barbieri JT (2004) How bacterial ADP-ribosylating toxins recognize substrates. *Nat Struct Mol Biol* **11**: 868–876
- Urano T, Emkey R, Feig LA (1996) Ral-GTPases mediate a distinct downstream signaling pathway from Ras that facilitates cellular transformation. *EMBO J* **15**: 810–816
- Wilde C, Barth H, Sehr P, Han L, Schmidt M, Just I, Aktories K (2002a) Interaction of the Rho-ADP-ribosylating C3 exoenzyme with RalA. *J Biol Chem* **277**: 14771–14776
- Wilde C, Chhatwal GS, Schmalzing G, Aktories K, Just I (2001) A novel C3-like ADP-ribosyltransferase from *Staphylococcus aureus* modifying RhoE and Rnd3. *J Biol Chem* **276**: 9537–9542
- Wilde C, Just I, Aktories K (2002b) Structure-function analysis of the Rho-ADP-ribosylating exoenzyme C3stau2 from *Staphylococcus aureus*. *Biochemistry* **41**: 1539–1544