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The guanine nucleotide exchange factor Rlf interacts with SH3 domain-containing proteins via a binding site with a preselected conformation

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ABSTRACT

Rlf is a guanine nucleotide exchange factor for the small G-proteins RalA and RalB and couples Ras- to Ral-signalling. Here the crystal structure of the catalytic module of Rlf consisting of a REM- and a CDC25-homology domain is determined. The structure is distinguished by an extended three stranded β-sheet called the flagpole. The flagpole is a conserved element in the RalGDS family of guanine nucleotide exchange factors and stabilises the orientation of the REM-domain relative to the CDC25-homology domain. A proline-rich sequence in the flagpole is unique to Rlf and several proteins that interact with this sequence by SH3 domains are identified. Conformational pre-selection results in a gain of affinity and contributes to the establishment of SH3 domain selectivity.

1. Introduction

The members of the Ras-family of small G-proteins are involved in the regulation of such diverse effects as proliferation, differentiation, adhesion and exocytosis. They do so by acting as molecular switches cycling between an off- and an on-state, whereby the latter mediates downstream signalling. The off-state corresponds to the GDP-bound version and the on-state to the GTP-bound version of the G-protein. Transition to the on-state is caused by the exchange of GDP for GTP. Nucleotide exchange is catalysed by guanine nucleotide exchange factors (GEFs). Switching off is achieved by the hydrolysis of GTP to GDP. The intrinsically low GTPase activity of small G-proteins is enhanced by GTPase activating proteins (GAPs), which terminate signalling. Effector proteins selectively interact with the GTP bound conformation of the G-protein and are responsible for transmitting the signal (Vetter and Wittinghofer, 2001; Bos et al., 2007; Wennerberg et al., 2005).

The small G-protein Ral is involved in the control of exocytosis (Moskalenko et al., 2002), endocytosis (Jullien-Flores et al., 2000), gene regulation (de Ruiter et al., 2000) and cellular transformation (Urano et al., 1996;White et al., 1996). The RalGDS family of GEFs connects signalling of the small G-proteins Ras and Ral and consists of four members in human: Rgl1, Rlf/Rgl2, Rgl3, and RalGDS. In all family members an N-terminal REM-domain is followed by a CDC25 homology domain (CDC25-HD) and a C-terminal RA-domain. CDC25-HDs are the catalytic domains found in GEFs for members of the Ras-family of small G-proteins (Quilliam et al., 2002). The CDC25-HDs of the RalGDS family are selective for the small G-proteins RalA and RalB (Albright et al., 1993; Wolthuis et al., 1997; Ferro et al., 2008). REM-domains co-occur with CDC25-HDs. REM-domains mainly stabilise the fold of the CDC25-HD (Quilliam et al., 2002) but the REM-domain of SOS, a GEF for Ras, is also involved in allosteric regulation (Sondermann et al., 2004). RA-domains bind to the GTP-bound form of Ras- and Rap-proteins (Herrmann, 2003). Thereby GEFs of the RalGDS family act mainly as effectors of Ras-signalling, as they are recruited by Ras•GTP to membrane compartments where Ral is localised as well.

SH3 domains interact with proline-rich sequences with the minimal consensus being PxxP (Kay et al., 2000). About 300 SH3 domains are found in the human genome. This number highlights the evolutionary success in using small domains as modules, which can be shuffled and integrated into many genes, as a tool to create protein interactions. This process is further eased by the need of only a short stretch of about seven residues containing a PxxP motif as the requirement for SH3 domain binding in the target

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protein. On the other hand this raises the question whether elements other than the primary structure surrounding the proline residues contribute to the establishment of selectivity.

Here the crystal structure of the catalytic module of Rlf is determined. An exposed SH3 domain binding site is predicted from the structure and indeed, several SH3 domain-containing proteins are identified in a yeast two-hybrid screen and confirmed to interact with the exposed binding site in Rlf.

2. Material and methods

2.1. Constructs

The SH3 domains of PLCγ-1 (PLCG1, *homo sapiens*, aa 790–848) referred to as PLCγ-1^{SH3}, myosin-1E (MYO1E, *homo sapiens*, aa 1054–1108) and intersectin-2 (ITSN2, *homo sapiens*, aa 1117–1185) were cloned into the pGEX4T3 expression vector. Amino acid residues 1–778, 1–735, 50–735 or 50–514 of Rlf (Rgl2, *mus musculus*) were cloned into the pGEX6P3 expression vector. cDNAs for PLCγ-1 (IRAKp961M0798Q) and myosin-1E (IRATp970C10100D) were obtained from Source BioScience and cDNAs for intersectin-2 (Addgene plasmid 25174) from Addgene.

2.2. Protein purification

Ral and the SH3 domains of PLCγ-1, myosin-1E and intersectin-2 were expressed and purified as GST fusion proteins according to standard procedures, if required, the GST-tag was removed by thrombin cleavage. H-Ras (*homo sapiens*, aa 1–166) was expressed from the ptac plasmid and loaded with the hydrolysis resistant GTP analogue GppNHp as described (Herrmann et al., 1996).

Rlf proteins were expressed as GST fusion proteins in the bacterial strain CK600K upon induction with 100 μM IPTG at 25 °C over night in Standard I medium (Merck). The bacteria were harvested by centrifugation, re-suspended in buffer containing 50 mM Tris HCl pH 7.5, 50 mM NaCl, 5 mM EDTA, 5% glycerol, 5 mM β-mercaptoethanol and 0.1 mM PMSF and lysed by sonication. The lysate was cleared by centrifugation at 50,000*g* and loaded to a GSH column (Pharmacia) equilibrated with the previous buffer. The column was washed with 6 column volumes of buffer containing 50 mM Tris HCl pH 7.5 400 mM NaCl, 5% glycerol and 5 mM β-mercaptoethanol, with 20 column volumes of buffer containing 50 mM Tris HCl pH 7.5, 100 mM KCl, 10 mM $MgCl_2$, 5% glycerol, 5 mM β-mercaptoethanol and 0.25 mM ATP at low flow rate, with two column volumes of 50 mM Tris HCl pH 7.5, 50 mM NaCl 2,5% glycerol and 5 mM β-mercaptoethanol (buffer A) and eluted with buffer A containing 20 mM glutathione. The protein containing fractions were pooled, concentrated, supplemented with 1 mg homemade GST-PreScission Protease per 100 mg protein, dialysed against buffer A and re-applied to a GST column equilibrated with buffer A, concentrated and further purified by gel filtration on a Superdex75 26/60 column (GE-Healthcare) in buffer A.

2.3. Crystallography

Rlf^{fl}, Rlf$^{1-735}$, Rlf$^{50-735}$ and Rlf$^{50-514}$ were subjected to crystallisation trials. Only Rlf$^{50-514}$ resulted in crystals. The initial hit was optimised and final crystals were grown at 289 K in sitting drops using a reservoir solution containing 0.1 M Bis–Tris propane, pH 6.5, 0.2 M $NaNO_3$ and 12% PEG 3350. Crystals were cryoprotected in a solution containing the mother liquor supplemented with 20% glycerol and flash cooled in liquid nitrogen. Data were collected at 100 K at beamline ID23eh1 of ESRF and processed with XDS (Kabsch, 1993). Molecular replacement was carried out in MOLREP (Vagin and Teplyakov, 2000) using the CDC25-HD of RalGPS1 (residues 23–289) and RasGRF1 (1028–1262) (residues 482–991) as poly-alanine search model. The program O (Jones et al., 1991) was used to build the model into 2Fo-Fc and Fo-Fc maps and refinement was carried out with REFMAC (Murshudov et al., 1997). Residues 195–203, 215–230, 273–282, 389–396, 403–426 and 513–514 of molecule A and 50–52, 75–81, 195–203, 218–233, 389–398 and 403–426 of molecule B are not visible. The Ramachandran plot depicts 94.7% of main chain torsion angles in the most favoured and 5.3% in additional allowed regions with 0 residues in disallowed regions.

Figures were generated using the programs Molscript (Kraulis, 1991), Bragi (Schomburg and Reichelt, 1988) and Raster3D (Merritt and Murphy, 1994).

2.4. Determination of GEF activity

RalB was loaded with the fluorescent GDP analogue 2′-/3′-O-(N-methylanthraniloyl)-guanosine diphosphate (mGDP) as described previously for Rap1B (Rehmann, 2006). The fluorescence intensity of Ral bound mGDP is approximately twice as intense as of free mGDP and thus nucleotide exchange can be observed as decay in fluorescence upon addition of an excess unlabelled GDP. All reactions were performed in buffer containing 50 mM TrisHCl pH7.5, 50 mM NaCl, 5 mM $MgCl_2$, 5 mM DTT, 5% glycerol and 20 μM GDP at 20 °C, with 200 nM Ral•mGDP and 50 nM Rlf as described (Rehmann, 2006). Ras•GppNHp and PLCγ-1^{SH3} were added at concentration as indicated in the figure legends.

2.5. Yeast two-hybrid

A human placenta cDNA library was screened for Rlf interacting proteins by Hybrigenics, SA, Paris, France.

2.6. Isothermal titration calorimetry

Titrations were performed in a NANO ITC 2G (TA Instruments) equipped with a 1 ml cell at 20 °C. In a typical experiment a solution of 190 μM Rlf$^{50-514}$ or GST-Rlf$^{79-91}$ was titrated with a solution of 1.5 mM PLCγ-1^{SH3} in steps of 6 μl. Experiments were performed in buffer containing 50 mM TrisHCl pH 7.5, 50 mM NaCl, 2.5% glycerol and 0.5 mM Tris(2-carboxyethyl)phosphine hydrochloride. All proteins were brought into buffer of the same preparation either by gel filtration or by extensive dialysis. Data were analysed by the manufacturer's software.

2.7. Co-precipitation assays

GST or GST-fusion proteins from lysates of bacteria overexpressing the respective proteins were coupled to glutathione beads and washed three times with buffer containing 50 mM Tris-HCl pH 7.5, 50 mM NaCl, 2% glycerol and 5 mM β-mercaptoethanol. 20 μg of Rlf$^{50-514}$ or Rlf$^{50-514}$R87E, or buffer control, were added to the pre-coupled beads and incubated for 20 min at room temperature under gentle tumbling. The beads were washed three times with buffer as before and eluted by application of 18 μl of Laemmli loading buffer and heating to 95 °C for three minutes. The samples were subjected to analysis by SDS–PAGE on 12.5% gels and protein bands were stained by Coomassie Brilliant Blue.

2.8. Coordinates

Coordinates and structure factors have been deposited in the Protein Data Bank with the accession code 4JGW.