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GTP-Ras Disrupts the Intramolecular Complex of C1 and RA Domains of Nore1

Elena Harjes,[1,3,6,7] Stefan Harjes,[1,3,6,7,*] Sabine Wohlgemuth,[2] Karl-Heinz Müller,[1,3] Elmar Krieger,[5] Christian Herrmann,[4] and Peter Bayer[1,3]

[1] Molecular and Structural Biophysics
[2] Department of Structural Biology
Max-Planck Institute of Molecular Physiology
Otto-Hahn Strasse 11
D-44227 Dortmund
Germany
[3] Interdisciplinary Center of Magnetic Resonance (IZMR)
D-44227 Dortmund
Germany
[4] Physikalische Chemie 1
Ruhr-Universität Bochum
D-44780 Bochum
Germany
[5] Center for Molecular and Biomolecular Informatics
Radboud University Nijmegen
P.O. Box 9101
6500 GL Nijmegen
The Netherlands

Summary

The novel Ras effector mNore1, capable of inducing apoptosis, is a multidomain protein. It comprises a C1 domain homologous to PKC and an RA domain similar to the Ras effectors AF-6 and RalGDS. Here, we determine the affinity of these two domains to the active forms of Ras and Rap1 using isothermal calorimetric titration. The interaction of Ras/Rap1-GTP with the RA domain of mNore1 is weakened significantly by direct binding of the C1 domain to the RA domain. In order to analyze this observation in atomic detail, we solved the C1 solution structure by NMR. By determining chemical shifts and relaxation rates, we can show an intramolecular complex of C1-RA. GTP-Ras titration and binding to RA disrupts this complex and displaces the C1 domain. Once the C1 domain tumbles freely in solution, a lipid binding interface becomes accessible. Furthermore, we provide evidence of phosphatidylinositol 3-phosphate binding of the free C1 domain.

Introduction

Small GTPases of the Ras family are central regulators of cellular signal transduction processes leading to cell proliferation, cell differentiation, and apoptosis (Shields et al., 2000; Feig and Buchsbaum, 2002; Cox and Der, 2003). Ras signaling is mediated by Ras effectors, including Raf, the RalGEF family member RalGDS, phosphatidylinositol (PI) 3-kinase, AF-6, and the novel Ras effector Nore1. These multidomain proteins contain a Ras association (RA) domain that tightly binds to active Ras/Rap-GTP, but not inactive Ras/Rap-GDP. In addition, the effectors Raf and RalGDS contain catalytic and signal transducing domains, whereas the effectors AF-6 and Nore1 more likely function as adaptors, attracting other proteins (Radziwill et al., 2003).

Nore1 was first discovered in a yeast two-hybrid screen of a mouse cDNA library by using Ras as the bait (Vavvas et al., 1998). There are two human splice variants, Nore1A and Nore1B, which have been assigned to the Ras association family (RASSF). Within the primary sequence of Nore1, five regions can be assigned: the N-terminal proline-rich region, able to bind RASSF1A (Ortiz-Vega et al., 2002), is followed by a cysteine-rich domain. Next, there is a region of ~100 amino acids, which, by using deletions, was implicated in Ras-independent inhibition of tumor cell growth (Aoyama et al., 2004). Adjacent to this stretch of residues, the RA domain can be found. The C terminus putatively forms a coiled-coil structure. It has been used to immunoprecipitate the kinase Mst1, known to be responsible for promotion of apoptosis (Khokhlatchev et al., 2002).

The cysteine-rich domain shares homology with the C1 domains of PKC and Raf. C1 domains have been implicated in intramolecular complexes, which mediate autoinhibition of these kinases (Medkova and Cho, 1999; Cutler et al., 1998). Additionally, C1 domains can interact with membrane lipids. Typical C1 domains bind diacylglycerol or phorbol ester, whereas atypical domains have other specificity. Residues involved in phorbol ester binding of PKCγ or PKCδ, for example, have been identified by using NMR spectroscopy (Xu et al., 1997) and crystallography (Zhang et al., 1995), respectively. A diacylglycerol binding consensus sequence could be derived (Hurley and Misra, 2000). There has been controversy about direct binding of the Raf C1 domain to Ras (Hu et al., 1995; Cutler et al., 1998). A better understanding of the role of the C1 domain may shed light on mechanisms of effector activation and/or translocation.

In this study, we resolve the structure of the C1 domain of mNore1 by heteronuclear NMR spectroscopy. Further, we investigate its interaction with the RA domain and with Ras by NMR titration, as well as by isothermal titration calorimetry (ITC). We find no direct binding of C1 to Ras; instead, we can demonstrate an intramolecular complex of the C1 and RA domains of Nore1. Titration of Ras disrupts this intramolecular complex, allowing the C1 domain to tumble freely in solution and to bind other interaction partners. Using a protein-lipid overlay assay, we further demonstrate phosphatidylinositol 3-phosphate and sulfatide binding of the free C1 domain of Nore1.

Results

Structure Description

We determined the structure of the C1 domain of Nore1 (residues Pro95–Arg166) by using high-resolution, heteronuclear, multidimensional NMR spectroscopy.

*Correspondence: stefan.harjes@uni-greifswald.de
[6] These authors contributed equally to this work.
[7] Present address: Greifswald University, Institute of Biochemistry, Department of Biotechnology, Soldmannstrasse 16, D-17487 Greifswald, Germany.

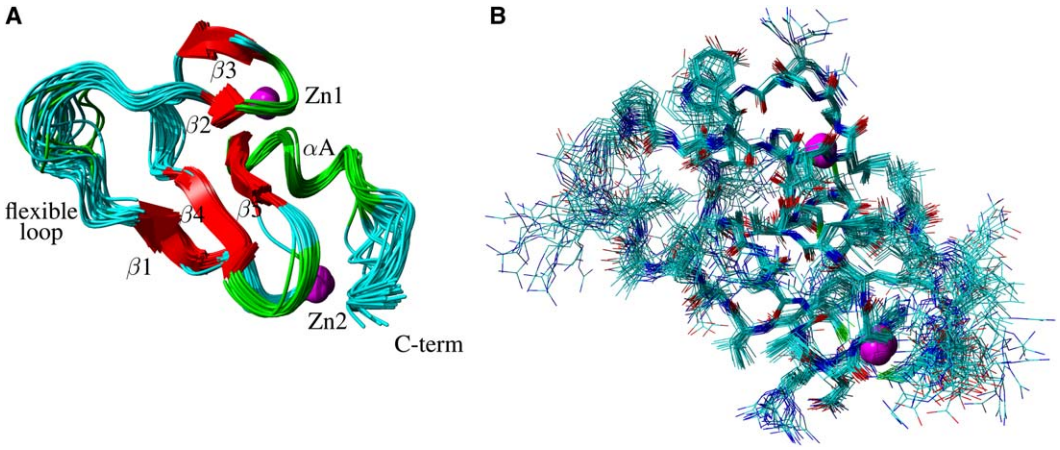

Figure 1. The Structure of the C1 Domain of mNore1

(A) Ribbon representation of 30 calculated structures superpositioned onto the average structure.
(B) The same ensemble, but shown as a stick representation.

Figure 1 shows the superposition of the 30 energetically most favorable C1 models. The well defined fold of the C1 domain (residues Cys118–Cys165) consists of a distorted five-stranded, antiparallel β sheet with a helix on one side of the sheet. The fold is stabilized by two zinc clusters, connecting the helix and the sheet (Figure 1). The five-stranded β sheet comprises a three- and a two-stranded part. The three-stranded region consists of strands 1, 4, and 5 (residues Val121–Leu123, Ala143–Arg145, and Phe151–Cys153, respectively), while the second part encompasses strands 2 and 3 (residues Gly130–Cys132 and Arg137–Val139, Figure 2). Strands 2 and 3 as well as 4 and 5 are connected by β turn elements, which are found on the same side of the β sheet. The first of these two β turns is stabilized by hydrogen bonds between the carbonyl oxygen of Cys132 and the backbone amide groups of Cys135 and Gly136. The second β turn is stabilized by the same pattern, where Cys146 binds to Cys149 and Lys150. Strands 1 and 2 as well as 3 and 4 are connected by long loops which can be found on the opposite side of the β sheet (Figure 1). The first of these two loops seems to be flexible, since it shows large conformational variations (Figure 1). The helix (Gln156–Ile161, Figure 2) is only weakly defined by NOEs. The hydrophobic core is formed by amino acids His118, Phe120, Cys132, Val139, Ala143, Leu144, Cys146, Cys149, Cys153, and Ile161. Additionally, the side chains of Lys150 (β, γ, and δ methylen groups) and Phe151 contribute to the hydrophobic core. The tetrahedral zinc coordination is conserved among C1 domains (Mott et al., 1996; Chen et al., 2000). Zn1 connects β strands 2 and 3 (S-Cys132 and S-Cys135) with the N terminus of the helix (Nδ-His154 and S-Cys157). Adoption of a different rotamer by evolutionary weakly conserved Cys153 would allow for alternate Zn1 coordination (mean S-Cys153 Zn1 distance of 6.6 Å). The second zinc ion attaches S-Cys165, located five amino acids from the C terminus of the helix, to β strands 1, 4, and 5 (Nδ-His118, S-Cys146, and S-Cys149 respectively).

Without considering the flexible parts, the ensemble superpositions well, with a backbone rmsd of 0.41 Å.

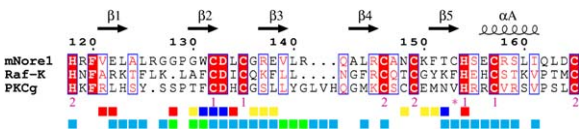

Figure 2. Alignment of C1 Domains

The sequence of the C1 domain of mNore1 was aligned by using the program pileup (Wisconsin Package 9.1, Genetics Computer Group). By using structural information, the alignment was refined with the program SPV (Guex and Peitsch, 1997). The secondary structure of the mNore1 C1 domain, deduced from the NOE and hydrogen bonding network, is display above the alignment. The residues marked in red, yellow, and blue correspond to the RA binding interface of the mNore1 C1 domain, defined by changes during Ras titration by using NMR. The green and cyan boxes below the alignment mark residues of PKCγ that interact tightly with phorbol ester (green) or change their NMR signals during lipid micelle titration (cyan) (Xu et al., 1997).

Table 1. Statistics of Structure Calculation

Number of NOE distance constraints	
Total	535
Long range (separated by more than 5 Å)	154
Torsion angle constraints	48
Rms deviation from experimental data	
Distance restraints (Å)	0.0068 ± 0.0007
Dihedral restraints (°)	2.4 ± 1.2
Rms deviation from ideal geometry[a]	
Bonds (Å)	0.015 ± 0.001
Angles (°)	1.76 ± 0.08
Dihedrals (°)	16.2 ± 1.4
Rms deviation from the structure with the lowest energy for residues 118–122 and 131–161[b] (Å)	
Backbone atoms	0.42
All atoms except hydrogen	1.13
Ramachandran plot[c] (%)	
Most favored regions	73.9
Additionally allowed regions	24.4
Generously allowed regions	1.7
Disallowed regions	0.0

[a] Calculated with the program CNS (Brunger et al., 1998).
[b] Calculated with the program molmol (Koradi et al., 1996).
[c] Calculated with the program procheck (Laskowski et al., 1993).