Parallel Coiled-coil Association of the RhoA-binding Domain in Rho-kinase*

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Rho-kinase is a serine/threonine protein kinase that regulates cytoskeletal events in cells. The enzyme activity of Rho-kinase is auto-inhibited in the free state but is activated through direct binding to the small GTPase Rho in the GTP-bound form. The crystal structure of the Rho-binding domain (RhoBD) of Rho-kinase has been determined at 1.8 Å resolution by the multi-wavelength anomalous dispersion technique. The structure shows that RhoBD dimerizes to form a parallel coiled-coil with long consecutive α-helices extended to ~97 Å and suggests that free Rho-kinase can also form a dimer through parallel self-association. At the middle region of the coiled-coil, the polypeptide chains are flexible and display loose “knobs-into-holes” packing of the side chains from both chains. RhoBD residues that have been shown to be critical for Rho-binding are spread in the positively charged C-terminal region. The parallel coiled-coil structure of our Rho-kinase RhoBD in the free form is different from the anti-parallel coiled-coil structure of RhoBD of protein kinase N when complexed with RhoA. Implications derived from these structural studies in relation to the mechanism of Rho-kinase activation will be addressed with previously reported experimental data.

Evidence has accumulated to suggest that the small GTPase Rho plays crucial roles in cytoskeletal rearrangements for cytoskeleton, cell motility, and cell adhesion (1–4). A number of Rho effectors have been identified which associate specifically with the GTP-bound forms of Rho GTPases, including Rho-kinase/ROK. Rho effectors have been identified which associate specifically with RhoA. Implications derived from these structural studies in relation to the mechanism of Rho-kinase activation will be addressed with previously reported experimental data.

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† The atomic coordinates and structure factors (code 1UXI) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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The abbreviations used are: PKN, protein kinase N; RhoBD, Rho-binding domain; DMPK, myotonic dystrophy kinase; MRCK, myotonic dystrophy kinase-related Cdc42-binding kinase; GST, glutathione S-transferase; GTPyS, guanosine 5′-O-(thiotriphosphate).
crystallized and solved the structure of the Rho-binding domain that consists of 69 amino acid residues (hereafter referred to as RhoBD-(69)). We show that RhoBD-(69) in the free state forms long consecutive a-helices dimerized in a parallel coiled-coil. Because RhoBD-(69) is part of the coiled-coil domain of Rho-kinase, our structure suggests a dimer form of Rho-kinase in the free state through parallel strand association in solution. We found that the coiled-coil contains several irregular structural features. In particular, the polypeptide chains of the middle region of the coiled-coil are flexible with poor inter-helical contacts between two chains. The parallel coiled-coil structure of our Rho-kinase RhoBD is different from the anti-parallel coiled-coil structure of PKN RhoBD complexed with RhoA. Implications derived from these structural studies in relation to the mechanism of Rho-kinase activation will be addressed with previously reported biochemical and structural data.

MATERIALS AND METHODS

Expression, Purification and Crystallization of RhoBD-(69)—The expression, purification, and crystallization of RhoBD-(69) from Rho-kinase were performed as reported previously (33). RhoBD-(69) encoding residues 979–1047 of bovine Rho-kinase was expressed as a fusion protein with glutathione S-transferase (GST) and subsequently purified using matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy from the thrombin cleavage site. The protein was verified using matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy (Applera Biosystems) of the resulting sample revealed the presence of an Se-Met protein with glutathione S-transferase(GST) and subsequently purified using matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy (Applera Biosystems). The presence of labeled RhoA. Labeled bands were visualized by an image analyzer (Fuji).

Data Collection, Processing, and Structure Determination—Crystals were directly mounted in a cryoloop from drops and flash frozen in a stream of nitrogen gas at 100 K. All of the data sets were processed with DENVOL and SCALEPACK (34). The crystals belong to the C2 space group (a = 148.0; b = 26.1; c = 39.6 Ǻ; β = 90.4°) with a Vn value (35) of 2.36 Å³ Da⁻¹, assuming two molecules in the asymmetric unit. Attempts to find heavy atom derivatives failed due to their high order non-isomorphism. Multiple-wavelength anomalous dispersion data using selenium-Met RhoBD-(69) were collected using a macromolecular oriented Weissenberg camera (36) with a cassette radius of 430 mm on large x-ray image plates (Fuji film, 400 x 800 mm) at BL-18B of the Photon Factory (Tsukuba, Japan). Diffraction data was collected at the absorption edge (λ1), peak (λ2), and high (λ3) and low (λ4) energy remote points. Oscillations of λ of 8° were used with a speed of 2°/s. The total oscillation ranges were 360° for peak, low remote, and native datasets, and 180° for edge and high remote datasets, respectively. Relatively low completeness in the outer shell might be derived from the experimental setting, irrespective of high mean I/σ values. Selenium positions were identified using the SnB program (37), which showed three prominent peaks even though each chain of RhoBD-(69) contains only one Met residue. This has been found to be due to two conformers of one Met residue in the determined structure. Phase calculation and heavy atom refinement using SHARP (38) resulted in a final overall figure of merit of 0.35 from 30 to 1.8 Å. Solvent flattening using Solomon (39) resulted in an electron density map of good quality in which a nearly complete model could be built. The built model was refined through alternating cycles using the program O (40) and CNS (41) programs, respectively. After several cycles of refinement with REFMAC (42) considering TLS treatment, the model finally converged, resulting in a crystallographic R value of 19.3% and a free R value of 23.6% for all of the diffraction data up to 1.8 Å resolution. The choice of the resolution is judged from the completeness in the outer shell (~70%), although mean I/σ around 1.8 Å is relatively high. The current model contains residues 979–1045 and 979–1044 for each chain including 174 water molecules. A summary of structure determination statistics is given in Table I. There is no residue in the disallowed region in PROCHECK (43). Because of the weak electron density, the 21 residues were modeled as Ala residues.

Structure Inspection—Identification of "knobs-into-holes" packing discussed by P. H. C. Crick was analyzed using the SOCKET program (44). Accessible surface areas were calculated using the NACCESS program (45). The figures displayed are those derived from the GRASP (46), MolScript (47), and Raster3d (48) programs.

RESULTS

The RhoA-binding Domain 69 Fragment Binds RhoA-GTP/γS—The RhoA-binding regions have previously been shown to correspond to residues 970–1059 in ROKa and 934–1015 in p160ROCK2 (3), which corresponds to residues 979–1068 and 964–1045 of Rho-kinase, respectively (5, 31). We found that RhoBD-(69), encompassing residues 979–1047 of Rho-kinase, represents a minimum construct sufficient for Rho-binding. Overlay assays clearly showed that RhoBD-(69) retains the binding ability for RhoA-GTP/γS (Fig. 1B).

| Item | Native (λ = 1.000) | Edge (λ₁ = 0.9784) | Peak (λ₂ = 0.9778) | High remote (λ₃ = 0.9600) | Low remote (λ₄ = 1.000) |
|------|-------------------|-------------------|-------------------|------------------------|------------------------|
| Phasing | Resolution (Å) | 1.8 | 1.9 | 1.9 | 1.9 |
| Reflections | Measured | 139.215 | 26.293 | 53.024 | 28.161 | 44.959 |
| Unique | 12.896 | 10.783 | 10.924 | 10.909 | 11.434 |
| Completeness (%) | 89.06/84.9 | 87.786 | 89.38/88.4 | 88.5/86.0 | 93.2/90.2 |
| Mosaicity | 0.301 | 0.273 | 0.420 | 0.284 | 0.290 |
| R_mer | 4.5/8.2 | 3.5/10.3 | 5.6/12.1 | 4.5/12.1 | 2.2/5.0 |
| Mean I/σ | 18.2/14.8 | 15.2/9.8 | 15.2/10.9 | 13.9/8.8 | 17.8/14.1 |
| Mean FOM | 0.35 | | | | |
| Refinement | Resolution range | 39.5–1.80 | | | |
| Number of residues | 137 | | | | |
| Atoms included | 1,003 for protein (174 water molecules) | | | | |
| R cryst-factor/R_free-factor (%) | 19.3/23.6 | 21.9 (protein), 30.8 (water molecules) | | | |
| Mean B-factor (Å²) | 0.018 Å⁻¹, 1.511, 1.713 | | | | |
RhoBD-(69) Dimerizes into a Coiled-coil Structure—The current model determined at high resolution (1.8 Å) displays well defined structures for two of the RhoBD-(69) chains, whereas the three C-terminal residues of each chain are unstructured. The RhoBD-(69) chains form two long consecutive α-helices that are wound around each other in a ~97 Å-long parallel coiled-coil structure (Fig. 2A). The total buried accessible surface area is ~2900 Å². This parallel coiled-coil structure is different from the ACC finger structure of the PKN RhoBD domain in the RhoA/PKN complex (Fig. 2B) (32). In the typical parallel coiled-coils, hydrogen-bonded salt bridges would occur between oppositely charged residues at g and succeeding e' positions (where prime denotes the other chain). However, in the RhoBD-(69) coiled-coil, only one possible g-to-e' pair is apparent.

Interfaces between the RhoBD-(69) Helices Forming the Parallel Coiled-coil—At the N-terminal region (residues 979–1002) of RhoBD-(69), the inter-helical interactions are similar to those previously observed in leucine zipper and coiled-coil structures encoded by a characteristic seven-residue repeat (abcdefg)ₙ (Figs. 3A and B). In fact, leucines or hydrophobic residues are located at most of the d and a positions in this region. The helices display canonical knobs-into-holes packing in which the side chains of the residues at the a and d positions form successive layers and make contact with four side chains from the opposing helix. The inter-helix distances between two α-helices in this region are comparable to the averaged value (9.6 Å) of typical helix bundles (Fig. 3C).

In contrast to closed packing of side chains at the N-terminal region, the middle region (residues 1003–1023) exhibits loose coiled-coil packing. At both ends of this region, Leu-1003 at the a position and Phe-1020 at the d position zip two α-helices by knobs-into-holes packing, whereas this side-chain packing is lost at Leu-1006 (at the d position), Glu-1010(a), Ala-1013(d), and Lys-1017(a) (Figs. 3E and 4). The inter-helix distances reveal an abnormally large value (>12 Å) for the coiled-coil in the middle of this region (Fig. 3C). Ordered solvent molecules are not observed among the helices where Ala-1013 fails to fill the space. Correspondingly, the average B factor (40.5 Å²) in this region is ~2-fold higher than the overall B-factor (21.9 Å²) (Fig. 3D). Moreover, the buried accessible surface area of the middle region is 2-fold lower than that of the N-terminal region. Generally, hydrophobic residues tend to appear at a and d positions with low accessibility (<30%). In the middle region, however, the accessibility of the residues at these positions is high (>40%) because of the loose packing. Interestingly, sequence alignment of Rho-kinase with its homologs shows an insertion/deletion in the middle region (Fig. 4).

At the C-terminal region (residues 1024–1044), tight side-chain packing is again retained, although the inter-helix distances in this region are varied and shorter than the averaged value of typical helix bundles. Two charged residues in this region, Glu-1027 and Lys-1031, occupy rather exceptional d and a positions, respectively. These residues may affect the irregularity of the helix. Notably, the side chains of these residues form a salt bridge within the same chain. The presence of the charged residues at the d and a positions might account for the finding that the MULTICOIL program (49) predicted that the C-terminal region (residues 1024–1047) has a low probability to form a coiled-coil structure.

Mapping of Mutations—Mutation analysis has identified several residues as being critical for Rho binding (18, 31). The Glu-1008 mutation of p160ROCK significantly weakens Rho binding, whereas the Ile-1041 mutation completely abolishes Rho binding (numbering scheme corresponds to Rho-kinase in Fig. 4). Double mutations consisting of Glu-1027+Arg-1028, Lys-1031+Gln-1033, and Asn-1036+Lys-1037 (ROKα) showed a reduction in RhoA binding. These residues are localized at the C-terminal region (Fig. 5A). The electrostatic potential surface of RhoBD-(69) shows that the C-terminal half is positively charged, whereas the N-terminal half is negatively charged.
charged (Fig. 5B). In the RhoA/PKN complex, complementary electrostatic potential exists at the PKN-RhoA interface where the positively charged ACC finger domain of PKN makes contact with the negatively charged region of RhoA (32).

**DISCUSSION**

Our RhoBD-(69) structure supports the notion that intact Rho-kinase would exist in a dimer form resulting from parallel association through the coiled-coil domain in solution. The
structure is consistent with results of gel filtration chromatography that showed a peak, which corresponds to a dimer. Evidence for parallel association has been obtained for MRCK from a biochemical experiment that revealed homotropic interactions of each subdomains from the coiled-coil domain (30). Moreover, hydrodynamic studies utilizing the Stokes radius and sedimentation coefficient showed that kinectin exists as a dimer through parallel self-association (50). Several lines of evidence have suggested oligomerization of each member of the DMPK family including Rho-kinase. Gel filtration and chemical cross-linking experiments have revealed the multimeric nature of ROK/H9251 (51), DMPK (29), and MRCK (30). It is likely that further self-association of the dimer forms of Rho-kinase and its closely related kinases takes place to yield a higher order oligomer such as a tetramer.

The RhoA-binding regions of RhoA GTPase effectors such as ROCKs, citron, and kinectin are all found in postulated extended α-helical or coiled-coil domains. It is probable that these RhoBDs form parallel coiled-coils resembling that of RhoBD-(69) from Rho-kinase. It would be interesting to determine whether the binding mode of RhoA to Rho-kinase is similar to that observed in the RhoA/PKN complex (32). It should be noted that the parallel coiled-coil structure of RhoBD-(69), which is distinct from the PKN ACC finger structure in the RhoA/PKN complex, is formed in an uncomplexed state with RhoA. The helical wheel diagram of RhoBD-(69) reveals that residues occupying each position (α–g) do not coincide with the residues critical for PKN binding in the RhoA/PKN complex (32). Recently, the structure of p21-activated kinase 1 (PAK1) that is regulated by Cdc42 or Rac has been solved in the auto-inhibited form (52). These studies suggest critical roles of the GTPase-binding site in dimerization and the subsequent induction of the auto-inhibitory form. GTPase binding could trigger a series of conformational changes, thus inducing disruption of the PAK1 dimer and resulting in kinase activation. As in the case of PAK1, RhoA of Rho-kinase might induce a structural change of the RhoBD coiled-coil, which may be similar to that of the PKN ACC finger for RhoA binding. We should point out that the flexible middle region in the coiled-coil of RhoBD-(69) corresponds to the turn region between two long α-helices of the PKN ACC finger, implying possible structural changes within this region upon RhoA binding. This induced fit mechanism for Rho-kinase is attractive in accounting for the kinase activation and auto-inhibition. However, further crystallographic investigations of RhoBD complexed with RhoA are required to understand the detailed molecular recognition mechanism of Rho-kinase for Rho.

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