High Resolution Crystal Structure of Human Rab9 GTPase
A NOVEL ANTIVIRAL DRUG TARGET*

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Liqing Chen‡§, Enrico DiGiammarino‡§, Xiaoyin E. Zhou‡§, Yujun Wang‡§, Diana Toh‡, Thomas W. Hodge‡§, and Edward J. Meehan‡§§

From the ‡Laboratory for Structural Biology, §Department of Chemistry, Graduate Programs of Biotechnology, Chemistry and Materials Science, University of Alabama in Huntsville, Huntsville, Alabama 35899 and ¶National Center for HIV, STD, and TB Prevention, Centers for Disease Control and Prevention, Atlanta, Georgia 30333

Rab GTPases and their effectors facilitate vesicular transport by tethering donor vesicles to their respective target membranes. Rab9 mediates late endosome to trans-Golgi transport and has recently been found to be a key cellular component for human immunodeficiency virus-1, Ebola, Marburg, and measles virus replication, suggesting that it may be a novel target in the development of broad spectrum antiviral drugs. As part of our structure-based drug design program, we have determined the crystal structure of a C-terminally truncated human Rab9 (residues 1–177) to 1.25-Å resolution. The overall structure shows a characteristic nucleotide binding fold consisting of a six-stranded β-sheet surrounded by five α-helices with a tightly bound GDP molecule in the active site. Structure-based sequence alignment of Rab9 with other Rab proteins reveals that its active site consists of residues highly conserved in the Rab GTPase family, implying a common catalytic mechanism. However, Rab9 contains seven regions that are significantly different in conformation from other Rab proteins. Some of those regions coincide with putative effector-binding sites and switch I and switch II regions identified by structure/sequence alignments. The Rab9 structure at near atomic resolution provides an excellent model for structure-based antiviral drug design.

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‡® To whom correspondence should be addressed. Tel.: 256-824-6533; Fax: 256-824-6349; E-mail: meehane@uah.edu.

© The abbreviations used are: HIV, human immunodeficiency virus; MES, 4-morpholineethanesulfonic acid; r.m.s., root mean square; GppNHp, guanosine-5′-[(γ-3H)imidotriphosphate]; Gppsp, 5′-guanosine-diphosphate-monothiophosphate.

The inactive conformation usually has displaced and mobile hydrophobic interface between the switch I and II regions (5, phosphate-binding loop and switch I region as well as an extensive rounded by five hydrogen bonds from the switch I and switch II regions significantly different in conformation from other Rab proteins, the largest subfamily of the Ras-like small GTPase superfamily, serve as molecular switches mediating vesicle cargo selection protein TIP47, which has been shown to bind the cytoplasmic tail of the HIV1 envelope glycoprotein subunit gp41 (13). By targeting Rab9 mRNA for degradation with small interfering RNA, Rab9 has just been identified to be a key cellular component for HIV-1, Ebola, Marburg, and measles virus replication, suggesting that inhibitors of Rab9 function, if developed, might prove useful in the control of those viruses. As part of a new structure-based antiviral drug design program, we have determined the crystal structure of a C-terminally truncated human Rab9 (residues 1–177) to near atomic resolution of 1.25 Å.

EXPERIMENTAL PROCEDURES

Cloning and Expression—The gene for human Rab9 (GenBank™ accession number NM_004251) was obtained from the IMAGE clone collection (IMAGE ID number 4139714) through distribution by Open-Biosystems. A C-terminally truncated fragment coding for residues 1–177 (20.1 kDa) was PCR subcloned using primers 5′-5′-ACA GCT AGC ATG GCA GGC AAA TCA TCA CTT TTT AAA G-3′ and 5′-GAT CCT TCA GTC CTC GGT AGC AAG AAC TCT TC-3′ into the NheI/BamHI restriction sites of pET28b (Novagen); the resulting construct encodes for a Rab9-(1–177) protein product with an N-terminal His6-containing fusion (MGSSHHHHHHSSGLVPRGSHMAS). The pET28-Rab9-(1–177) vector was transformed into Escherichia coli BL21(DE3) (Novagen), and overexpression of the fusion protein was induced at an A600 of ~2.0 with 1 mM isopropyl-1-thio-β-D-galactopyranoside at 310 K for 2 h; the cells were harvested by centrifugation and frozen at 253 K.

Protein Purification—The cell pellet was resuspended in nickel buffer A (20 mM Tris, pH 8.0, 500 mM NaCl, 5 mM imidazole), lysed by sonication, and centrifuged at 20,000 g for 20 min at 277 K. The soluble fraction was filtered through a 0.45-μm filter and applied to chelating Sepharose (Amersham Biosciences), which had been previously charged with 50 mM NiSO4 and equilibrated with nickel buffer A. The column was then washed with nickel wash buffer (20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 55 mM imidazole), and the His6-Rab9-(1–177) fusion protein was eluted with nickel elution buffer (20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 350 mM imidazole). The His6-Rab9-(1–177) fusion protein was then dialyzed against Thr buffer (20 mM Tris-HCl, pH 8.4, 200 mM NaCl, 10% glycerol) and concentrated by ultrafiltration to ~5 mg/ml. The protein sample was then subjected to gel filtration on a Superdex 200 10/30 column (Amersham Biotek) equilibrated in Thr buffer and concentrated to 5 mg/ml.

The active site consists of residues highly conserved in the Rab GTPase family, implying a common catalytic mechanism. However, Rab9 contains seven regions that are significantly different in conformation from other Rab proteins. Some of those regions coincide with putative effector-binding sites and switch I and switch II regions identified by structure/sequence alignments. The Rab9 structure at near atomic resolution provides an excellent model for structure-based antiviral drug design.
150 mM NaCl, 2.5 mM CaCl₂) at 277 K, and precipitate was removed by centrifugation at 20,000 g for 20 min at 277 K. To the soluble fraction, 1 unit of thrombin protease (Novagen) was added per milligram of fusion protein, and the His tag was removed by digestion for 4 h at 298 K (thrombin cleavage results in a Rab9a-(1–177) protein with an N-terminal GSHMAS extension). The thrombin cleavage reaction was diluted (1:3, v/v) with 20 mM MES, pH 6.5, and applied to Q-Sepharose (Amersham Biosciences), which had been previously equilibrated with Q buffer A (20 mM MES, pH 6.5). Native Rab9-(1–177) was eluted from Q-Sepharose with a 50–750 mM NaCl linear gradient in MES, pH 6.5; fractions containing native Rab9-(1–177) were identified by denaturing gel electrophoresis and pooled. The pooled Q fractions were then further purified by gel filtration on Sephacryl S-200 (Amersham Biosciences) in MES, pH 6.5, 150 mM NaCl; fractions containing Rab9-(1–177) were pooled and concentrated by ultrafiltration.

Crystallization and Data Collection—The stock protein solution used for crystallization contained 20 mM MES buffer, pH 6.5, and 150 mM sodium chloride with a protein concentration of 10 mg/ml. Crystals were grown at 277 K by the hanging-drop vapor diffusion method with 100 mM sodium acetate buffer, pH 5.0, 5% (v/v) polyethylene glycol 4000 as
crystallization solution. Crystals formed in space group P2₁ with a = 38.40 Å, b = 45.62 Å, c = 51.22 Å, α = 99.8°, β = 107.2°, and γ = 101.8° and contained two monomers in the unit cell. X-ray diffraction data to 1.25-Å resolution were collected at beamline 22-ID in the facilities of the South East Collaborative Access Team at the Advanced Photon Source, Argonne National Laboratory. The statistics for data collection and processing are summarized in Table I.

**Structure Determination and Refinement**—The orientation and position of the Rab9 dimer in the P2₁ unit cell were determined using the molecular replacement protocols in Crystallography & NMR System (14) starting from the structure of Rab11a (PDB code 1IVF (15)) as the search model. The composite omit map was calculated to guide electron density fitting of the model. Energy-restrained crystallographic refinement was carried out with maximum likelihood algorithms implemented in Crystallography NMR software (14). Refinement proceeded through several cycles in combination with manual checking by subjecting the structure to cycles of isotropic conjugate gradient minimization and also used for structure-based sequence alignment (18, 19). Ribbon diagrams were prepared by the program MOLSCRIPT (20).

**RESULTS**

**Structure Determination**—The human Rab9 variant we used for crystal structure determination included residues 1–177, lacking its last 24 residues (Fig. 1). Known as the C-terminal hypervariable region, the amino acid sequence of this region in Rab9 is poorly conserved with respect to other Rab proteins. Therefore, we excluded the C-terminal 24 residues from our cloning and crystallographic studies. We will refer below to this truncated form of the protein as Rab9.

Rab9 bound to GDP was crystallized, and its structure was determined by molecular replacement (Table I). The structure was refined against 1.25-Å resolution data, making it one of the highest resolution structures in the Rab protein family. Both N and C termini (residues 1 and 176–177 of monomer A and residues 1–4 and 176–177 of monomer B) and some loop re-
regions (residues 34–38 of both monomers and residues 111–114 of monomer B) were disordered and could not be seen in the experimental electron density map. The final refined model, which includes residues 2–34 and 39–175 of monomer A, residues 5–34, 39–110, and 115–175 of monomer B, 2 GDP molecules, and 508 ordered water molecules, has a working R value of 0.139 and a free R value of 0.196. The stereochemistry is excellent with r.m.s. deviations for bond lengths and angle distances of 0.013 Å and 0.032 Å, respectively (Table I). The Ramachandran plot statistics showed that 93.2% of the backbone dihedral angles were in the most favored regions, 6.8% in the additional allowed regions, and none of the non-glycine residues were in the disallowed regions. The two crystallographically unique Rab9 molecules in the crystal unit cell have almost identical structures with the r.m.s. deviation between the 161 equivalent Cα atoms of 0.40 Å. We will use monomer A in our description of Rab9 structure. Overall Structure of Rab9—Like other members of the Rab GTPase family, Rab9 adopts a classical nucleotide binding fold consisting of a six-stranded β-sheet surrounded by five α-helices (Figs. 2 and 3). The five α-helices (H1–H5) and six β-strands (B1–B6) connect with a B1-H1-B2-B3-H2-B4-H3-B5-H4-B6-H5 topology containing 30.5% (54/177) α-helix, 28.8% (51/177) β-sheet, 37.3% (66/177) turn/loop, and 3.4% (6/177) other (Figs. 294x733)
Active Site Structure—The overall structure of Rab9 is very similar to the prototype Ras protein p21Ras (21) and several Rab proteins (Table III). Among those, Rab9 has the highest sequence identity with Ypt7p (54% over 153 equivalent positions), followed by Rab11a (43% over 161 equivalent positions). The structural similarity Z-scores (18, 19) range from 26.6 to 23.2 with r.m.s. deviations of equivalent positions in the range of 1.4–2.0 Å. Structure-based sequence alignment reveals that the active site of Rab9 consists of residues highly conserved in the Rab GTPase family (Fig. 5), implying a common catalytic mechanism. However, Rab9 contains seven hypervariable regions that are significantly different in conformation from other Rab proteins (Figs. 5 and 6). Some of those regions coincide with putative effector-binding sites and conformational switch I and switch II regions identified by earlier crystallographic studies of other Rab proteins. Regions II and IV correspond to the switch I and switch II, respectively, whereas regions I, V, and VII correspond to the three effector-binding sites/complementary determining regions. These seven hypervariable regions in Rab9 structure may serve as sites for antiviral drug binding and provide an excellent target for structure-based drug design and development.

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