High Resolution Crystal Structures of Human Rab5a and Five Mutants with Substitutions in the Catalytically Important Phosphate-binding Loop*

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GTPase domain crystal structures of Rab5a wild type and five variants with mutations in the phosphate-binding loop are reported here at resolutions up to 1.5 Å. Of particular interest, the A30P mutant was crystallized in complexes with GDP, GDP + AlF₃, and authentic GTP, respectively. The other variant crystals were obtained in complexes with a non-hydrolyzable GTP analog, GppNHp. All structures were solved in the same crystal form, providing an unusual opportunity to compare structures of small GTPases with different catalytic rates. The A30P mutant exhibits dramatically reduced GTPase activity and forms a GTP-bound complex stable enough for crystallographic analysis. Importantly, the A30P structure with bound GDP plus AlF₃ has been solved in the absence of a GTPase-activating protein, and it may resemble that of a transition state intermediate. Conformational changes are observed between the GTP-bound form and the transition state intermediate, mainly in the switch II region containing the catalytic Gln¹⁹⁸ residue and independent of A30P mutation-induced local alterations in the P-loop. The structures suggest an important catalytic role for a P-loop backbone amide group, which is eliminated in the A30P mutant, and support the notion that the transition state of GTPase-mediated GTP hydrolysis is of considerable disassociative character.

As essential regulators of intracellular vesicle trafficking between subcellular compartments of eukaryotic cells, Rab proteins comprise the largest branch in the monomeric Ras-related GTPase superfamily (1, 2) and mediate membrane fusion in endocytosis (23–25) and possibly the budding process (3–7). This group of 20–25 kDa proteins share ~30% amino acid sequence identity (8). Like other Ras-related GTPases (small GTPases), Rab proteins serve as molecular switches by cycling between GTP-bound (on/active) and GDP-bound (off/inactive) conformations. Upon GTP binding, an extensive hydrophobic interface forms between two so-called switch regions (I and II) (9), resulting in presentation of ordered structural features characteristic for the active state that binds and responds to effectors/regulators (10, 11). The inactive form usually has displaced and mobile switch regions (11, 12). The off-to-on process requires dissociation of GDP, which is an intrinsically slow and reversible process, and association of GTP. This process can be accelerated by guanine nucleotide exchange factors (GEF) (13, 14) and regulated by other proteins such as GDP dissociation inhibitors (GDI) (15). The on-to-off process is also an intrinsically slow but irreversible process, which involves hydrolysis of GTP to GDP and is stimulated by GTPase-activating proteins (GAP)¹ (16–20). Despite the conserved catalytic machinery, the intrinsic GTP hydrolytic rates in the Rab family vary by more than an order of magnitude. For example, Rab5a exhibits a rate 20-fold higher than that of Rab6 or Rab7 (21). The intrinsic GTPase hydrolytic rate of a GTPase is important for the association duration with its GTP-specific partners, and thus for its functions in vivo. The structural determinants responsible for the large variation in the intrinsic rates of GTP hydrolysis remain elusive. In addition to the common GTPase fold, Rab5a usually possesses hypervariable N- and C-terminal peptides; the C-terminal peptides are often isoprenylated for targeting to specific membranes (8, 22). These peptides may participate directly in protein-protein contacts with some effectors or regulators but are not essential for nucleotide binding or intrinsic GTP hydrolysis.

Rab5, a member of the Rab family, regulates early endosome fusion in endocytosis (23–25) and possibly the budding process (26). It is widely distributed in many tissues. Rab5 physically changes locations during its GTP hydrolysis cycle. In its active stage, Rab5 is localized on the cytoplasmic side of early endosomes, while in its inactive stage, Rab5 stays in cytosol presumably associated with GDI. The biological functions of Rab5 are evident from the following observations. Anti-Rab5 antibodies and dominant negative Rab5 mutants are inhibitory in several early endosome fusion assays reconstituted in vitro (24, 27, 28). GTP-bound, but not GDP-bound Rab5, can stimulate early endosome fusion in vitro (27, 29, 30). Expression of dominant negative Rab5 mutants in intact cells causes fragmentation of early endosomes and reduced endocytosis (23, 28). Furthermore, overexpression of wild-type (WT) Rab5 or a constitutively activated Rab5 mutant results in enlargement of early endosomes and increased endocytosis (23, 28, 30). The intrinsic GTPase hydrolytic rate human Rab5a (one of three Rab5 isoforms) has been reported to be about 0.1 min⁻¹ (21), which is in the high range among Rab family members.

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¹ The abbreviations used are: GAP, GTPase-activating protein; GppNHp, guanosine-5’-(β,γ)-imidotriphosphate; WT, wild type; P-loop, phosphate-binding loop; rmsd, root mean square deviation; PDB, protein data bank; MES, 4-morpholineethanesulfonic acid.
Structures of Rab5 Complexed with Various Nucleotides

Structures of a number of small GTPases and effects of mutations have been investigated over the last two decades (31, 32). Besides their similar overall folding, these proteins share a number of conserved structural motifs. Among them, the phosphate-binding loop (P-loop), i.e. the GXXGXXGK/S/T motif (where X stands for any amino acid residue), is found to play crucial roles in GTP hydrolysis. In Rab5a, the P-loop consists of residues 27GESAVGKS34, corresponding to residues 110GAGGVGKKS117 in the prototypic small GTPase Ras (residue numbers in brackets denote positions in Ras). Mutations at Ras Gly12 often reduce its GTPase activity and increase its biological activity in cellular transformation (39); however, some of these variations can be found at an equivalent position in other functional WT small GTPases. To investigate structural roles of P-loop in GTP hydrolysis and biological function, Rab5a has been used as a model system to replace both Ser30(12) and Ala30(13) with all the other 19 amino acids (34, 35).

The choice of mutation sites was partly made based on their proximity to the γ-phosphate group of the substrate GTP. The resulting variants have been analyzed for GTP hydrolysis, GTP binding, GAP dissociation, and biological activity. At position 30 (13), only the substitution with proline reduces the GTPase activity significantly (at least 12-fold) (35). Whereas most of the other substitutions at this position show either a small negative effect or no effect on the GTPase activity, the arginine substitution stimulates the intrinsic GTP hydrolysis by 5-fold. It was proposed that this introduced arginine residue may mimic the function of an arginine finger motif (35), which enhances GTPase activity in trimeric GTPases (36, 37) and small GTPase-GAP complexes (38) by positioning the positively charged guanidinium group close to the GTP γ-phosphate group.

Despite considerable kinetic, structural, and theoretical studies on the reaction mechanism of small GTPases, there still lacks definitive structural evidence to resolve different mechanistic hypotheses. It has been shown that GTP hydrolysis in solution occurs via a dissociative, metaphosphate-like transition state (39). Such a reaction pathway implies that there is little bond formation between the nucleophilic water and GTP but substantial cleavage of the bond between the γ-phosphoryl moiety and the GDP-leaving group. Recent results from Foutrier transform infrared spectroscopy experiments suggest that the transition state seems to have a considerable amount of dissociative character (40). Binding of GTP to Ras has been shown to shift negative charge from the γ to β-phosphate (41), which is a characteristic feature of dissociative-like transition states, and such a shift can be enhanced by GAP binding (42). The charge shift is interpreted as a key factor contributing to catalysis by Ras in addition to correct positioning of the nucleophilic water. These observations seriously challenge the long-held dominant mechanistic hypothesis that GTP hydrolysis occurs via an associative-like pathway. A fully associative mechanism involves a trigonal bipyramidal intermediate, followed by the departure of the leaving group. It is characterized by an accumulation of negative charge on the γ-phosphate in the transition state. Structural observations, that catalytically important residues in GTPases interact extensively with the trigonal bipyramidal or hexacoordinating transition site analog GDP+AlF4− (where x is 3 or 4), form a cornerstone of associative transition state hypothesis. A compromising view has been put forward to describe the phosphoryl transfer by a structure somewhere between dissociative and associative extremes (40). However, there is still a controversial debate as to what extent the reaction proceeds via a dissociative or an associative mechanism. The dissociation transition state hypothesis of GTPase catalysis predicts that the enzyme stabilizes accumulation of negative charge at the β-γ bridge oxygen in the transition intermediate through an important hydrogen bond between this oxygen and the backbone amide group at the residue equivalent to Ras Gly12 (43). In short, the associative transition hypothesis emphasizes the catalytic roles of the nucleophilic water and structural features that stabilize negatively charged GTP γ-phosphate (44, 45), whereas the dissociative transition hypothesis proposes that the P-loop is crucial for the reaction (41, 43). A few crystal structures of Ras variants with mutations at Gly12 of the P-loop have been reported (44, 46), which in general support the notion that correct alignment of the nucleophilic water is critical for the GTP catalytic hydrolysis. To identify further the structural determinants in the P-loop that may regulate the intrinsic GTPase activity and potentially, the GAP-accelerated GTP hydrolytic rates, we have carried out a comprehensive crystallographic study to analyze a number of crystal structures of human Rab5a and five variants. Particularly, we have determined crystal structures of a variant that contains an Ala30(13) to Pro substitution (A30P) in its GTP-bound, (GDP+AlF4−)-bound, and GDP-bound forms, taking advantage of its very low hydrolytic activity. High resolution crystal structures of WT and other P-loop mutants have also been solved in complexes with a non-hydrolyzable GTP analog; they provide a clean background for discussion of the novel structural features observed in A30P complexes. Our results support the dissociation transition state hypothesis (43).

EXPERIMENTAL PROCEDURES

Expression and Purification of Recombinant Rab5 Proteins from Escherichia coli—Human Rab5a WT and P-loop mutants with Arg, Lys, Glu, Leu, and Pro at position 30 were subcloned into the bacterial expression vector pET11 from the corresponding pGEX-3X constructs containing the Rab5a cDNAs described previously (35). The native Rab5 protein consists of 215 residues. After comparison with the canonical folding of small GTPases, we deleted the N- and C-terminal hypervariable regions to promote crystallization. As a result, the recombinant proteins contain a starting codon-derived methionine residue, followed by residues 15–184 of Rab5a or its mutants. The recombinant proteins were expressed in the BL21 strain of E. coli, and purified as soluble proteins from the cytoplasm. Briefly, cell cultures were grown at 30 °C to an OD600 of 0.6–0.9, and then induced with isopropyl-β-D-thiogalactoside at final concentration of 0.5 mM, with an additional 3 h of growth at 35 °C. Cells were harvested by centrifugation and resuspended in 4 °C TN buffer (50 mM sodium phosphate, 50 mM NaCl, 0.5 mM EDTA, 0.1% Tween 20, 10 mM NaCl) containing EDTA-free protease inhibitor mixture (Roche Molecular Biochemicals, Mannheim, Germany). Cells were then lysed with lysozyme and a freeze-thaw cycle and treated with DNase in the presence of 1 mM MgCl2. The cell lysate was centrifuged, and the supernatant was loaded to a Sephacryl-200-HR sizing column (Amersham Biosciences) equilibrated in TN buffer containing 0.1% (v/v) β-mercaptoethanol and 0.02% (w/v) NaN3. High A280 fractions were pooled and loaded onto a Resource-Q ion-exchange column (Amersham Biosciences). Rab5a was eluted at ~100 mM salt concentration with a NaCl gradient.

Catalysis of Rab5—Rab5 recombinant proteins were crystalized using hanging drop vapor diffusion methods under conditions similar to those reported previously (10). The protein sample at ~15 mg/ml concentration in a buffer containing 20 mM MES (pH 6.0), 0.2 M NaCl, 1 mM MgCl2, and 0.1% (v/v) β-mercaptoethanol was mixed with an equal volume of the reservoir solution containing 10% (w/v) polyethylene glycol 6000 (PEG 6000), 50–100 mM NaCl, 0.2 mM MnCl2, and 0.1% (v/v) β-mercaptoethanol. The A30P mutant crystals were grown with GDP or GTP instead of GppNHp. In order to grow (GDP+AlF4−)-Rab5 complex crystals, NaCl in the above reservoir solution was replaced with 2 mM AlCl3 and 80 mM NaF. Crystals appeared 1 day after microseeding. Reservoir solution with additional 30% (v/v) glycerol (for WT, A30E, and A30L), 30% PEG 6000 (for A30K), or 20% 2-methyl-2,4-pentanediol (for A30R) served as the cryoprotection solution, and crystals were then cooled in a 100 K nitrogen gas stream for data collection.

Data Collection and Structure Refinement—A complete data set of Rab5a complexed with GppNHp was collected at 1.5 Å resolution from our in-house MAR345 image-plate data collection system (Mar Research
The Overall Structure—A previous biochemical study comparing the GTP hydrolytic rates of Rab5a WT and P-loop mutants replacing the Ala30[13] residue with 19 different amino acid residues showed a range of over 50-fold variation in their intrinsic GTPase activities (35). We selected five mutants from this group, namely A30P, Lys, Leu, Glu, and Arg, as well as the WT, for further structural studies. To facilitate crystallization, we deleted the hypervariable N- and C-terminal peptides (14 and 31 residues, respectively). The resulting recombinant proteins had comparable GTPase activities as their full-length counterparts (data not shown). Since both termini are located at one end of the β-sheet that is opposite from the nucleotide-binding site, the truncations are unlikely to contribute to the conformational changes we observed in the different mutant crystal structures. In the following, we will refer to these truncation constructs as WT Rab5a and its mutants. All constructs were overexpressed in E. coli as soluble proteins and purified for structural studies using X-ray crystallography. The intrinsic GTP hydrolytic rates of these mutants ranged from the highest (A30R) to the lowest (A30P) among the original 20 variants. The purification yields of these variants were about the same as the WT (5–10 μg per liter of cell culture). The recombinant proteins of all variants were able to be crystallized under the same or very similar conditions. Of particular interest, three A30P complex crystals were obtained with GDP, (GDP + AlF3), and authentic GTP, respectively. For the other variants, crystals were obtained only in complexes with GppNHp. Like the GppNHp-Rab5a variant crystals, the GTP-A30P complex crystals were stable at 20 °C for at least 50 days; GDP-A30P crystals, however, appeared less stable (i.e., <10 days).

All crystals belonged to the P212121 space group with similar unit cell parameters. There was one Rab5a molecule per asymmetric unit, with a low Vm of 2.0 Å3/Da (50), reflecting a tight crystal packing. The crystal structures of Rab5a were refined at resolutions ranging from 1.8 to 1.5 Å. Statistics of data collection and structural refinement of the eight crystal structures are summarized in Table I. All coordinates have been deposited to the Protein Data Bank (see Table I for the PDB IDs). The overall structure of Rab5a (Fig. 1) possessed a typical Ras-like small GTPase folding (51) and was essentially the same as that of Rab5c, a Rab5a isofrom (10). In all structures, residues 18–181 were visible in the electron density map. The corresponding root mean square deviations (rmsd) for Ca atoms between WT structure and those of non-proline variants range from 0.10 (A30R) to 0.34 Å (A30E). The structures of GTP (or its analog) and Mg2+ ligands were essentially identical in all variant crystals. Even in the GDP- and (GDP + AlF3)-bound complex structures, the GDP, Mg2+ ion, and its ligand atoms were perfectly superimposable with the corresponding parts from other structures.

Summary of WT and Non-proline Substitution Variants—The mutation site, residue 30[13] in the P-loop, was not involved in any crystal contact in the present crystal form; the closest distance between CB of residue 30[13] and symmetry-related protein molecules was larger than 8 Å. In each structure, residues in the P-loop had lower-than-average backbone B-factors. They ranged between 9 and 16 Å2 for the P-loop, compared with those between 13 and 21 Å2 of the overall backbone B-factors. Structural differences between the non-proline mutants and WT were restricted to the mutated side chain only; the P-loop backbone rmsd was below 0.1 Å for all structures in this group. As an example, Fig. 2 shows the differences in electron density map between A30R and WT. The differences included the side chain mutation (the large blue density block) as well as the shift of a water molecule nearby. The P-loop contained a type-II tight turn (52) formed by residues 28[11]–31[14]. Atoms of the 29[12]–30[13] peptide plane and β-γ bridge nitrogen atom in GppNHp were located perfectly in the same plane. The β-γ bridge nitrogen atom hydrogen-bonded as an acceptor with the Ala30[13] amide group (N–N distance of 3.02 ± 0.03 Å averaged over the five structures ± S.D.) and as a donor with a water molecule (N–O distance of 2.90 ± 0.02 Å), respectively. Together with the β- and γ-phosphate atoms, they formed a nearly perfect tetrahedral geometry centered at the β-γ bridge atom. A similar network should form around the bridge oxygen atom if an authentic GTP molecule had been bound, except that the oxygen atom would function as an acceptor in each hydrogen bond. At the mutation site in each structure, the side chains were mobile beyond the Cγ atom because of lacking supportive interaction, while the χ1 torsion angles of A30E, A30K, A30L, and A30R variants all assumed a gauche+ rotamer. The side chain terminal group in both A30R and A30E pointed to solvent with a trans γ2 rotamer; and in A30K, the Nζ group of lysine side chain hydrogen bonded with the hydroxyl group of Ser51[13] in the switch I region. In no case did we see that a side chain at position 30 pointed to the nucleotide. Thus any influence of the non-proline mutations at this position on the GTPase activity would most likely be indirect in nature. In the following, we use the WT structure as a representative of this group for comparison with the A30P structures, unless otherwise specifically mentioned. Furthermore, in all GTP(agonist)-bound complex structures, including GTP-A30P, the nucleophilic water was located in the vicinity of the γ-phosphoryl group and hydrogen bonded with the backbone amide group of residue 79 (O–N distance of 3.2 ± 0.1 Å). The side chain of catalytic residue Gln79[61] protruded into solvent and became mobile at its carbonyl tip, although it might form a weak hydrogen bond with the Arg41[63] side chain.

P-loop and Nucleotide in A30P Complexes—We were able to obtain crystal structures for three A30P complexes, including GDP-A30P, (GDP + AlF3)-A30P, and GTP-A30P. In contrast to all other mutants, the Ala30[13] to Pro substitution results in significant changes in the local three-dimensional structure (Fig. 3A). The changes were, however, confined at Ser29[12] and Pro30[13], while the P-loop retained an overall low thermal B-factor; for example, the GTP-A30P structure had a 10 Å2 average backbone B-factor for the P-loop, compared with the 13 Å2 one of the overall backbone. Pro30[13] assumes a cis conformation, thus distorting the β-turn in which the mutated residue was at the third position (52). Because of the cyclic proline side chain, the backbone amide group was no longer available for hydrogen bonding with the β-γ bridge oxygen in GTP/GDP. The corresponding proline imide group moved 1.5 Å in the direction away from the nucleotide. The CB atom of Ser29[12] in the GTP-A30P complex moved 0.4 Å relative to the GTP-WT complex, tilted toward to the γ-phosphoryl group. In the GDP-complex, this CB atom moved even further (1.2 Å relative to WT) presumably due to loss of contact with the γ-phosphoryl group, and the hydroxyl group assumed double conformations (gauche+trans). In all three structures, the nucleotides had well defined electron densities, and the P-loop conformations...
### Table I

Crystallography data collection and refinement statistics

|                  | WT  + GppNHp | A30P + GDP | A30P + GDP + AIF₃ | A30P + GTP | A30R + GppNHp | A30K + GppNHp | A30E + GppNHp | A30L + GppNHp |
|------------------|--------------|------------|-------------------|------------|---------------|---------------|---------------|---------------|
| **Data statistics** |              |            |                   |            |               |               |               |               |
| Space group      | P2₁2₁2₁      |            |                   |            |               |               |               |               |
| Unit cell (Å)    |              |            |                   |            |               |               |               |               |
| a                | 35.7         | 35.9       | 35.8              | 35.7       | 35.8          | 35.7          | 35.5          | 35.7          |
| b                | 64.1         | 63.9       | 64.0              | 64.2       | 63.9          | 63.6          | 63.7          | 63.8          |
| c                | 66.1         | 66.1       | 65.9              | 65.9       | 66.0          | 65.6          | 65.9          | 65.7          |
| Resolution (Å)   | 20(1.56)     | 20(1.66)   | 20(1.61)          | 20(1.66)   | 20(1.65)      | 20(1.86)      | 20(1.60)      | 20(1.61)      |
| Rmerge (%)       | 3.5(14)      | 4.6(29)    | 4.9(35)           | 4.2(13)    | 6.4(43)       | 7.9(35)       | 4.6(26)       | 3.9(16)       |
| No. of observations | 134,293    | 109,238    | 116,591           | 156,201    | 88,810        | 83,171        | 125,870       | 169,336       |
| No. of unique reflections | 24,442     | 20,680     | 22,654            | 20,631     | 20,607        | 14,404        | 22,532        | 22,394        |
| Completeness (%) | 99.5(99.3)   | 99.8(99.4) | 99.6(99.6)        | 99.7(100)  | 99.7(99.3)    | 98.9(99.3)    | 99.6(99.8)    | 99.6(99.8)    |
| Redundancy       | 5.5          | 5.3        | 5.1               | 7.6        | 4.3           | 5.8           | 5.6           | 7.6           |
| I/Io              | 36.3(5.2)    | 32.7(5.5)  | 31.0(3.7)         | 41.1(15.9) | 21.5(3.3)     | 20.5(4.3)     | 37.8(6.6)     | 43.0(11.0)    |
| **Refinement statistics** |            |            |                   |            |               |               |               |               |
| Rworking (%)/# of reflections | 17.6/23,097 | 17.9/19,286 | 17.7/20,990      | 17.3/19,501 | 17.6/18,937   | 17.4/13,961   | 18.4/21,188   | 17.9/21,154   |
| Rfree (%)/# of reflections | 18.9/1,162  | 21.4/982   | 20.0/1,072       | 21.1/995   | 20.7/961      | 21.0/706      | 20.4/1,082    | 20.0/1,080    |
| No. of non-hydrogen atoms | 1,313       | 1,313      | 1,313             | 1,320      | 1,313         | 1,339         | 1,294         | 1,313         |
| Protein          |              |            |                   |            |               |               |               |               |
| Solvent          | 300          | 269        | 287               | 291        | 282           | 240           | 274           | 269           |
| Visible range in protein (residues) | 15–181      | 15–181     | 15–181            | 15–182     | 15–182        | 15–184        | 18–181        | 15–181        |
| Rms deviation from ideal values |              |            |                   |            |               |               |               |               |
| Bond length (Å)  | 0.009        | 0.010      | 0.011             | 0.010      | 0.010         | 0.009         | 0.010         | 0.010         |
| Bond angle (deg.) | 1.44         | 1.51       | 1.56              | 1.48       | 1.49          | 1.43          | 1.46          | 1.52          |
| Average B-factor (Å²) | 16.4(18.8)  | 17.0(20.7) | 15.9(19.6)       | 15.1(18.1) | 18.1(20.6)    | 22.7(21.6)    | 17.6(21.7)    | 15.1(19.6)    |
| Protein Data Bank ID | 1N6H         | 1N6I       | 1N6K              | 1N6L       | 1N6N          | 1N6O          | 1N6P          | 1N6R          |

*Numbers in parentheses are the corresponding numbers for the highest resolution shells.

*Reflections of |Fobs| > 0.0.

*Numbers in parentheses are B-factors from the Wilson plot.
were similar but not identical to each other (Fig. 3B), suggesting a structural adjustment associated with nucleotide binding. In the GTP-bound structure, B-factors of all atoms in GTP, including the γ-phosphate group, ranged between 6 and 12 Å² and were lower than that of the average protein backbone (13 Å²), indicating that no significant hydrolysis occurred inside the crystal.

**Shift of the Switch I Region in A30P Structures**—In the structures of all three A30P complexes, part of the switch I region (i.e. residues 49–51) shifted away from the nucleotide by 0.5–0.8 Å relative to WT (Fig. 3A). This displacement was plausibly caused by a solvent network rearrangement between the P-loop and switch I region as a consequence of the A30P mutation. In the WT structure, the water molecule that hydrogen bonded with the β-γ bridge nitrogen atom in GppNHp simultaneously formed another hydrogen bond with an α-phosphoryl oxygen atom but did not interact with the switch I region directly. In A30P structures, this water molecule was not seen because of a close contact from the Prn[50][13] Cβ atom. It was replaced by two new water molecules at slightly shifted positions. One of them formed hydrogen bonds in a tetrahedral geometry with the α-phosphoryl oxygen, the backbone carboxyl oxygen of Gln49[32] in the switch I region, and two water molecules including the other new one. Similarly, the second water molecule formed four hydrogen bonds with a γ-phosphoryl oxygen atom, the hydroxyl group of Ser51[34] side chain again in the switch I region, and two water molecules. Through interactions with residues 49[32] and 51[34], this new solvent network resulted in a shift in the switch I region, making the nucleotide-binding pocket slightly more open, which was consistent with the higher GTP dissociation rate (3-fold) of this mutant than that of WT (35).

Located in the switch I region, the Ser51[34] side chain assumed a trans rotamer in the WT structure and formed a hydrogen bond with a γ-phosphoryl oxygen atom. In A30K, Ser51[34] switched to the gauche+ rotamer, which might weaken the hydrogen bond with the γ-phosphoryl oxygen; the potential hydrogen bond with Lys79[61] in A30K might provide some compensation for the energy loss. Furthermore, in A30R, A30E, and A30L, the Ser51[34] side chain appeared to have a double conformation, switching between trans and gauche+. Collectively, the greatest difference was observed in A30P, where the hydrogen bond between a γ-phosphoryl oxygen and the hydroxyl group of Ser51[34] was abrogated, accompanied by the backbone shift of the switch I region and a Ser51[34] side chain rotamer change to gauche+.

**Comparison of GDP-, (GDP-AlF3)-, and GTP-bound A30P Structures**—The overall structures of GDP-A30P complexes both with and without AlF3 were similar to those of other Rab5a variants, and their switch I regions were essentially identical to that in the GTP-A30P complex. However, noticeable conformational changes were found in the (i) γ-phosphate-binding site, (ii) Gln79[61] side chain, (iii) backbone of the switch II region, and (iv) Lys33[16] side chain, accompanying deformation/removal of the γ-phosphoryl group (see Fig. 3B). First, in the GDP-A30P complex, the γ-phosphoryl group was replaced by two water molecules, including one that coordinated with Mg²⁺ at the position of a GTP γ-phosphoryl oxygen. In the (GDP+AlF3)-bound complex, the aluminum ion coordinated with a hexavalent octahedral symmetry, namely three fluoride ions, the hydroxyl group of Ser29[12], one β-phosphoryl oxygen, and a nucleophilic water (Fig. 3C). The rmsd of the aluminum and three fluoride ions and Ser29[12] Oy atom from an ideal plane was 0.07 Å, and the rmsd values of the other two principal planes of the octahedral were 0.09 and 0.18 Å. Two of the fluoride ions occupied similar positions to those of γ-phosphoryl oxygen atoms that bind with the Mg²⁺ and Lys33[16] side chain, respectively. These two fluoride ions formed covalent bonds with the aluminum ion (1.83 ± 0.04 Å), while the third one was 2.1 Å from the aluminum ion and had a 23 Å² B-factor, which was almost twice as high as that of the other two fluoride ions. Thus, this third ion could be interpreted as a water molecule, although it is reported here as a fluoride ion for simplicity. Distances from the aluminum ion to the β-phosphoryl oxygen, Ser29[12] hydroxyl group, and the nucleophilic water molecule were 2.0, 2.1, and 2.4 Å, respectively. Secondly, while Gln79[61] retained the same gauche+ rotamer in its χ₁ torsion angle in both GDP and (GDP+AlF3) complexes as in the WT structure, its χ₂ rotamer changed so that the side chain tip plunged into the phosphate-binding pocket. The side chain carboxyl oxygen
of Gln\textsuperscript{79} simultaneously formed hydrogen bonds with its own backbone amide group and the nucleophilic water, replacing the hydrogen bond between the latter two. The water molecule was pushed \(1.5\) \(\text{Å}\) toward the \(-\text{phosphate atom as well as its symmetry axis along which the nucleophilic attack would occur. In the GDP-bound form, the water molecule further hydrogen bonded with a Mg\textsuperscript{2+} coordinating water molecule; in the (GDP+AlF\textsubscript{3})-bound form, the bound water molecule mimicked the nucleophile and coordinated to the aluminum ion. The coordination of both Mg\textsuperscript{2+} and Al\textsuperscript{3+} (except the Al-F bonds) are shown in thin lines. Orientations are the same as that of Fig. 2.}

A, reduce structural comparison between GppNHp\textsubscript{WT} and GTP-A30P\textsubscript{WT} is shown in red and A30P in blue. The water hydrogen-bond network around the active site and Mg\textsuperscript{2+} coordination are shown in dashed lines. Mg\textsuperscript{2+} and ordered water molecules are shown as large and small crosses, respectively. B, structural comparison among the three A30P complexes. The active sites of GTP\textsuperscript{−} (blue), (GDP+AlF\textsubscript{3})- (red), and GDP- (yellow) forms of A30P are superimposed on each other. C, active site structure of the (GDP+AlF\textsubscript{3})A30P complex. 2F\textsubscript{obs}−F\textsubscript{calc} map was contoured at 1.0 \(\sigma\) and superimposed with a ball-and-stick model of the final refined structure. Carbon (yellow), nitrogen (blue), oxygen (red), phosphate (white), aluminum (cyan), fluoride (magenta), and magnesium (green) ions are colored, respectively. The coordination of both Mg\textsuperscript{2+} and Al\textsuperscript{3+} (except the Al-F bonds) are shown in thin lines. Orientations are the same as that of Fig. 2.

Other Structural Features—In addition to the structural changes in the vicinity of the nucleotide-binding site, we observed some alternative packing in a hydrophobic cluster among the variant Rab5a structures. In A30E, A30K, and A30L, the side chain of Phe\textsubscript{21} was partially exposed to solvent. However, in other crystal structures, this side chain assumed mainly a buried conformation inside a hydrophobic core. The Phe\textsubscript{71} side chain had to make a 20\textdegree \(\chi\) adjustment to accommodate the insertion of Phe\textsubscript{21}. Considering the fact that the Ca–Ca distance between residues 21 and 30 was over 25 \(\text{Å}\), these structural changes more likely reflect intrinsic flexibility of Rab5a GTPase domain rather than response to the mutations introduced. Whether this hydrophobic repacking was of any functional significance, for example in the presence of N- and C-terminal peptides, remains an open question.

Fig. 3. Structures of A30P mutant. A, reduce structural comparison between GppNHp\textsubscript{WT} and GTP-A30P\textsubscript{WT} is shown in red and A30P in blue. The water hydrogen-bond network around the active site and Mg\textsuperscript{2+} coordination are shown in dashed lines. Mg\textsuperscript{2+} and ordered water molecules are shown as large and small crosses, respectively. B, structural comparison among the three A30P complexes. The active sites of GTP\textsuperscript{−} (blue), (GDP+AlF\textsubscript{3})- (red), and GDP- (yellow) forms of A30P are superimposed on each other. C, active site structure of the (GDP+AlF\textsubscript{3})A30P complex. 2F\textsubscript{obs}−F\textsubscript{calc} map was contoured at 1.0 \(\sigma\) and superimposed with a ball-and-stick model of the final refined structure. Carbon (yellow), nitrogen (blue), oxygen (red), phosphate (white), aluminum (cyan), fluoride (magenta), and magnesium (green) ions are colored, respectively. The coordination of both Mg\textsuperscript{2+} and Al\textsuperscript{3+} (except the Al-F bonds) are shown in thin lines. Orientations are the same as that of Fig. 2.
DISCUSSION

The biological and medical importance of GTPases continue to stimulate structural studies on their functional specificity and interactions with effectors/regulators. Despite considerable mutagenesis, kinetic and structural efforts, a consensus remains to be reached regarding key mechanistic aspects of catalysis by small GTPases. Here, we report high resolution crystal structures of a number of Rab5a mutants with substitutions in the catalytically important P-loop, which provide new insight into the GTPase catalysis. Whereas some of the variants (e.g., the A30R mutant) demonstrate noticeable difference in their intrinsic GTPase activity, the structural differences between non-proline substitution variants are marginal and mainly restricted to the mutation site, with a uniform gauche$^\pm$ $\chi_1$ rotamer. The equivalent position in Ras is a glycine. Mutations that add a side chain at position Gly$^{13}$ in Ras have little effect on the intrinsic GTPase activity (53). On the other hand, Rab3A has a serine at this position, which assumes a gauche$^\pm$ rotamer to avoid clashing with the side chain of Phe$^{12}$ from the switch I region. In Rab5a, the corresponding Gln$^{32}$ side chain in the switch I region points in a different direction, thus having no interaction with whatever residue is at position 30$^{13}$ in the P-loop.

Structural flexibility in solution, especially for a side chain at position 30$^{13}$ in the P-loop, may modify the static picture we have obtained here from the crystal structures, thus contributing to the variation in hydrolytic rates. For example, A30R hydrolyzes GTP with a 5-fold higher rate than WT (35). It was hypothesized that Arg$^{30}$ may accelerate the hydrolysis by contributing an arginine finger-like motif to the catalytic site. Such a motif might stabilize the transition state intermediate through electrostatic interaction with negatively charged groups in either the $\beta$-$\gamma$ bridge oxygen or $\gamma$-phosphoryl group. Our crystal structure of the A30R mutant argues against a static model of such a structural motif, because no hydrogen bond or salt bridge was observed between the Arg$^{30}$ side chain and the nucleotide or catalytic apparatus. However, favorable interactions of a similar nature, directly or indirectly through a solvent network, may occur transiently in solution where the Arg$^{30}$ side chain may exhibit dynamic movement. One could argue that the Arg$^{30}$ guanidine group might not attack the $\beta$-$\gamma$ bridge atom in the crystal structure because of the GTP analog used; an authentic GTP has an oxygen atom at this position, which may function as an acceptor to two hydrogen bonds and allow an interaction with the guanidine group in addition to the hydrogen bond with P-loop. However, it is noteworthy that the rate increase of A30R is significantly smaller than the 200–10$^3$-fold increase stimulated by an arginine finger from GAPs (54, 55). It indicates that the arginine side chain introduced by the single point mutation is far from optimal for such a catalytic function. Another possibility is that a mutation at position 30$^{13}$ may change the association/dissociation rate of the substrate/product, thus modifying the apparent hydrolytic rate. This might happen to A30R as observed for A30I and A30V (34).

Our GTP-A30P complex crystal structure provides a high resolution picture of the binding of an authentic GTP molecule to a GTPase, confirming that GTP assumes a similar conformation to GppNHp inside the nucleotide-binding site, thus justifying most conclusions obtained from structural studies on the GTP analog-GTPase complexes. Given the fact that A30P can crystallize with both GTP and GDP in the same crystal form, it raises the question of why WT and other Rab5a variants do not crystallize in the GDP-bound form. One possible explanation is that A30P possesses a quasi-stable active conformation that does not require $\gamma$-phosphosphate. This is consistent with the observation that the A30P mutant exhibits dominant positive phenotype (i.e. it is locked in the active conformation) in stimulating endocytosis (34). Furthermore, the altered water network observed in the A30P crystal structures may favor such a conformation, which is associated with a small (–0.5 Å) and localized (residues 48–53) shift in the switch I region.

The A30P structures support the dissociative transition state hypothesis for GTPase-catalyzed GTP hydrolysis (43). One important prediction from this hypothesis is that the backbone amide group of Gly$^{13}$ in Ras (equivalent of Rab5a Ala$^{30}$) plays the critical role in stabilizing a negative charge accumulation during the catalysis. In all GDP-small GTPase complex structures that we have examined (PDB files 1a0f, 1d16, 1d5c, 1k3y, 1q21, 1rrf, 1rrg, and 4q21), this amide group forms a hydrogen bond with the $\beta$-phosphoryl oxygen corresponding to the bridge oxygen in GTP, consistent with the charge-shifting theory. NMR spectroscopic studies of Rab in solution also suggest that the Gly$^{13}$ amide proton forms a hydrogen bond in a GTP[S]-Ras complex (56). Our GTP-A30P complex crystal structure provides direct evidence supporting the dissociative transition state hypothesis from a different angle. In this case, the proposed important hydrogen bond between the backbone amide group and the $\beta$-$\gamma$ bridge oxygen does not exist because of the nature of the mutation. Elimination of the backbone amide group of residue 30$^{13}$ in A20P is accompanied by the loss of GTPase activity, strongly supporting the mechanism requiring a hydrogen bond to the $\beta$-$\gamma$ bridge oxygen of GTP (43). The fact that we have been able to obtain the complex crystal of GTP-A30P confirms that the GTP hydrolytic rate must be very low and suggests that other hydrolytic mechanisms are unlikely to function in the absence of this hydrogen bond. Inside the crystals, the most noticeable structural change in GTP-A30P complex relative to GppNHp-WT complex is located in residues 29$^{12}$ and 30$^{13}$. Therefore, the decrease in the intrinsic GTPase activity can be explained solely by loss of the key catalytic component, particularly the peptide plane between residues 29$^{12}$ and 30$^{13}$. This conclusion is consistent with the observation that a proline substitution at Rab5 Ser$^{29}$ does not reduce the intrinsic GTPase activity (34). This residue is the closest neighbor to Ala$^{30}$ but whose backbone atoms assume no direct role in catalysis. Furthermore, our GTP-A30P structure shows no conformational change in the rest of the P-loop, including residues 31$^{14}$–33$^{16}$, which were proposed to be important for a charge shift from the $\gamma$-to $\beta$-phosphate on GTP hydrolysis (41); thus, it is unlikely that the A30P mutation might disrupt the catalysis via this region. In addition, unlike the backbone amide group of Ras Gly$^{13}$ that retains its hydrogen bond with the $\beta$-phosphoryl oxygen in GDP (9), the Lys$^{33}$ side chain in GDP-A30P moves away from GDP. It suggests that this lysine residue is less important than the backbone amide group of residue 30$^{13}$ in the charge shift during GTP hydrolysis. Based on the WT Rab5a structure, both the Ala$^{30}$ amide group and the water molecule that hydrogen bonds to the $\beta$-$\gamma$ bridge group are likely to contribute to catalysis, supporting the dissociative transition state hypothesis (43). This water molecule has been observed in a number of small GTPase crystal structures, e.g. those of Ypt51 (PDB file 1ek0) (57) and ARF1 (58), but is disrupted in others often because of interference from the switch I region. The water molecule occupies a position similar to the arginine finger from the GAP protein in the Ras-GAP complex crystal structure (45); its catalytic effect is likely, however, to be weaker.

Another crucial residue in the GTPase catalysis is the frequently conserved Gin$^{32}$, which is located in the switch II region. A mutation of this residue to leucine in a variety of...
GTPases has been repeatedly shown to abrogate the GTPase activity, and thus maintains the protein in its GTP-bound form (59). It is widely believed that the function of this glutamine residue is to align the nucleophilic water molecule in position for attacking the γ-phosphoryl group from the axial direction (9, 43, 44, 46, 60, 61), although it has been argued that activation of the nucleophilic water is not the rate-limiting step in the hydrolytic reaction (44). In our GTP(analog)-Rab5α crystal structures, the Gln79(H11001) side chain interacts with the nucleophilic water molecule only through a Van der Waals contact. Therefore, the residue specificity is unlikely to function at this stage. In contrast, in both GDP- and (GDP+AlF3)-bound forms of A30P, the Gln79(H11001) side chain carboxyl group points to the nucleoside-hinging pocket. Particularly, in the (GDP+AlF3)-bound form, the Gln79(H11001) side chain carboxyl group forms a hydrogen bond ideal for orientating the nucleophilic water molecule to attack the γ-phosphoryl group analog, in this case the AlF3 moiety. The formation of such an intermediate is clearly vulnerable to a mutation at Gln79(H11001). Given the structural integrity of this part of the catalytic apparatus in (GDP+AlF3)-A30P, the dramatically reduced intrinsic GTPase activity of A30P strongly argues that a perfect alignment of the nucleophilic water is not sufficient for the catalysis. Furthermore, because the complex structure preserves all required structural features predicted by an associative transition-state mechanism, yet no hydrolysis is detectable, such a mechanism is unlikely to function in A30P, and possibly neither in WT Rab5 and other small GTPases.

We have successfully solved the structure of a small GTPase complexed with GDP and AlF3. While similar transition state intermediate analog conformations have been observed in a number of crystal structures of (GDP+AlF3)-bound forms of small GTPases complexed with GAPs (PDB files 1grn, 1k5d, 1xc4, 1wq1, and 2ngr) and of trimeric GTPases (PDB files 1agr and 1tx4), the AlF3 moiety. The formation of such an intermediate is clearly vulnerable to a mutation at Gln79(H11001). Given the structural integrity of this part of the catalytic apparatus in (GDP+AlF3)-A30P, the dramatically reduced intrinsic GTPase activity of A30P strongly argues that a perfect alignment of the nucleophilic water is not sufficient for the catalysis. Furthermore, because the complex structure preserves all required structural features predicted by an associative transition-state mechanism, yet no hydrolysis is detectable, such a mechanism is unlikely to function in A30P, and possibly neither in WT Rab5 and other small GTPases.

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In conclusion, our study has emphasized the importance of P-loop in catalysis. Particularly, the amide group of residue 30 is likely to play a critical role in the dissociative-like transition state, because elimination of this group seems to be a major, if not the only, reason for the A30P mutant to lose GTP hydrolytic activity. Structurally, the (GDP+AlF3)-A30P complex resembles the reaction intermediate, although not necessarily in the sense of an associative transition state. It provides an unusual opportunity to visualize the transition state intermediate in a small GTPase in the absence of stabilization by a GAP partner.

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High Resolution Crystal Structures of Human Rab5a and Five Mutants with Substitutions in the Catalytically Important Phosphate-binding Loop
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