GTPase mechanism and function: new insights from systematic mutational analysis of the phosphate-binding loop residue Ala³⁰ of Rab5

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Structural and biochemical data indicate the importance of the phosphate-binding loop residues Gly¹² and Gly¹³ of Ras both in the GTP hydrolysis reaction and in biological activity, but these two residues are not conserved in other Ras-related GTPases. To gain a better understanding of this region in GTP hydrolysis and GTPase function, we used the Ras-related Rab5 GTPase as a model for comparison, and substituted the Ala³⁰ residue (the equivalent of Gly¹³ of Ras) with all the other 19 amino acids. The resulting mutants were analysed for GTP hydrolysis, GTP binding, GTP dissociation and biological activity. Only the substitution of alanine with proline reduced the GTPase activity by an order of magnitude. This effect is in sharp contrast with the observation that a proline substitution at the neighbouring position (Gly¹² of Ras) has little effect on the GTPase activity. Whereas most other substitutions showed either a small negative effect or no effect on the GTPase activity, the arginine substitution surprisingly stimulated the GTPase activity by 5-fold. Molecular modelling suggests that this built-in arginine mimics the catalytic arginine residues found in trimeric GTPases and GTPase-activating proteins in providing the positive charge to facilitate the GTP hydrolysis reaction. We investigated further the biological activity of the Rab5 mutants in relation to stimulating endocytosis. When expressed in cultured baby hamster kidney cells, both arginine and proline mutants, like wild-type Rab5, stimulated endocytosis. However, the arginine mutant was a more potent stimulator than the proline mutant (3-fold stimulation as against 1.7-fold). The tryptophan mutant, on the other hand, was completely deficient in activity in terms of the stimulation of endocytosis, demonstrating the importance of the phosphate-binding loop in Rab GTPase function.

Key words: endocytosis, GTP hydrolysis, Rab, Ras.

INTRODUCTION

GTPases are universal molecular 'switches' that regulate diverse cellular functions, such as signal transduction, cytoskeletal organization and intracellular trafficking. The Ras-related GTPase family represents a large number of 20–30 kDa monomeric GTPases, including Ras, Rho/Rac, Ran, ADP-ribosylation factor and Rab proteins [1–4]. They serve as molecular switches by cycling between GTP- and GDP-bound conformations. The GTPase activity associated with these proteins is important for the cycling process in terms of converting the GTP-bound conformation into the GDP-bound conformation. However, the catalytic mechanism and biological significance of GTP hydrolysis remain to be firmly established.

The Ras-related GTPases contain a conserved GTP-binding domain consisting of four motifs: GXX¹²XVGKS/T, DTAGQ⁶¹E, N¹¹⁶KXD and E¹⁴³XSA (where single-letter amino acid codes are shown, 'X' indicates 'any residue', and numbers denote relative positions in Ras). Structural and biochemical data indicate that the conserved residue Gln⁶¹ functions in GTP hydrolysis [5] by positioning the hydrolytic water molecule in alignment with the γ-phosphoryl group of GTP [6–8]. The transition-state alignment is stabilized further by interaction with a GTPase-activating protein (GAP) [9,10]. Another important catalytic group is the so-called 'arginine finger' [11]. The guanidinium group of an arginine residue is suggested to interact with a γ-phosphoryl oxygen and/or the β–γ bridge oxygen of GTP in the transition state, thereby enhancing the catalytic rate of GTP hydrolysis. This catalytic arginine residue is conserved in trimeric GTPases [12,13], but is not found in Ras-related monomeric GTPases. In the latter case, however, GAPs fulfil this role by providing the arginine residue in trans [9,10].

The phosphate-binding loop (i.e. the first GTP-binding motif) residues are also important in GTP hydrolysis. For example, mutations at the Gly¹² residue of Ras often reduce its GTPase activity, and increase its biological activity, in cellular transformation [6,14]. Structural data indicate that side chains at this position may interfere with the formation of the transition state [6]. Several mutations at the neighbouring residue, Gly¹³, also affect the GTPase activity [15,16], but the mechanism is not understood. Gly¹³ was identified as an important catalytic residue in the dissociative transition-state model proposed recently [7], where the backbone amide group of Gly¹³ is suggested to donate a hydrogen bond to the β–γ bridge oxygen of GTP, thereby stabilizing the transition state and catalysing GTP hydrolysis. This hydrogen bond can be observed in the crystal structures of Ras and Rab3A [8,17–19]. NMR spectroscopic studies of the structure of Ras in solution also suggest that the amide proton of Gly¹³ forms a hydrogen bond in both the Ras–guanosine 5′-[γ-thio]triphosphate (GTP[S]) and Ras–GDP complexes [20–22]. Given the unusual phosphate-binding loop conformation and dihedral angles of Gly¹³ (the φ and ψ values allow only glycine to be present at this position, according to the Ramachandran plot), it is intriguing that neither Gly¹² nor Gly¹³ is conserved in other Ras-related GTPases.

To investigate whether the position of Gly¹³ (backbone amide and side chain) is important in GTP hydrolysis and biological function of other GTPases, we used Rab5, which is a member of

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the Ras-related GTPase family and a regulator of endocytosis [23,24], as a model GTPase, and substituted Ala[30] of Rab5 (the equivalent of Gly[13] in Ras) with all the other 19 amino acids. An analysis of these mutants in terms of GTP hydrolysis, GTP binding, GTP dissociation and biological activity provides new insights into the GTPase mechanism and function.

MATERIALS AND METHODS

Mutagenesis, expression and purification of recombinant proteins

The Rab5 cDNA was cloned previously into the bacterial expression vector pGEX-3X [25]. This pGEX-3X/Rab5 plasmid was used as a template to generate a complete set of mutation substitutions (a total of 19 mutations) at the Ala[30] position of Rab5 by using a PCR-based mutagenesis strategy [26]. Specific mutations were confirmed by direct DNA sequencing of both strands of the entire Rab5 cDNA using an automatic DNA sequencer (ABI377), and no errors resulting from PCR were found in other regions. The mutant cDNAs were subcloned back into the *Bam*HI site of pGEX-3X, and the resulting constructs were transformed into *Escherichia coli* strain MC1061. Recombinant Rab5 and mutant proteins were expressed as glutathione S-transferase (GST) fusion proteins, and then affinity-purified by using the glutathione–Sepharose 4B resin (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.) [25].

GTPase assays

Two GTPase assays were employed. The first assay was modified from a procedure reported previously [25], and was used to measure the GTPase activity of GST fusion proteins (see Figure 2). In these experiments, the wild-type (wt) and mutant GST fusion proteins (1 μM) that were bound to the glutathione–Sepharose 4B resin were incubated with [α-^{32}P]GTP (0.1 μM) (Amersham Pharmacia Biotech) for 30 min at 25 °C in 50 μl of loading buffer [20 mM Tris/HCl (pH 8.0)/2 mM EDTA/1 mM dithiothreitol (DTT)]. Unbound [α-^{32}P]GTP was then removed by washing the resin twice with the same buffer. The GTP hydrolysis reaction was initiated by resuspending the resin in reaction buffer [20 mM Tris/HCl (pH 8.0)/5 mM MgCl$_2$/1 mM DTT], and incubating at 37 °C. Samples were taken at the indicated times and immediately solubilized in elution buffer [0.2% (w/v) SDS/5 mM EDTA/5 mM GDP/5 mM GTP] by heating at 65 °C for 2 min. The eluted GTP and GDP were separated by TLC on polyethyleneimine-cellulose sheets (J. T. Baker, Phillipsburg, NJ, U.S.A.) with 0.75 M KH$_2$PO$_4$, pH 3.5, as the developing solvent. The radioactive GTP and GDP spots were revealed by autoradiography, and quantified by using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA, U.S.A.). The specific radioactivity of GDP was corrected as two-thirds that of GTP.

The second assay was used to measure the GTPase activity of free proteins (see Figure 3B). Free wt and mutant proteins were released from the GST fusion proteins by digestion with the Xa protease, and purified as described previously [25]. Each protein (1 μM) was loaded with [γ-^{32}P]GTP (0.1 μM; Amersham Pharmacia Biotech) for 10 min at 25 °C in 100 μl of the loading buffer. GTP hydrolysis was initiated by adding MgCl$_2$ to a final concentration of 5 mM, followed by incubation at 37 °C. Samples were taken at the indicated times, and the extent of GTP hydrolysis was determined by the release of [^{32}P]P$_i$, which was extracted into the isobutanol/toluene phase and quantified by liquid scintillation counting [27].

GTP-binding assay

The wt and mutant proteins (1 μM) were incubated with [^{35}S]GTP[S] (1 μM; Amersham Pharmacia Biotech) under the same loading conditions as those described for the GTPase assay above. At the indicated times, 10 μl of the GTP binding reaction was rapidly diluted into 1 ml of ice-cold stop buffer [20 mM Tris/HCl (pH 8.0)/100 mM NaCl/5 mM MgCl$_2$/1 mM DTT] and filtered through a HA-type nitrocellulose membrane (0.45 μm; Millipore, Bedford, MA, U.S.A.) using a vacuum manifold. After washing with 2 ml of the same buffer, the membrane was dried and the radioactivity retained on the membrane was quantified by liquid scintillation counting.

GTP dissociation assay

The wt and mutant proteins were loaded with [^{35}S]GTP[S] for 1 h, as described above for the GTP-binding assay. [^{35}S]GTP[S] dissociation was initiated by adding an equal volume of dissociation buffer [40 mM Tris/HCl (pH 8.0)/200 mM NaCl/ 10 mM MgCl$_2$/2 mM DTT/1 mM GTP]. The reaction was performed at 37 °C. Aliquots of the reaction mixture (20 μl) were removed at the indicated times, diluted in the stop buffer, filtered, and the radioactivity retained on the membrane was quantified by liquid scintillation counting, as described above for the GTP-binding assay.

Horseradish peroxidase (HRP) uptake assay

Confluent baby hamster kidney-21 (BHK-21) cell monolayers in 35-mm dishes ($\approx 5 \times 10^5$ cells/dish) were infected with either the vector virus as a negative control or the recombinant viruses capable of expressing wt and mutant Rab5 proteins. The procedures for generating the recombinant viruses and for infecting BHK cells were as described previously [24]. Cells were incubated in a 37 °C tissue-culture incubator. At 4 h post-infection, cells were washed once with serum-free α-minimal essential medium, followed by addition of 1 ml of the same medium containing 5 mg/ml HRP (Sigma, St. Louis, MO, U.S.A.) and 1% (w/v) BSA to each dish. The uptake of HRP was conducted at 37 °C for 30 min, and then stopped by washing the cell monolayers three times with ice-cold PBS containing 1% (w/v) BSA. After the final wash, cells were scraped into 2 ml of PBS and pelleted at 800 **g** for 3 min in a Beckman GPR centrifuge. The cell pellet was washed one more time by resuspension in 2 ml of PBS, followed by re-centrifugation. The final cell pellet was lysed in 500 μl of PBS containing 0.1% (v/v) Triton X-100 and the lysate was assayed for HRP activity. The enzyme assay was performed in a 96-well microplate (Costar Co., Acton, MA, U.S.A.) using the 2,2′-azidobis-(3-ethylbenzothiazoline-6-sulphonic acid), or ABTS, peroxidase substrate (Kirkegaard & Perry Inc., Gaithersburg, MD, U.S.A.). The reaction was started by adding 100 μl of the substrate to 5 μl of the lysate. The reaction was carried out at room temperature for 5 min, and stopped by adding 100 μl of 1% (w/v) SDS. The green–blue product was quantified by measuring A_{405} in a microplate reader. The protein content was quantified by using the Bio-Rad protein assay, according to the manufacturer's instructions.

Molecular modelling

A structural model of Rab5 was constructed by homology modelling, as described by the method of Greer [28], using the

software MAIN [29]. The template structure was Rab3A [Protein Data Bank (PDB) accession no. 3RAB] [17]. Any close contacts were resolved manually before energy minimization. Substitution mutations were then modelled into the Ala[30] position of the Rab5 model, and the equivalent Ser[32] position of the Rab3A structure. For comparison of the Arg[30] substitution in Rab5 with the catalytic residue Arg[789] in the Ras–GAP complex, only the side chain of Arg[30] was modelled into the equivalent Gly[13] position of the structure of Ras in the Ras–GAP complex (PDB accession no. 1WQ1) [10].

RESULTS

The resultant 19 mutants, in addition to wt Rab5, were expressed in *E. coli* as GST fusion proteins, and were affinity-purified via glutathione–Sepharose resin (Figure 1). To determine GTPase activity, the GST fusion proteins bound to the resin were loaded with [α-^{32}P]GTP, followed by resuspension in the reaction buffer and incubation at 37 °C. Samples were taken after 0, 10 and 20 min and analysed for the nucleotide products by TLC on polyethyleneimine-cellulose sheets (Figure 2). The relative amounts of GTP and GDP were quantified by using a PhosphorImager (Figure 2). Most substitutions (16 out of 19) at the Ala[30] position showed either no effect or a small negative effect ($< 50\%$) on the Rab5 GTPase activity (Figure 2A). However, these negative effects were reproducible in independent experiments with different protein preparations. For example, the aspartate substitution consistently resulted in a 50% decrease in GTPase activity. Substitutions with larger aliphatic side chains (leucine, valine, isoleucine) also led to decreased GTP hydrolysis, as well as GTP binding. Interestingly, two substitutions (threonine and arginine) showed a stimulatory effect on the GTPase activity (Figure 2B).

The most striking effect was seen with the proline substitution, which almost completely abolished the GTPase activity (Figure 2C). The GTPase activity of this Pro[30] mutant, as well as of the Arg[30] and Trp[30] mutants, was quantified further by a second GTPase assay that measured the GTPase activity of the free protein, rather than the GST fusion protein (Figure 3). The free protein was generated by cleaving the GST fusion protein with factor Xa (Figure 3A). The protein was then loaded with [γ-^{32}P]GTP, and GTP hydrolysis was determined by the release of [^{32}P]P$_i$. In this assay, the Pro[30] mutant showed a 12-fold lower GTPase activity in comparison with the wt protein (Figure 3B). The GTP-binding rate under the same loading conditions was determined by using a rapid-filtration method [30]. The Pro[30]

mutant showed rapid binding to [^{35}S]GTP[S] (a poorly hydrolysable analogue of GTP), and the initial binding rate was about 2-fold higher than that of wt protein (Figure 3C). Because purified Rab proteins often contain tightly bound nucleotides [31,32], the difference in binding rate might reflect a difference in nucleotide dissociation rate. This contention was confirmed by direct measurement of the GTP dissociation rate (Figure 3D). In this case, the wt and mutant proteins loaded with [^{35}S]GTP[S] were chased with a 1000-fold excess of unlabelled GTP. During the time course of the experiment, the amount of [^{35}S]GTP[S] that remained bound to the protein was determined by the rapid-filtration method. In comparison with the wt protein, the Pro[30] mutant showed a 3-fold increase in the GTP dissociation rate (Figure 3D). The other two mutants examined in these assays, i.e. the Arg[30] and Trp[30] mutants, were the same as the wt protein in terms of GTP binding and dissociation rates (Figures 3C and 3D). The ratio of GTP hydrolysis rate to GTP dissociation rate for Rab5 is high (0.0034 s^{-1} as against 0.0042 min^{-1}) [31]. The 3-fold increase in the GTP dissociation rate might contribute to, but cannot account for, the 12-fold decrease in the GTPase activity of the Pro[30] mutant. The data can be explained most simply by decreases in both GTP affinity and GTP hydrolysis.

In contrast with the Pro[30] mutant, the Arg[30] mutant showed a 5-fold stimulation of the GTPase activity (Figure 3B). This interesting observation that different mutations at the same position have opposite effects on the GTPase activity prompted us to investigate further the mechanism by molecular modelling. The proline and arginine, as well as other substitution mutations were modelled into the available Ras and Rab3A structures [8,17–19] at the equivalent Gly[13] (Ras) and Ser[32] (Rab3A) positions. Energy minimization suggested that the proline side chain adopts a different orientation from that of other side chains, which normally point towards the exterior of the molecule (inferred from the C$_\beta$ position). The proline side chain points inward and may cause steric hindrance of the bound GTP. In addition, only the proline substitution may change the backbone structure.

The specific stimulatory effect of the arginine substitution on Rab5 GTPase activity (the lysine substitution had no such effect) was qualitatively similar to the catalytic function of the conserved arginine residues in trimeric GTPases and GAPs [11,33,34]. Indeed, when the arginine substitution was modelled into the Gly[13] position of Ras in the Ras–GAP complex structure [10], energy minimization and simple rotation of the side chain directed its guanidinium group to a similar position as that of the 'arginine finger' (Arg[789]) inserted by the GAP, with no steric
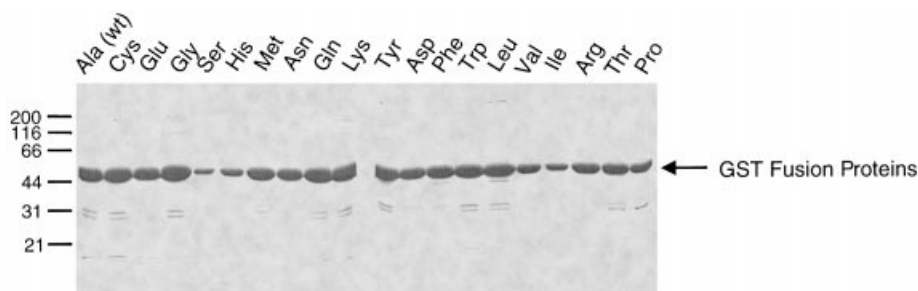

Figure 1   Expression and purification of Rab5 and its mutants as GST fusion proteins

Aliquots (2 μl) of the resin with bound GST fusion protein were solubilized with 20 μl of Laemmli sample buffer, followed by SDS/PAGE (10% acrylamide gels). Coomassie-Blue-stained gels are shown containing the purified GST fusion proteins. Molecular mass standards (in kDa) are indicated on the left. The three-letter amino acid codes are used to denote the substitutions at the Ala[30] (wt) residue.
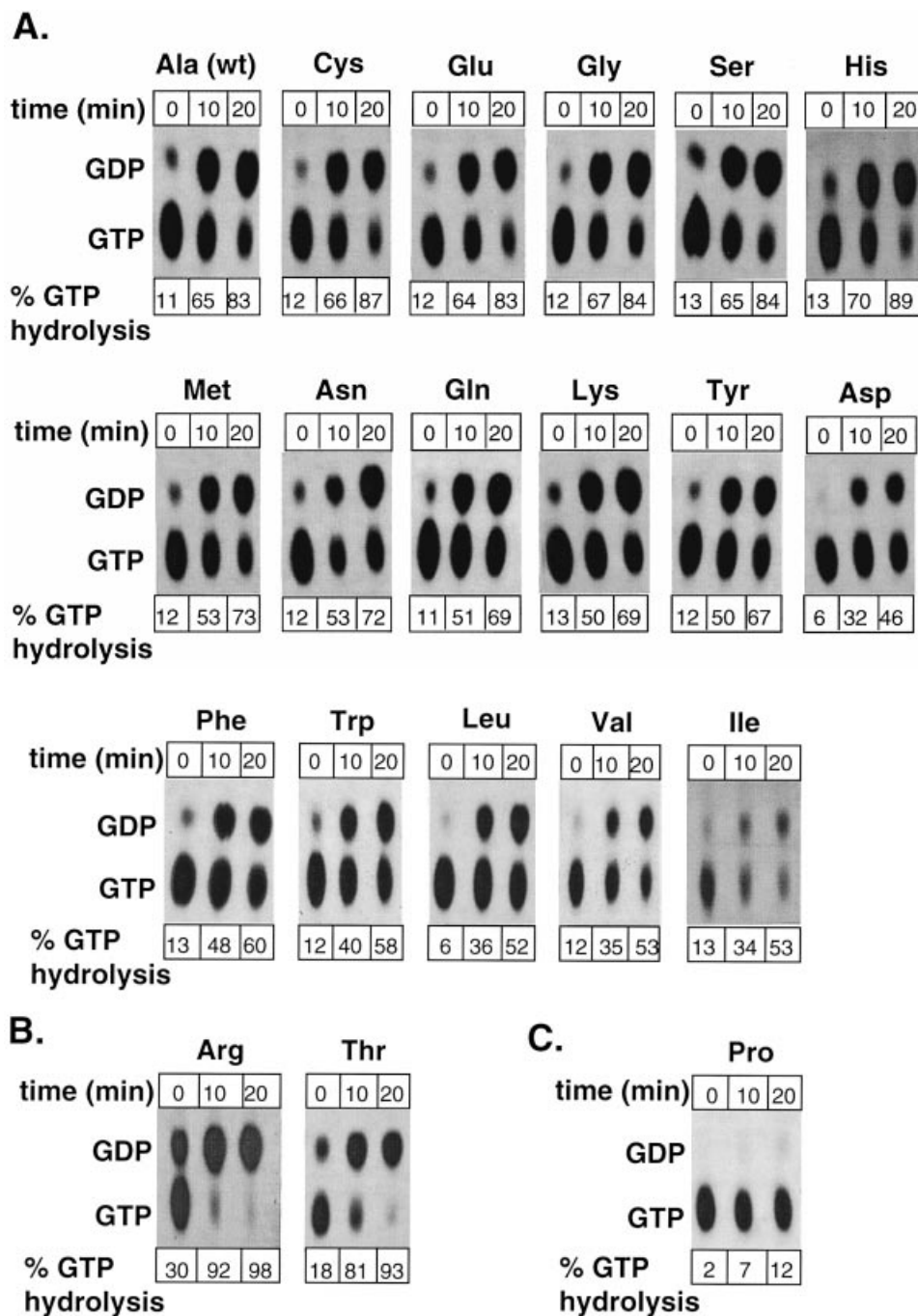
Figure 2   Single-step GTP hydrolysis by the GST fusion proteins of Rab5 and its mutants

Autoradiographs of the GTP hydrolysis products analysed by TLC at the indicated times are shown. The result was reproducible in two independent experiments, and the difference in the percentage of GTP hydrolysis was within 2%. (**A**) The majority of the mutants (16 out of 19) show either the same, or slightly decreased, GTPase activity in comparison with the wt protein. (**B**) The Thr[30] and Arg[30] mutants show increased GTPase activity. (**C**) The Pro[30] mutant shows drastically reduced GTPase activity.

hindrance (Figure 4). This structural model supports the contention that the Rab5 Arg[30] substitution stimulates GTPase activity by mimicking the catalytic function of the 'arginine finger' of the GAP.

We then determined the biological activity of the Arg[30], Pro[30] and Trp[30] mutants. The biological function of Rab5 is to regulate endocytosis by promoting early endosome fusion [31,35,36], and

possibly vesicle budding at the plasma membrane [37]. As a result, overexpression of Rab5 in cultured cells increased the endocytic activity by 2-fold, as determined by HRP uptake [23,24]. This stimulation of HRP uptake was used as a biological measure of Rab5 activity. The wt and mutant Rab5 proteins were expressed in BHK cells via a Sindbis virus expression vector, which ensured that almost all the cells expressed the
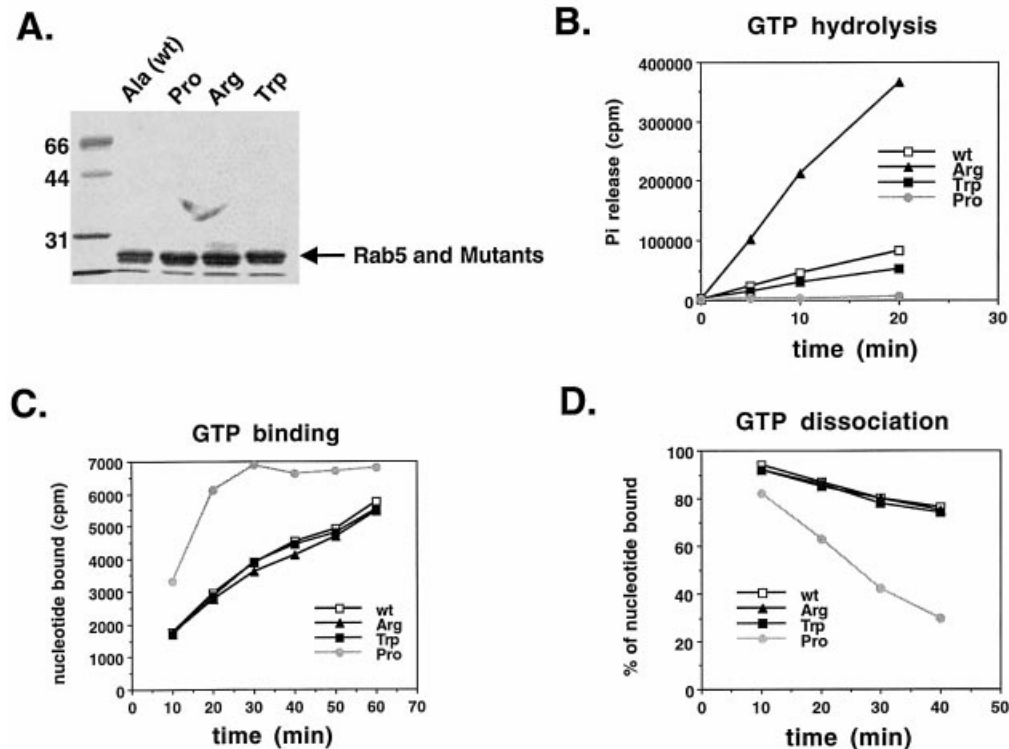
Figure 3   GTPase activities, GTP binding rates and GTP dissociation rates of free Rab5 and the mutants featuring Arg[30], Trp[30] and Pro[30] substitutions

Free wt and mutant proteins were released from the GST fusion proteins by digestion with the Xa protease, and were purified as described previously [25]. (**A**) A Coomassie-Blue-stained gel containing the purified proteins (5 μl loaded in each lane) is shown. Molecular mass standards (in kDa) are indicated on the left. (**B**) GTPase activity. The wt and mutant proteins were loaded with [γ-^{32}P]GTP, and GTP hydrolysis was determined by the release of [^{32}P]P$_i$. The results are shown as the means for duplicate samples, and were reproducible in four independent experiments. (**C**) GTP binding. The wt and mutant proteins were incubated with [^{35}S]GTP[S] for the indicated times, and the amount of bound [^{35}S]GTP[S] was determined by a rapid-filtration method modified from that described previously [30]. The results are shown as the means for duplicate samples, and were reproducible in three independent experiments. (**D**) GTP dissociation. The wt and mutant proteins were loaded with [^{35}S]GTP[S] for 1 h, as described in the Materials and methods section, followed by measurement of [^{35}S]GTP[S] dissociation in the presence of a 1000-fold excess of unlabelled GTP. The results are shown as the means for duplicate samples, and were reproducible in four independent experiments.

protein. When the expression level was about 10-fold greater than the endogenous level (4 h post-infection; Figure 5A), the endocytic activity of the cells was determined by uptake of HRP over 30 min (Figure 5B). Expression of wt Rab5 stimulated HRP uptake by about 2-fold. The Pro[30] mutant also stimulated HRP uptake, although it was less active than the wt (Figure 5B). Interestingly, the Arg[30] mutant showed the highest activity, and stimulated HRP uptake by 3-fold (Figure 5B). The Trp[30] mutant, on the other hand, completely lost its activity in terms of stimulating HRP uptake (Figure 5B).

DISCUSSION

We have investigated the biochemical and biological properties of Rab5 mutants that contain a complete set of 19 substitutions at the phosphate-binding loop residue, Ala[30]. The equivalent residue in Ras is Gly[13]. Several mutations at this position lead to altered Ras GTPase activity and biological activity [15,16], but the mechanism is not understood. In addition, Gly[13] has unusual dihedral angles that allow only glycine at this position, according to the Ramachandran plot. Therefore it is interesting to note that this glycine residue is not conserved in other Ras-related GTPases. Recent structural studies on Rab3A indicate that its phosphate-binding-loop conformation is essentially the same as that of Ras [17,18], even though it has Ser at the equivalent position (Gly[13]). In the present study, we have used Rab5 as a

model to understand how various side chains and backbone structures at the Ala[30] position, which is equivalent to Gly[13] of Ras, can affect GTPase activity and GTPase function. Our findings are discussed as follows.

Many substitution mutations at the Ala[30] residue reduce Rab5 GTPase activity, although the effect is usually less than 50 %. The most striking effect is seen with the proline substitution, which decreases the Rab5 GTPase activity by an order of magnitude. One explanation for the loss of GTPase activity could be that the Pro[30] mutant is misfolded and denatured. This is unlikely, however, for the following reasons. First, the purified recombinant Pro[30] mutant retains the ability to bind GTP, suggesting that it is properly folded. The bound GTP, however, is hardly hydrolysed, suggesting a defect in catalysis (Figure 2C). Secondly, the Pro[30] mutant is biologically active in stimulating endocytosis when expressed in cultured cells (Figure 5B), indicating that it is also properly folded *in vivo* in intact cells.

Modelling the proline and other substitutions into the available Ras and Rab3A structures [8,17–19] suggests that the proline side chain adopts a different orientation from other side chains, which normally point towards the exterior of the protein. The proline side chain points inwardly, and might cause steric hindrance with GTP.

The unusual backbone structure of proline might also contribute to the loss of GTPase activity. Proline differs from other amino acids in that proline cannot form the backbone amide
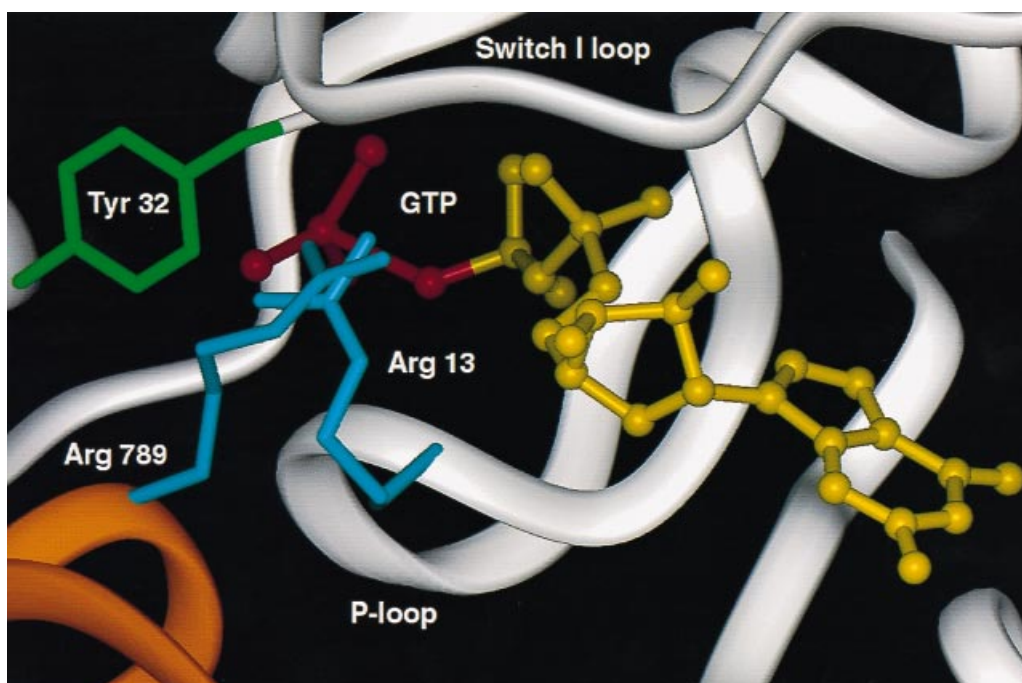
Figure 4   Molecular modelling of an Arg[13] substitution (equivalent of an Arg[30] substitution in Rab5) into the Ras structure of the Ras–GAP complex

The Ras–GAP complex structure (PDB 1WQ1) was modelled by using the software MAIN. Shown is the approximate superimposition of the arginine residues (in blue) from the GAP 'arginine finger' loop and the Ras phosphate-binding loop (P-loop). The backbones of Ras and GAP in the complex are represented by white and orange ribbons respectively. The bulky side chain of Tyr[32] in the Switch I loop, a potential steric barrier during the modelling, is shown in green. This model shows that the Arg[13] substitution can occupy almost the same topological space as that of the catalytic Arg[789] of GAP with respect to the γ-phosphate (shown in red) of the bound GTP (in yellow), with no steric clashes.

linkage in a polypeptide. Instead, it forms an imide linkage that cannot contribute a hydrogen bond. This can be disruptive for GTP hydrolysis in the dissociative transition-state model [7], where the backbone amide is suggested to donate a hydrogen bond to the β–γ bridge oxygen of GTP, thereby stabilizing the transition state and catalysing the GTP-hydrolysis reaction. We notice, however, that the proline substitution does not completely abolish the GTPase activity, suggesting that this backbone amide cannot be the sole catalytic group for the GTPase activity.

Another important finding is that two substitutions (threonine and arginine) actually stimulate the GTPase activity (Figures 2B and 3B). The equivalent threonine substitution in Ras has a similar 2-fold stimulatory effect [15]. The 5-fold stimulation by the arginine substitution, however, is an interesting novel finding and provides further insight into the GTPase mechanism. The arginine effect is specific in the sense that the conservative lysine substitution has no such effect on the GTPase activity (Figure 2A). A conserved arginine residue can be found in the trimeric GTPases, e.g. Arg[174] of transducin, and is responsible for their higher GTPase activity relative to Ras [33,34]. An equivalent arginine residue is absent in Ras and Ras-related monomeric GTPases. However, these GTPases can be stimulated by GAPs [38,39] that donate a catalytic arginine *in trans* to the active site [9,10]. These catalytic arginine residues of trimeric GTPases and GAPs stimulate the GTPase activity by 100-fold [33,34] and 1000-fold [11] respectively, by stabilizing a γ-phosphoryl oxygen and/or the β–γ bridge oxygen in the transition state [9,10,12,13]. Modelling an arginine into the Gly[13] position of the Ras structure in the Ras–GAP complex [10] (Figure 4) has revealed that its guanidinium group can occupy a similar position to that of the catalytic Arg[789] of the GAP, with no steric clashes. This structural

model suggests that the built-in Arg[30] of Rab5 may play a similar catalytic role in GTP hydrolysis to that of the arginine residues found in trimeric GTPases and GAPs. The quantitative difference in the catalytic power of these arginine residues might result from different structural contexts. The Arg[789] of GAP in the Ras–GAP complex is held in position by multiple interactions with Thr[785], Arg[903] of GAP, and Gln[61] of Ras [10]. These stabilizing interactions are not present to support the Rab5 Arg[30] substitution, which could explain its relatively low catalytic power.

In the crystal structure of Rab3A [17,18], the side chain orientation of the equivalent Ser[32] points away from the active site, as inferred from the C_β position. This orientation would lead to the prediction that side chains at this position should not affect GTPase activity. However, this prediction cannot fully explain our data. Many substitutions at this position decrease the GTPase activity to different degrees (Figure 2A). It is important to note the opposite effects of the charged amino acids. The negatively charged Asp substitution decreases the GTPase activity by about 50%, whereas the positively charged arginine substitution leads to a 5-fold stimulation of the GTPase activity (Figures 2 and 3B). Taken together, the data suggest that the side chains are pointing towards the active site in order to exert these positive and negative effects on GTPase activity, and this side chain conformation in solution is not easily predicted from the crystal structure.

The comprehensive mutational analysis of the Ala[30] residue of Rab5 has general implications for other Ras-related GTPases. The equivalent residue in Ras is Gly[13], and proline and arginine substitutions at this position are predicted to have the same effects on Ras as on Rab5, because of the conserved GTP-binding domain and hydrolysis mechanism. In the case of Ras,
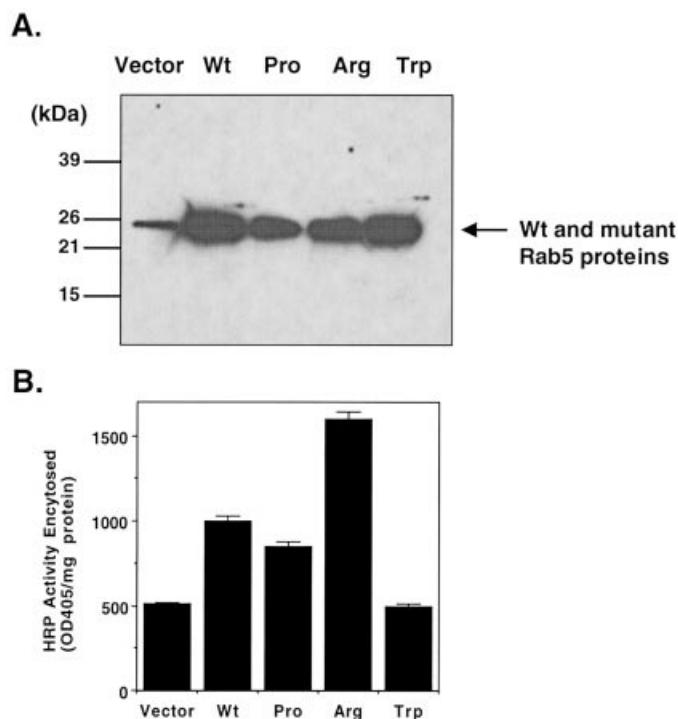
A.

B.

Figure 5   Effect of Pro[30], Arg[30] and Trp[30] substitutions on the biological activity of Rab5 in stimulating endocytosis

Wt and mutant Rab5 proteins were expressed in confluent BHK-21 cell monolayers via a Sindbis virus vector. At 4 h post-infection, one set of cells were lysed in 1 % (w/v) SDS. The proteins in the lysates were separated by SDS/PAGE, followed by immunoblot analysis to identify the Rab5 proteins with an anti-Rab5 monoclonal antibody (Transduction Laboratories). (**A**) The results were quantified by using a PhosphorImager. A second set of cells were used to determine the endocytic activity by a 30-min uptake of a fluid-phase endocytic marker HRP. The amount of HRP endocytosed was determined as described in the Materials and methods section, and the results are shown in the histogram (**B**). Each result is the mean for triplicate samples, and the calculated standard deviations are also shown (**B**). The results have been reproduced in three independent experiments.

the neighbouring Gly[12] has been studied in more detail, and both proline and arginine substitutions have been introduced at this position [14]. However, the Pro[12] substitution has little effect on the GTPase activity [15,38,40], whereas the Arg[12] substitution substantially reduces the GTPase activity [6]. These effects are in sharp contrast with those observed when the proline and arginine substitutions are created at the immediately adjacent residue (i.e. Ala[30] of Rab5) (Figures 2 and 3B), reflecting a remarkable precision in the conformation of the backbone amides and side chains at these two positions of the phosphate-binding loop. Structural studies of the newly identified Pro[30] and Arg[30] mutants by X-ray diffraction and NMR spectroscopic techniques should yield further information on the GTPase mechanism.

The availability of these Rab5 mutants with either decreased or increased GTP hydrolysis rates has allowed us to investigate further the relationship between GTPase activity and biological activity. The biological function of Rab5 is to promote early endosome fusion [31,35,36]. A recent study also suggests the involvement of Rab5 in vesicle budding from the plasma membrane [37]. As a result, overexpression of Rab5 in cultured cells stimulates both receptor-mediated and fluid-phase endocytosis [23,24]. In the present study, we have shown that the GTP-hydrolysis-defective Pro[30] mutant is biologically active in stimu-

lating endocytosis, supporting the notion that GTP-bound Rab5 is the active form in promoting endosome fusion [41,42] and, possibly, vesicle budding. Interestingly, the Arg[30] mutant, which shows a 5-fold increase in the GTP-hydrolysis rate, is almost twice as active as the Pro[30] mutant in terms of stimulating endocytosis (Figure 5B), demonstrating the importance of GTP hydrolysis in the function of newly synthesized Rab5 molecules. Although GTP hydrolysis is not required for the functioning of Rab5 in endosome fusion, it is important in converting GTP-bound Rab5 into the GDP-bound form, so that the Rab5 protein can interact more efficiently with the Rab geranylgeranyl-transferase [43,44], and be escorted/targeted to the early endosomes. This more efficient membrane targeting might contribute to the high biological activity of the Arg[30] mutant in stimulating endocytosis.

The finding that the Trp[30] mutant completely loses its activity in stimulating endocytosis is rather unexpected, since the tryptophan substitution has only a small effect on the biochemical properties of Rab5 in comparison with the proline and arginine substitutions. In cells, however, newly synthesized Rab5 needs to interact with other protein factors in order to exert its stimulatory effect on endocytosis. Although not all of these factors have been identified and characterized, recent studies [3,45] support the notion that they include a Rab geranylgeranyltransferase that prenylates and escorts Rab5 to the early endosomes, a GDP-dissociation-inhibitor (GDI)-displacement factor that displaces the escort protein, a guanine-nucleotide-exchange factor that promotes GTP loading, and an effector (e.g. early-endosomal antigen 1) that binds to GTP-bound Rab5 and promotes endosome fusion. Our results indicate that the tryptophan substitution, with its bulky aliphatic side chain, may disrupt one or more of these essential protein interactions, leading to the loss of biological activity.

REFERENCES

1   Bourne, H. R., Sanders, D. A. and McCormick, F. (1990) Nature (London) **348**, 125–132

2   Moss, J. and Vaughan, M. (1998) J. Biol. Chem. **273**, 21431–21434

3   Novick, P. and Zerial, M. (1997) Curr. Opin. Cell Biol. **9**, 496–504

4   Pfeffer, S. R. (1994) Curr. Opin. Cell Biol. **6**, 522–526

5   Der, C. J., Finkel, T. and Cooper, G. M. (1986) Cell **44**, 167–176

6   Krengel, U., Schlichting, L., Scherer, A., Schumann, R., French, M., John, J., Kabsch, W., Pai, E. F. and Wittinghofer, A. (1990) Cell **62**, 539–548

7   Maegley, K. A., Admiraal, S. J. and Herschlag, D. (1996) Proc. Natl. Acad. Sci. U.S.A. **93**, 8160–8166

8   Privé, G. G., Milburn, M. V., Tong, L., de Vos, A. M., Yamaizumi, Z., Nishimura, S. and Kim, S.-H. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 3649–3653

9   Rittinger, K., Walker, P. A., Eccleston, J. F., Smerdon, S. J. and Gamblin, S. J. (1997) Nature (London) **389**, 758–762

10   Scheffzek, K., Ahmadian, M. R., Kabsch, W., Wiesmuller, L., Lautwein, A., Schmitz, F. and Wittinghofer, A. (1997) Science **277**, 333–338

11   Ahmadian, M. R., Stege, P., Scheffzek, K. and Wittinghofer, A. (1997) Nat. Struct. Biol. **4**, 686–689

12   Coleman, D. E., Berghuis, A. M., Lee, E., Linder, M. E., Gilman, A. G. and Sprang, S. R. (1994) Science **265**, 1405–1412

13   Sondek, J., Lambright, D. G., Noel, J. P., Hamm, H. E. and Sigler, P. B. (1994) Nature (London) **372**, 276–279

14   Seeburg, P. H., Colby, W. W., Capon, D. J., Goedel, D. V. and Levinson, A. D. (1984) Nature (London) **312**, 71–75

15   Chung, H.-h., Benson, D. R. and Schultz, P. G. (1993) Science **259**, 806–809

16   Lowy, D. R. and Willumsen, B. M. (1993) Annu. Rev. Biochem. **62**, 851–891

17   Dumas, J. J., Zhu, Z., Connolly, J. L. and Lambright, D. G. (1999) Structure **7**, 413–423

18   Ostermeier, C. and Brunger, A. T. (1999) Cell **96**, 363–374

19 Pai, E. F., Krengel, U., Petsko, G. A., Goody, R. S., Kabsch, W. and Wittinghofer, A. (1990) EMBO J. **9**, 2351–2359

20 Campbell-Burk, S., Papastavros, M. Z., McCormick, F. and Redfield, A. G. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 817–820

21 Miller, A. F., Papastavros, M. Z. and Redfield, A. G. (1992) Biochemistry **31**, 10208–10216

22 Redfield, A. G. and Papastavros, M. Z. (1990) Biochemistry **29**, 3509–3514

23 Bucci, C., Parton, R. G., Mather, I. M., Stunnenberg, H., Simons, K., Hoflack, B. and Zerial, M. (1992) Cell **70**, 715–728

24 Li, G. and Stahl, P. D. (1993) J. Biol. Chem. **268**, 24475–24480

25 Liu, K. and Li, G. (1998) J. Biol. Chem. **273**, 10087–10090

26 Chen, B. and Przybyla, A. E. (1994) BioTechniques **17**, 657–659

27 Bollag, G. and McCormick, F. (1995) Methods Enzymol. **255**, 161–170

28 Greer, J. (1990) Proteins Struct. Funct. Genet. **7**, 312–334

29 Turk, D. (1992) Ph.D. Thesis, Technische Universitaet, Muenchen, Germany

30 Kabcenell, A. K., Goud, B., Northup, J. and Novick, P. J. (1990) J. Biol. Chem. **265**, 9366–9372

31 Hoffenberg, S., Sanford, J. C., Liu, S., Daniel, D. S., Tuvin, M., Knoll, B. J., Wessling-Resnick, M. and Dickey, B. F. (1995) J. Biol. Chem. **270**, 5048–5056

32 Shapiro, A. D., Riederer, M. A. and Pfeffer, S. R. (1993) J. Biol. Chem. **268**, 6925–6931

33 Freissmuth, M. and Gilman, A. G. (1989) J. Biol. Chem. **264**, 21907–21914

34 Landis, C. A., Masters, S. B., Spada, A., Pace, A. M., Bourne, H. R. and Vallar, L. (1989) Nature (London) **340**, 692–696

35 Gorvel, J.-P., Chavrier, P., Zerial, M. and Gruenberg, J. (1991) Cell **64**, 915–925

36 Li, G., Barbieri, M. A., Colombo, M. I. and Stahl, P. D. (1994) J. Biol. Chem. **269**, 14631–14635

37 McLauchlan, H., Newell, J., Morrice, N., Osborne, A., West, M. and Smythe, E. (1998) Curr. Biol. **8**, 34–45

38 Gibbs, J. B., Schaber, M. D., Allard, W. J., Sigal, I. S. and Scolnick, E. M. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 5026–5030

39 Trahey, M. and McCormick, F. (1987) Science **238**, 542–545

40 Eccleston, J. F., Moore, K. J., Morgan, L., Skinner, R. H. and Lowe, P. N. (1993) J. Biol. Chem. **268**, 27012–27019

41 Barbieri, M. A., Li, G., Colombo, M. I. and Stahl, P. D. (1994) J. Biol. Chem. **269**, 18720–18722

42 Stenmark, H., Parton, R. G., Steele-Mortimer, O., Lutcke, A., Gruenberg, J. and Zerial, M. (1994) EMBO J. **13**, 1287–1296

43 Sanford, J. C., Pan, Y. and Wessling-Resnick, M. (1993) J. Biol. Chem. **268**, 23773–23776

44 Seabra, M. C. (1996) J. Biol. Chem. **271**, 14398–14404

45 Schimmöller, F., Simon, I. and Pfeffer, S. R. (1998) J. Biol. Chem. **273**, 22161–22164