Phosphate-binding loop and Rab GTPase function: mutations at Ser²⁹ and Ala³⁰ of Rab5 lead to loss-of-function as well as gain-of-function phenotype

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Ras-like GTPases contain a structurally conserved GTP-binding domain. An important element of the GTP-binding domain is the phosphate-binding loop, which contains two Gly residues (Gly¹² and Gly¹³) in Ras. Because the two Gly residues are crucial for normal Ras function, it is intriguing that they are not conserved in other Ras-like GTPases, including the Rab GTPases; for example, the equivalent residues in Rab5 are Ser²⁹ and Ala³⁰. The present study builds on earlier biochemical characterizations of the Rab5 mutants containing substitutions at Ala³⁰ and provides a comprehensive analysis of the structure–function relationship of the Rab5 phosphate-binding loop. We have generated 19 new mutants containing amino acid substitutions at Ser²⁹ and determined whether these Ser²⁹ mutants, as well as the Ala³⁰ mutants, remain able to stimulate the endocytosis of horseradish peroxidase in baby hamster kidney cells. A total of 11 mutants lose the activity of stimulating endocytosis.

Of these 11 mutants, 9 are defective in membrane association. In contrast, 27 mutants remain able to stimulate endocytosis. Five of them induce a novel cellular phenotype: cell rounding and detachment from culture dishes. They also induce super-large early endosomes such as the constitutively activated Rab5:Q79L mutant. Biochemical results suggest that the constitutive activation of Rab5 requires an increased nucleotide exchange rate and/or decreased GTPase activity. This study establishes functional significance for the phosphate-binding loop of Rab5 and shows that mutations in this region lead to either a loss-of-function or a gain-of-function phenotype, indicating a structure–function relationship distinct from that of Ras.

Key words: endocytosis, GTP binding, GTP hydrolysis, Ras, structure–function relationship.

INTRODUCTION

The Ras-related GTPase superfamily contains a large number of 20–30 kDa proteins, including the Ras, Rho/Rac, Ran, ADP-ribosylation factor ('ARF') and Rab proteins [1–4]. They cycle between GTP-bound and GDP-bound conformations and thereby regulate diverse cellular functions such as signal transduction, cytoskeleton organization and intracellular trafficking. The structural basis for the GTP/GDP cycle resides in a conserved GTP-binding domain, which contains four motifs: GXX¹²XVGKS/T, DTAGQ⁶¹E, N¹¹⁶KXD and E¹⁴³XSA (single-letter amino acid codes; superscript numbers denote amino acid positions in Ras) [5]. The first motif forms the phosphate-binding loop structure and interacts with the phospho groups of GTP [6,7]. Of interest are the Gly¹² and Gly¹³ residues in Ras. Replacement of Gly¹² with any other amino acid except proline decreases intrinsic and GTPase-activating protein (GAP)-catalysed GTPase activity [8], leading to the constitutive activation of Ras and cell transformation [9]. Some mutations at Gly¹³ have a similar effect on Ras function [10,11]. In this regard, oncogenic Ras mutations in human tumours are often replacements of the Gly residues [5,12]. The structural explanation for the requirement of Gly¹² is that side chains at this position would interfere with the formation of the transition state and consequently decrease the GTPase activity [6–8,10].

Given such an important role, it is unclear why neither Gly residue is conserved in other Ras-related GTPases, including the Rab GTPases that regulate intracellular trafficking. Rab5 in particular regulates early endosome fusion during endocytosis [13–18]; in it Ser²⁹ and Ala³⁰ substitute for the two Gly residues

in the phosphate-binding loop [19]. This raises interesting questions about the structure–function relationship of the phosphate-binding loop and Rab function. Why does a wild-type (wt) Rab contain phosphate-binding loop side chains that can constitutively activate the Ras function? Would mutations in the phosphate-binding loop lead to constitutive activation of the Rab function or other functional phenotypes? Would mutations in the phosphate-binding loop have the same biochemical effect on the Rab GTPase activity as on the Ras GTPase activity?

To address the role of the phosphate-binding loop in Rab function, we have generated a series of Rab5 mutants with amino acid substitutions at Ser²⁹ and Ala³⁰ and determined their functional and biochemical properties. In contrast with the effect on Ras, many of the mutations abolish Rab5 function in endocytosis, whereas some mutations (only one at Ser²⁹) lead to the constitutive activation of the Rab5 function. We show that overexpression of the constitutively activated Rab5 mutants induces gross changes in cell morphology, resulting in the detachment of cells from culture dishes. Further experiments on the membrane association and biochemical properties of these Rab5 mutants have shed light on the mechanisms.

MATERIALS AND METHODS

Generation and expression of the phosphate-binding-loop mutants

The pGEX-3X/Rab5 plasmid [20] was used as a template to replace Ser²⁹ and Ala³⁰ of Rab5 with all of the other 19 amino acids by using a PCR-based mutagenesis strategy [21,22]. The resulting 38 mutant cDNA species were subcloned back into the *Bam*HI site of pGEX-3X, and the desired mutations were

Abbreviations used: BHK, baby hamster kidney; GAP, GTPase-activating protein; GST, glutathione S-transferase; HRP, horseradish peroxidase; MEM, minimal essential medium; wt, wild-type.

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confirmed by direct DNA sequencing of the entire Rab5 cDNA with an automatic sequencer (ABI377) (The DNA Sequencing Facility, University of Oklahoma Health Sciences Center, Oklahoma City, OK, U.S.A.). No PCR errors were found in other regions. These pGEX-3X constructs were transformed into the *Escherichia coli* strain MC1061, in which wt and mutant Rab5 proteins were expressed as glutathione S-transferase (GST) fusion proteins on induction with 1 mM isopropyl β -D-thiogalactoside. The GST fusion proteins were affinity-purified by using glutathione-Sepharose 4B resin (Amersham Pharmacia Biotech) in accordance with the manufacturer's instructions.

For expression in baby hamster kidney-21 (BHK-21) cells, wt and mutant Rab5 cDNA species were subcloned into the double-subgenomic Sindbis virus vector pToto1000:3'2J [17,23]. The resulting plasmid constructs were used to generate recombinant virus stocks as described previously [17]. The recombinant viruses were able to express the corresponding wt and mutant Rab5 proteins on infection of fresh BHK cells.

Horseradish peroxidase (HRP) uptake experiments

Confluent BHK-21 cell monolayers were grown in α -minimal essential medium (α -MEM) (Life Technologies) containing 5% (v/v) fetal bovine serum (Life Technologies) in six-well Costar culture plates (approx. 5×10^5 cells per well). The cells were infected with recombinant Sindbis viruses at a multiplicity of infection of 50 plaque-forming units per cell and incubated at 37 °C in a tissue-culture incubator with air/CO₂ (19:1) for 4 h before the HRP uptake assay. This high multiplicity of infection ensured that nearly all of the cell population expressed the desired Rab5 protein. HRP uptake was initiated by replacing the growth medium with 1 ml of serum-free α -MEM containing 5 mg/ml HRP (Sigma) and 1% (w/v) BSA, followed by incubation at 37 °C for 30 min. The uptake was stopped by washing the cell monolayers three times with ice-cold PBS containing 1% (w/v) BSA. Cells were then scraped into 2 ml of PBS with a cell scraper (Fisher) and pelleted at 800 g for 3 min in a Beckman GPR centrifuge. The cell pellet was washed once more by resuspension in 2 ml of PBS, followed by re-centrifugation. The cell pellet was lysed and assayed for HRP activity as described previously [22].

Subcellular fractionation

BHK-21 cell monolayers were grown and infected with recombinant Sindbis viruses capable of expressing wt or mutant Rab5 proteins as described above. Cells were incubated for 4 h at 37 °C in a tissue-culture incubator with air/CO₂ (19:1). The growth medium was then aspirated and the cell monolayers were rinsed once with ice-cold PBS (1 ml per well), followed by scraping the cells into 300 μ l of PBS with a cell scraper (Fisher). Cells were homogenized by passing them 20 times through a 26-gauge needle attached to a 1 ml syringe. Cell homogenates were first centrifuged at 800 g for 5 min to remove nuclei and cell debris. Post-nuclear supernatants were then subjected to ultra-speed centrifugation at 200 000 g for 6 min in a Beckman TL-100 centrifuge to separate the membrane fraction (pellet) from the cytosol fraction (supernatant). The membrane pellet was resuspended in the same volume of PBS as the cytosol fraction, and SDS was added to both fractions at a final concentration of 1% (w/v). Proteins in each fraction (10 μ l) were analysed by SDS/PAGE, followed by immunoblot assay with a monoclonal anti-Rab5 antibody (Transduction Laboratories) and enhanced chemiluminescence reagents (Amersham Pharmacia Biotech).

Metabolic labelling with [³H]mevalonolactone

Confluent BHK-21 cell monolayers in six-well Costar plates were infected with either the vector virus as a control or recombinant viruses expressing wt or mutant Rab5 proteins as indicated. At 2 h after infection, the growth medium was replaced with the labelling medium (α -MEM containing 5% (v/v) fetal bovine serum, 200 μ Ci/ml [³H]mevalonolactone and 20 μ M lovastatin). [³H]Mevalonolactone and lovastatin were obtained from NEN and Merck Sharp and Dohme Research Laboratories respectively. Labelling was performed at 37 °C for 5 h. Cells were then lysed in 1% (w/v) SDS, followed by immunoprecipitation with the anti-Rab5 antibody, SDS/PAGE and autoradiography. The gel was treated with 1 M salicylic acid for 30 min, dried and exposed to a Kodak XAR-5 film for 1 week.

Confocal immunofluorescence microscopy and phase-contrast microscopy

BHK-21 cells were grown on coverslips and infected with recombinant Sindbis viruses capable of expressing wt or mutant Rab5 proteins (see above). At 4 h after infection, cells were rinsed three times with PBS and fixed for 30 min in 4% (w/v) paraformaldehyde (in PBS) at room temperature. Cells were then permeabilized for 5 min with 0.1% Triton X-100 (in PBS) and incubated for 30 min with the monoclonal anti-Rab5 antibody (Transduction Laboratories) in Hanks balanced salt solution containing 1% (w/v) BSA. Cells were rinsed three times with PBS to remove unbound primary antibody and were then incubated for 30 min with the secondary antibody [goat anti-mouse IgG conjugated with Alexa 488 (Molecular Probes)]. The coverslips were rinsed twice with PBS and mounted in SlowFade (Molecular Probes) on glass slides. The fluorescence was observed via a Leica confocal laser scanning microscope. To observe phase-contrast images of cell morphology, coverslips were prepared 6 h after infection and viewed on an Olympus AX-70 microscope with a $\times 40$ objective. The images were saved on a computer by using a digital camera.

GTPase assay

The GST fusion proteins of wt and mutants (1 μ M) were bound to glutathione-Sepharose 4B resin and incubated with 0.1 μ M [α -³²P]GTP (Amersham Pharmacia Biotech) for 30 min at 25 °C in 50 μ l of loading buffer [20 mM Tris/HCl (pH 8)/2 mM EDTA/1 mM dithiothreitol]. Unbound [α -³²P]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)/5 mM MgCl₂/1 mM dithiothreitol] and incubating at 37 °C. Samples were taken at the indicated times and immediately solubilized in elution buffer (0.2% 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) with 0.75 M KH₂PO₄, pH 3.5, as the developing solvent. The radioactive GTP and GDP spots were detected by autoradiography and quantified by using a PhosphorImager (Molecular Dynamics). The specific radioactivity of GDP was corrected as two-thirds that of GTP.

GDP dissociation assay

Free wt and mutant Rab5 proteins were released from the GST fusion proteins by digestion with factor Xa protease (Amersham

Figure 1 Eleven phosphate-binding-loop mutants lose the activity to stimulate HRP uptake in BHK-21 cells

Shown are bar graphs indicating a 30-min HRP uptake in cells expressing the wt Rab5, the mutants, or mock (vector). (A) Stimulatory activities of the Rab5 mutants containing substitutions at Ser²⁹. (B) Stimulatory activities of the Rab5 mutants containing substitutions at Ala³⁰. Abbreviation: OD405, A_{405} .

Figure 2 Loss-of-function Rab5 mutants are defective in membrane association

Subcellular fractionation was used to separate the membrane (mem) and cytosol (cyt) fractions. Shown is an immunoblot analysis of the wt and mutant Rab5 proteins in each fraction. (A) Membrane/cytosol distribution of wt and mutants containing substitutions at Ser²⁹. (B) Membrane/cytosol distribution of the mutants containing substitutions at Ala³⁰.

Pharmacia Biotech) and dialysed in GDP loading buffer [50 mM Tris/HCl (pH 7.4)/150 mM NaCl/1 mM EDTA/1 mM dithiothreitol]. The Rab5 proteins (1 μ M) were then loaded with 20 μ M [³H]GDP (NEN) for 30 min at 37 °C in 100 μ l of loading buffer. The dissociation of [³H]GDP was initiated by diluting the reaction with 900 μ l of loading buffer containing unlabelled GDP (final concentration 2 mM) and different amounts of MgCl₂ (final concentration 5 or 10 mM, as indicated). The reaction was conducted at 37 °C. Samples of the reaction mixture (150 μ l) were taken at the indicated times and rapidly filtered through HA-type nitrocellulose membranes (0.45 μ m pore size; Millipore) with a vacuum manifold. The membranes were washed with 2 ml of loading buffer and air dried; the radioactivity retained on the membrane was quantified by liquid-scintillation counting.

RESULTS

It has been shown previously that overexpression of Rab5 in BHK cells is able to stimulate endocytosis [14,17] by increasing

Figure 3 Loss-of-function Rab5 mutants are not defective in isoprenylation

Shown is an autoradiogram indicating [³H]mevalonolactone-labelled (isoprenylated) proteins (arrowed). BHK-21 cells expressing wt Rab5, the indicated mutants or mock (vector) were metabolically labelled with [³H]mevalonolactone, followed by immunoprecipitation of the cell lysates with the anti-Rab5 antibody, SDS/PAGE and autoradiography. The positions of molecular mass standards are indicated at the left.

early endosome fusion [15,24] and possibly vesicle budding from the plasma membrane [25]. The stimulation of endocytosis provided a functional measure of Rab5 activity in the cell. The

Figure 4 Five phosphate-binding-loop mutants show a gain-of-function phenotype as demonstrated by inducing the production of super-large early endosomes in the same way as Rab5:Q79L

Shown are confocal immunofluorescence images of Rab5-positive endosomes in BHK-21 cells expressing wt Rab5 and the indicated Rab5 mutants. Scale bar, 10 μ m.

38 mutants and wt Rab5 were expressed in BHK cells via a Sindbis virus vector and the effect on endocytosis was determined by monitoring HRP uptake. The expression level for each mutant was similar and was approx. 10-fold over the endogenous level at 4 h after infection as determined by immunoblot analysis (results

not shown). The HRP uptake was conducted at 37 °C for 30 min. Overexpression of wt Rab5 stimulated HRP uptake 2-fold (Figure 1). In contrast with the gain-of-function phenotype of the equivalent Ras mutants, seven Rab5 mutants with substitutions at Ser²⁹ completely lost biological activity in terms of

Figure 5 Gain-of-function Rab5 mutants induce cell rounding and detachment from culture plates

Shown are phase-contrast images of BHK-21 cells expressing wt Rab5, the indicated mutants or mock (Vector). The apparent morphological changes caused by expressing each of the indicated gain-of-function mutants are not seen in cells expressing wt Rab5 (WT) or mock (Vector). Scale bar, 20 μm .

stimulating HRP uptake (Figure 1A). All substitutions with aliphatic amino acids except Pro and Ala abolished Rab5 activity (Figure 1A). Replacement with the negatively charged Glu or the bulky Tyr also abolished Rab5 activity (Figure 1A). Furthermore, replacement of Ser²⁹ with Arg decreased Rab5 activity by approx. 60 % (Figure 1A). In addition, four mutants with substitutions at Ala³⁰ also lost the activity to stimulate HRP uptake, including replacements by Leu, Trp, Asp and Ser (Figure 1B).

The loss-of-function phenotype exhibited by the Rab5 mutants was investigated further to understand its mechanism. The membrane association and isoprenylation of the Rab5 mutants were examined because they are the first necessary steps by which newly synthesized Rab5 molecules gain function. BHK cells expressing each mutant were homogenized and subjected to sub-cellular fractionation to separate the membrane and cytosol fractions, followed by SDS/PAGE. The Rab5 mutants in each fraction were identified by immunoblot analysis. The wt Rab5 protein was mostly in the membrane fraction (80 %) (Figure 2A). In contrast, all Rab5 mutants that lost activity were more than 80 % in the cytosol fraction, except for the two mutants containing replacements by negatively charged amino acids [Ser²⁹ → Glu (S29E) and Ala³⁰ → Asp (A30D)] (Figure 2). The Rab5 mutants that retained biological activity were mostly membrane-associated, like wt Rab5 (Figure 2, and results not shown). Because isoprenylation was required for Rab5 to associate with the membrane, we determined whether the loss-of-function mutants were defective in isoprenylation. BHK cells expressing the mutants were metabolically labelled with [³H]mevalonolactone, a precursor of the prenyl group. Cell lysates were immunoprecipitated and analysed by SDS/PAGE and autoradiography. The mutants were readily labelled and the steady

level of isoprenylation was similar to that of wt Rab5 (Figure 3), suggesting that the defective membrane association exhibited by these mutants was not due to defective isoprenylation. An alternative explanation for the defect in membrane association was that the mutations disrupted the Rab5 interaction with its acceptor on the endosomal membranes, i.e. the putative membrane recruitment machinery.

The finding that 11 mutations (12 if the partly inactive S29R mutant was included) led to a loss-of-function phenotype of Rab5 was unexpected because the equivalent mutations had exactly the opposite effect on Ras, leading to a gain-of-function phenotype. In this regard, we investigated whether any of the remaining biologically active Rab5 mutants would exhibit a gain-of-function (constitutively active) phenotype. The HRP uptake assay was not suitable for distinguishing between the wt and constitutive active mutants because they would stimulate the uptake to similar extents. A more direct measure of early endosome fusion in the cell was to examine early endosome morphology by confocal fluorescence microscopy. Constitutive activation of Rab5 would lead to excessive endosome fusion, leading to the enlargement of early endosomes. Super-large early endosomes were first observed in cells overexpressing the Rab5: Q79L mutant [18], which was the only known constitutively activated Rab5 mutant; it had a lower GTPase activity [16–18]. By using Rab5:Q79L as a control for comparison, we expressed the phosphate-binding-loop mutants in BHK cells and examined the morphology of early endosomes in BHK cells by confocal immunofluorescence microscopy with an anti-Rab5 antibody. This screen revealed that five mutants (A30P, A30I, A30V, A30K and S29K) exhibited a gain-of-function phenotype, as demonstrated by their ability to induce super-large endosomes in the same way as the Rab5:Q79L mutant (Figure 4).

Figure 6 Single-step GTP hydrolysis by the GST fusion proteins of wt and mutant Rab5 proteins

Shown are autoradiographs of the GTP hydrolysis products analysed by TLC at the indicated times. **(A)** GTP hydrolysis rates of wt and mutant Rab5 proteins with Ser²⁹ replaced by aliphatic amino acids. **(B)** GTP hydrolysis rates of the mutants with Ser²⁹ replaced by charged amino acids. **(C)** GTP hydrolysis rates of the mutants with Ser²⁹ replaced by polar amino acids. The result was reproducible in two independent experiments; the difference in the percentage of GTP hydrolysis was within 2%.

In the process of characterizing these gain-of-function mutants, we found that overexpression of them caused interesting changes in cell morphology, leading to cell rounding and detachment from culture dishes (Figure 5). The morphological changes were induced by all the gain-of-function mutants described above, but not by wt Rab5 (Figure 5) or any of the other mutants (results not shown). The appearance of the morphological changes was

a late event during transient expression of the gain-of-function mutants. When these mutants were expressed in BHK cells via the Sindbis virus vector, enlargement of early endosomes (Figure 4) occurred early (3–4 h after infection), whereas cell rounding (Figure 5) did not appear until 5–6 h after infection. The potency of each gain-of-function mutant was different. The most potent mutant was A30P, which induced cell rounding at 5 h after

infection, followed by Q79L, A30I and A30V; the least potent mutants were S29K and A30K, which induced cell rounding at approx. 6 h after infection. It had been shown previously that the constitutively activated Rab5:Q79L mutant affected actin organization in NIH 3T3 and Madin-Darby canine kidney cells [26,27]. Taken together, the change in cell morphology induced by the gain-of-function mutants was probably a result of uncontrolled excessive endosome fusion and/or an effect on the actin cytoskeleton.

To understand the biochemical basis of the functional phenotypes, we determined the GTPase activity of the mutants and compared it with that of wt Rab5. All the mutants were expressed in *E. coli* as GST fusion proteins and purified on glutathione-Sepharose resin. To determine GTPase activity, the proteins were loaded with [α - 32 P]GTP and incubated at 37 °C for the indicated durations; the products were analysed by TLC and autoradiography (Figure 6). All the Ser²⁹ substitution mutants, except S29G and S29P, showed decreased GTPase activity to various extents. The most marked effects were seen with amino acid substitutions containing bulky aliphatic side chains (S29W, S29F, S29I and S29L) (Figure 6A), positively charged side chains (S29R and S29K) (Figure 6B) and bulky polar side chain (S29Y) (Figure 6C). These mutations resulted in as much as an order of magnitude decrease in Rab5 GTPase activity. In contrast, the S29G and S29P mutants showed a small increase in the GTPase activity (Figure 6A). We had previously determined the GTPase activity of the Ala³⁰ substitution mutants [22]. Most of these mutants exhibited a small decrease (less than 2-fold) in GTPase activity, but the A30P mutation decreased Rab5 GTPase activity by an order of magnitude. The A30R and A30T mutants, in contrast, showed an increased GTPase activity in comparison with wt Rab5.

Gain-of-function phenotypes of GTPase mutants are often explained by their decreased GTPase activity. Whereas the A30P and S29K mutants showed a substantial decrease in GTPase activity (Figure 6B), the A30I, A30V and A30K mutants had only a small effect (less than 2-fold) on the GTPase activity [22]. An alternative method of keeping these mutants constitutively in the GTP-bound active form could be to increase the rate of GDP dissociation, thereby increasing the chance of GTP loading. To test this mechanism, the GDP dissociation rates of the mutants were determined and compared with that of wt Rab5. Purified GST fusion proteins were cleaved by factor Xa to release wt and mutant Rab5 proteins. The free proteins were loaded with [3 H]GDP and then chased with 1000-fold excess of unlabelled GDP. Samples were taken at different times and the amount of [3 H]GDP remaining bound to the protein was determined by a rapid filtration method [28]. In comparison with the wt Rab5, three gain-of-function mutants (A30P, A30I and A30V) exhibited faster GDP dissociation rates (Figure 7). Indeed, the apparent affinity of the A30P mutant for GDP was so low (several orders of magnitude) that there was only background [3 H]GDP loaded and it was difficult to perform the GDP dissociation assay for this mutant (results not shown). At a relatively low MgCl₂ concentration (5 mM), the S29K and A30K mutants exhibited a slow GDP dissociation rate, like that of wt Rab5 (Figure 7A). However, the A30V and A30I mutations increased the GDP dissociation rate by 3-fold and 4-fold respectively (Figure 7A). At high MgCl₂ concentration (10 mM), GDP dissociation from wt Rab5 and the S29K and A30K mutants was blocked (Figure 7B), which was consistent with a previous study [29]. However, the dissociation of GDP from the A30I and A30V mutants was not blocked (Figure 7B). As a further control, we determined the GDP dissociation rates of two additional mutants (S29A and A30M), which were active in stimulating HRP uptake but did

Figure 7 GDP dissociation rates of wt and mutant Rab5 proteins

Free wt (□) and mutant proteins (▲, A30V; ■, A30I; △, A30K; ●, S29K) released from the GST fusion proteins were loaded with [3 H]GDP, followed by the measurement of [3 H]GDP dissociation at 37 °C in the presence of a 1000-fold excess of unlabelled GDP. Results were calculated as percentages of maximal [3 H]GDP loaded and are means for triplicate samples. [3 H]GDP dissociation rates are shown for wt and mutant Rab5 proteins in the presence of 5 mM MgCl₂ (A) and 10 mM MgCl₂ (B).

not exhibit a gain-of-function phenotype. These mutants showed similar GDP dissociation rates to that of wt Rab5 (results not shown).

The results suggested that the gain-of-function phenotype of the A30I and A30V mutants was due to their increased GDP dissociation rate, which became independent of Mg²⁺ concentration and possibly independent of the Rab5 guanine nucleotide exchange factor(s) (e.g. Rabex5) [30]. However, the marked decrease in GTPase activity could contribute to the gain-of-function phenotype of S29K. The most potent gain-of-function mutant, A30P, exhibited a combination of increased GDP dissociation rate and decreased GTPase activity. The gain-of-function phenotype of the A30K mutant, which showed little change in the intrinsic GTPase activity and GDP dissociation rate, suggested that a Rab5 GAP was important for the normal Rab5 function in the cell. The A30K mutant might be insensitive to the GAP and thereby kept in the GTP-bound active conformation. In this regard, two proteins (tuberin and the p120 GAP) were reported to contain Rab5 GAP activity [20,31].

DISCUSSION

This study has investigated the structure–function relationship of the phosphate-binding loop of Rab5 and revealed its distinct role in Rab5 function compared with that of Ras. Biochemical and structural results on Ras have suggested that the two Gly residues (Gly¹² and Gly¹³) in the phosphate-binding loop are required for its normal function, because side chains at these positions can decrease the GTPase activity by interfering with the transition state and lock Ras in its active GTP-bound conformation [6–8,10]. The resulting gain-of-function phenotype of the Ras mutants is cell proliferation/transformation [9]. It is therefore surprising that these two Gly residues are not conserved in other Ras-like GTPases such as the Rab GTPases. By using Rab5 as a model for comparison, we have generated a series of substitution mutations at the equivalent phosphate-binding loop residues, Ser²⁹ and Ala³⁰. These mutations alter the GTPase activity of Rab5 in the same way that they affect Ras, reflecting the conserved GTP-binding domain structure and GTP hydro-

lysis mechanism. However, the effect on biological activity is quite different. In contrast with Ras, many of the mutations result in a loss-of-function phenotype of Rab5, reflecting the functional diversity of similar GTPase structures and their interaction with distinct sets of factors.

Rab5 functions as a molecular switch regulating endocytosis by facilitating early endosome fusion [15,16,24] and possibly the budding of endocytic vesicles from the plasma membrane as well [25]. Overexpression of Rab5 in cultured cells stimulates endocytosis [14,17] and this provides a functional assay for Rab5. For newly synthesized Rab5 molecules to obtain biological activity, they first need to be modified by isoprenylation, then escorted to endosomal membranes where they undergo nucleotide exchange to become the active GTP-bound conformation. These processes have been suggested to be facilitated by a Rab5-specific membrane recruitment machinery and a guanine nucleotide exchange factor [30]. The activated Rab5 recruits effectors to promote the docking of endosomes [32], which ultimately leads to membrane fusion. Our study has identified 11 phosphate-binding-loop mutants that fail to stimulate endocytosis, of which 9 are defective in membrane targeting. However, this defect is not due to deficient isoprenylation, suggesting that the mutants interact normally with the Rab escort protein REP (a subunit of the Rab geranylgeranyl transferase) [33]. Our results suggest that these mutants are incompetent for interacting with specific factors on the endosomal membrane, i.e. the putative membrane recruitment machinery for Rab5. In this regard, the results shed light on the membrane topography of Rab, namely that the phosphate-binding loop should face the membrane and be in close contact with the membrane factors. Replacements of Ser²⁹ with amino acids with bulky aliphatic side chains (except Pro) all disrupt the interaction of Rab5 with the membrane. In addition, Tyr or Arg (but not Lys) at this position also significantly decreases the membrane association of Rab5. Replacements of the neighbouring Ala³⁰ with Leu, Trp or Ser have the same effect, namely blocking the interaction of Rab5 with the membrane. It is surprising that the substitution of Ser for Ala results in defective membrane association and a loss-of-function phenotype of Rab5, considering that another Rab (Rab3) normally contains Ser at the equivalent position (Ser³²) [19,34,35]. These results reveal individual differences between the Rab proteins in terms of membrane targeting. The nature of Rab5 and membrane interaction remains to be established; these Rab5 mutants, which uncouple the isoprenylation and membrane-targeting steps, should be useful reagents in identifying the components of the membrane recruitment machinery.

It is interesting to note that two of the loss-of-function mutants remain able to associate with the membrane efficiently. These two mutants (S29E and A30D) contain a negatively charged side chain in the phosphate-binding loop. Remarkably, at the Ser²⁹ position, only the Glu side chain, but not Asp, inactivates the Rab5 function, whereas at the Ala³⁰ position the opposite is true. The loss-of-function phenotype of these two mutants can be explained if the mutations disrupt the downstream interaction with a guanine nucleotide exchange factor or an effector that is necessary for the Rab5 function. Taken together, the results suggest that the phosphate-binding loop of Rab5 is important for interacting with the membrane recruitment machinery and other membrane-associated factors such as a nucleotide exchange factor. In this regard, it is important to make a comparison with Ras, in which some of the equivalent phosphate-binding-loop mutants have been generated and characterized. None of the Ras mutants is defective in membrane association or shows a loss-of-function phenotype [9], suggesting that its phosphate-binding loop is not important for interacting with the membrane, the

guanine nucleotide exchange factor or the effector. Therefore, despite their similar structures [7,35,36], the phosphate-binding loops of Rab and Ras are distinct in terms of their contribution to the respective functions of Rab and Ras.

Aside from the 11 loss-of-function mutants (12 if the partly inactive S29R is included), the remaining 26 mutants are biologically active, like wt Rab5, in terms of stimulating endocytosis. Only five mutants show a gain-of-function phenotype as demonstrated by their ability to induce super-large endosomes in BHK cells in the same way as the Rab5:Q79L mutant. In the process of characterizing the gain-of-function mutants, we find that overexpression of these mutants induces a novel cellular phenotype: cell rounding and detachment from culture dishes. This phenotype occurs later than the enlargement of early endosomes, suggesting that it is a result of excessive early endosome fusion and/or an effect on actin organization [26,27]. This phenotype is not due to the Sindbis expression vector used in this study, because the same cell phenotype is not induced by either the vector itself or the same vector expressing wt Rab5. The change in cell morphology provides a simple assay for identifying constitutively activated Rab5 mutants in the cell. In addition, this finding might explain the reported difficulty of establishing cell lines expressing high levels of the gain-of-function mutant (Rab5:Q79L) [18,26].

Among the five newly identified gain-of-function mutants, A30I, A30V and A30P show a faster GDP dissociation rate than wt Rab5. The GDP dissociation step is necessary for the subsequent GTP-loading and activation of Rab5, a process normally facilitated by a nucleotide exchange factor that is suggested to disrupt the Mg²⁺ co-ordination in the GDP-binding pocket [37]. GDP dissociation from the A30I, A30V and A30P mutants is independent of Mg²⁺ concentration and possibly no longer requires the nucleotide exchange factor, thereby enhancing the chance of GTP loading (the intracellular concentration of GTP is much higher than that of GDP) [37] and contributing to their gain-of-function phenotype. Both the A30P mutant and the S29K mutant show a substantial decrease (an order of magnitude) in intrinsic GTPase activity, which might contribute to keeping the mutants in the active GTP-bound conformation. The A30K mutant, in contrast, shows little change in either GDP dissociation rate or intrinsic GTPase activity in comparison with wt Rab5. The gain-of-function phenotype of the A30K mutant suggests that normal Rab5 function in the cell requires a GAP to facilitate GTP hydrolysis and that the A30K mutation diminishes the interaction with the GAP and thus keeps the mutant in the active GTP-bound conformation.

In conclusion, the phosphate-binding loop of Rab5 is functionally distinct from that of Ras. Substitution mutations in this region can lead to either a loss-of-function or a gain-of-function phenotype, depending on the nature of the mutation. It is suggested that the loss-of-function phenotype results from the failure to interact properly with Rab5-specific membrane-associated factors, whereas the gain-of-function phenotype is due to enhanced nucleotide exchange rate and/or decreased GTPase activity.

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