Small G proteins of the Rab family are regulators of intracellular vesicle traffic. Their intrinsic rate of GTP hydrolysis is very low but is enhanced by specific GTPase-activating proteins (GAPs) that switch G proteins to their inactive form. We have characterized the activity of recombinant Rab3-GAP on Rab3A in solution. The $K_m$ and $K_v$ values (75 $\mu$M) indicate a low affinity of Rab3-GAP for its substrate. The affinity is higher for the transition state analog Rab3A:GDP:AlF$_4^-$ (15 $\mu$M). The $k_{cat}$ (1 s$^{-1}$) is within the range of values reported for other GAPs. A mutation in the switch I region of Rab3A disrupted the interaction with Rab3-GAP. Furthermore, Rabphilin, a putative target of Rab3, inhibited the activity of Rab3-GAP on Rab3. Therefore, the Rab3-GAP-binding site involves the switch I region of Rab3 and overlaps with the Rabphilin-binding domain. Substitution of a single arginine residue (Arg-728) of Rab3-GAP disrupted its catalytic activity but not its interaction with Rab3A. We propose that Rab3-GAP, like Ras- and Rho-GAPs, stabilizes the transition state of Rab3 and provides a critical arginine residue to accelerate the GTPase reaction.

Rab3 has been implicated in the exocytosis of hormones and neurotransmitters (3, 4). Increasing the amount of GTP-bound Rab3A results in a reduction of the magnitude of the secretory responses (5–8). Controlling the amount of Rab3:GTP or the kinetics of the GTPase reaction might therefore be of prime importance in the physiological regulation of the exocytosis process.

In yeast, several GAPs for Ypt/Rab proteins have been identified (9–12). In mammals, only two Rab-GAPs are currently known: GAPCenA that acts on Rab6 (13) and Rab3-GAP that is specific for Rab3 subfamily members (14). GAPCenA displays some homology with yeast Rab-GAPs, but the sequence of Rab3-GAP seems to be totally different and is not related to any other known protein. Previous studies have shown that the catalytic domain of Rab3-GAP is localized in the last 400 (out of 981) amino acids and that Rab3-GAP is firmly bound to another subunit of 150 kDa, which is not required for the catalytic activity of Rab3-GAP in vitro (15).

In this study, we have further characterized the biochemical properties of recombinant full-length Rab3-GAP. We show that the binding affinity of Rab3-GAP for its substrate in solution is low and that the mechanism of action of Rab3-GAP involves a critical arginine residue and an interaction with the switch regions of Rab3.

**EXPERIMENTAL PROCEDURES**

**Materials**—Nucleotides were purchased from Roche Molecular Biochemicals and other chemicals from Sigma. Calmodulin was from Calbiochem.

**Expression Vectors**—The cDNA encoding KIAA0066 (Rab3-GAP), kindly provided by Dr. T. Nagase, was subcloned in frame into pRSETB (Invitrogen) using polymerase chain reaction (PCR). A two-step PCR strategy was followed to mutate different arginine residues of Rab3-GAP. The corresponding sequences were cloned in pRSETB, pET-3A-Rab3A and pGEX-2T-Rab3A (1–217) have been described previously (16, 17). pGEX-2T-Rabphilin (1–206) was a kind gift of Dru S. H. Chung and R. W. Holz. To express His$_6$-tagged Rab3A (1–185), a stop codon was introduced by PCR into the sequence of hu-Rab3A, followed by in-frame subcloning into pET-15B (Novagen).

The sequences generated by PCR were verified by dideoxy sequencing.

**Purification of Recombinant Proteins**—Rab3-GAP was found to be highly toxic in *Escherichia coli* BL21 or DH5$a$. For expression, freshly transformed C41 cells (a strain kindly provided by B. Miroux and J. Walker, which derived from BL21 DE3 (18)) were grown in LB medium at 37°C. Isopropyl-$\beta$-d-thiogalactopyranoside (1 mM) was added at $A_{600}$ ~0.7, and the culture was grown overnight at 25°C. Bacterial pellets were resuspended in 20 mM Tris/Cl, pH 8.0, 300 mM NaCl, 5 mM $\alpha$-methyl aminopropionitrile, 5 mM leupeptin, 5 mM pepstatin, 0.5 mM Pefabloc, 0.5 mM diisopropyl fluorophosphate and rapidly frozen in liquid nitrogen. After thawing, the cells were incubated with lysozyme (1 mg/ml) for 15 min at 4°C and briefly sonicated. Cell extracts were centrifuged for 30 min at 100,000 $\times$ g. Ammonium sulfate (10%, w/v) was added to the supernatant, which was stirred on ice for 1 h and centrifuged at 20,000 $\times$ g for 30 min. Ammonium sulfate (40%, final concentration) was added to the supernatant, which was stirred on ice for 1 h and spun at 20,000 $\times$ g for 30 min. The pellet was resuspended...
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RESULTS

Catalytic Properties of Rab3-GAP—His<sub>6</sub>-tagged full-length Rab3-GAP (981 amino acids in length) was found to be highly toxic in E. coli. However, it was readily expressed in a soluble form in the mutant strain C41 (18). The protein was purified to near homogeneity by affinity chromatography onto cobalt-agarose. Recombinant His<sub>6</sub>-tagged Rab3-GAP was able to accelerate the hydrolysis of [γ-<sup>32</sup>P]GTP bound to Rab3A, as measured in a GAP binding assay (Fig. 3). Preincubation experiments did not reveal major differences between prenylated and nonprenylated Rab3A. Therefore, nonprenylated Rab3A proteins expressed in E. coli were used in this study. Rab3-GAP was found to be active either on full-length Rab3A-(1–220) or on COOH-terminally truncated Rab3A proteins (Fig. 1). Most of the experiments were performed with His<sub>6</sub>-tagged Rab3A-(1–185).

To determine K<sub>m</sub>, 50 nM Rab3-GAP was incubated with 1 μM [γ-<sup>32</sup>P]GTP-loaded Rab3A and increasing concentrations of GTP-loaded Rab3A, for 0–10 min at 25 °C. The initial rate of GTP hydrolysis was determined and plotted as a function of Rab3-GAP. As illustrated in Fig. 2, high amounts of Rab3-GAP were required to saturate the activity of Rab3-GAP on [γ-<sup>32</sup>P]GTP-loaded Rab3A. Values for K<sub>m</sub>, (75 ± 10 μM; mean ± S.D.) and k<sub>cat</sub> (1 ± 0.3 s<sup>−1</sup>; mean ± S.D.) of Rab3-GAP were derived from four independent experiments. Since the intrinsic GTP hydrolysis rate of Rab3A is ~3 × 10<sup>−4</sup> s<sup>−1</sup> (data not shown), one can conclude that Rab3-GAP increases GTP hydrolysis rate by a factor of ~3000. This is significantly less than the acceleration factor determined for Gyps, the GAPS for yeast Ypt/Rab proteins (20), and Ras-GAPs. However, the k<sub>cat</sub> values are within the range of values reported for the different GAPS (1–19 s<sup>−1</sup> (20, 21)). The lower acceleration factor observed for Rab3-GAP may be partly due to the high intrinsic GTP hydrolysis rate of Rab3A.

Binding Affinity of Rab3 for Rab3-GAP—Next, we determined the binding affinity of Rab3 to Rab3-GAP. Increasing amounts of Rab3A loaded with the nonhydrolyzable analog GTP<sub>S</sub> were added into a standard GAP reaction (Fig. 3A). Here, the IC<sub>50</sub> value was taken as a measure of the K<sub>d</sub>. A value of 75 ± 28 μM (mean ± S.D.) was derived from six independent experiments. This value is similar to that of K<sub>D</sub>. Accordingly, Rab3A-GTP and Rab3A:GTP<sub>S</sub> behave similarly in competition experiments (Fig. 3A). Rab3A Q61L was also used to determine the affinity of Rab3A for Rab3-GAP. Glutamine 81 in Rab3A corresponds to glutamine 61 in Ras that plays a critical role in the nucleophilic attack of the γ-phosphate of GTP. Accordingly, this mutated protein has no intrinsic GTase activity. Even in the presence of large amounts (up to 300 nm) of Rab3-GAP, no GTP hydrolysis could be detected (Fig. 4B). GTP-loaded Rab3A Q61L competes with wild type Rab3A in a GAP assay (Fig. 3B) with an IC<sub>50</sub> value of 5.4 ± 0.4 μM (mean ± S.D., n = 3). In contrast, that Rab3A Q81L:GTP is a 10-fold more potent inhibitor of Rab3-GAP than GTP-S-loaded Rab3A suggests a difference in their conformational state. Such a difference has been observed previously in the case of Ras. Mutations of Gln-61, and especially Q61L, increase the affinity between p21-Ras:GTP and GAP (21). It was also reported that p120-GAP has a higher affinity for Ras:GDP:AlF<sub>3</sub> than for the triphosphate state (ground state) of Ras (22). Structural and biochemical studies led to the conclusion that...
the ternary complex formed by Ras:GDP, Ras-GAP, and AlF₃ is a transition state analog (23, 24). We tested the ability of Rab3:GDP to interact with Rab3-GAP in the presence of aluminum fluoride. As shown in Fig. 3B, Rab3A:GDP:AlFₓ is a potent inhibitor of the activity of Rab3-GAP on Rab3A-GTP. The IC₅₀ value determined in these experiments was 15 ± 9 μM (mean ± S.D., n = 3). It seems likely that this value, which is very similar to that determined with Rab3A Q81L, is a measure of the affinity of Rab3A, in the transition state, for Rab3-GAP.

Rabphilin but Not Ca²⁺/Calmodulin Interferes with Rab3-GAP Activity—GTP hydrolysis is responsible for the disruption of the interaction of G proteins with their effectors. We investigated whether Rab3-GAP was able to act on Rab3A complexed with an effector molecule. Rabphilin is one of the putative effectors of Rab3A (4). As illustrated in Fig. 4A, the Rab3-binding domain of Rabphilin inhibited Rab3-GAP activity. This indicates that Rab3-GAP- and Rabphilin-binding domains overlap. Rab3A interacts with Rabphilin via its switch I region, which encompasses the so-called effector domain, and via other regions, termed Rab-complementary determining regions, which contact a conserved SGAWFF motif in Rabphilin (25). In agreement with the ability of Rabphilin to compete with Rab3-GAP, the mutation V55E in the switch I region of Rab3A was found to disrupt the interaction with Rab3-GAP (Fig. 4, B and C).

Another putative effector of Rab3 is calmodulin. In the presence of calcium ions, calmodulin (CaM) solubilizes isoprenylated membrane-bound Rab3 (26). Furthermore, binding of Ca²⁺/CaM to Rab3 correlates with the effect of Rab3A on the secretory activity of endocrine cells (27). The addition of Ca²⁺/
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To ascertain that the reduced activity of Rab3-GAP R728A was not due to improper folding, its interaction with Rab3A was assayed. First, Rab3-GAP R728A was found to compete with wild type Rab3-GAP (Fig. 8A). Second, Rab3-GAP R728A was able to inhibit the binding of GTP-loaded Rab3A to GST-Rabphilin (Fig. 8B). In these experiments (Fig. 8, A and B), IC₅₀ values were in good agreement with the affinity of Rab3A for Rab3-GAP. At high concentrations, Rab3-GAP R728A had very low but detectable GAP activity (Fig. 8D). However, this activity was too weak to account for the diminished signal in the Rab3A/Rabphilin binding assay (i.e. the γ32P[GTP bound to Rab3A and retained on the filters). The binding of Rab3-GAP to Rab3A Q81L was also assayed. In sharp contrast to wild type Rab3-GAP, the mutated protein did not interact with the GTPase-deficient Rab3A mutant (Fig. 8C).

DISCUSSION

Ras-GAPs and Rho-GAPs act by ordering the switch regions of the GTP-binding protein such that the catalytic glutamine residue is positioned properly and by supplying an arginine residue to the active site (23, 28). ARF-GAP only stabilizes switch II, but coatomer (an effecter of ARF) might provide catalytic residues, since it contributes a further ~1000-fold acceleration of the reaction rate (29). Ga subunits possess an arginine finger very similar to that of Ras-GAP, which is responsible for the higher intrinsic rate of GTP hydrolysis, compared with small GTP-binding proteins (30, 31). RGS proteins are GAPs for Gₐ GTPases, which act by “stabilizing” the switch regions (32, 33). Therefore, the mechanism responsible for the acceleration of GTP hydrolysis seems to be universal, although the work is shared out differently in each family of GTPases.

Our results suggest that Rab3-GAP is functionally related to Ras-GAPs and Rho-GAPs. First, the overlap between Rab3-GAP and Rabphilin-binding sites on Rab3A and the insensitivity of Rab3A V55E to Rab3-GAP argue for an interaction between GAP and the Rab3A switch regions. The higher affinity of Rab3-GAP for the transition state, as mimicked by Rab3: GDP:AlFx, than for the ground state (represented by Rab3: GTPγS) further suggests that Rab3-GAP stabilizes the transition state conformation of the switch regions. Second, the lack of catalytic activity of Rab3-GAP R728A is consistent with Rab3-GAP introducing an arginine finger into the catalytic site. Since Rab3-GAP R728A interacts normally with wild type Rab3A, its loss of function cannot be accounted for by disruption of binding to the substrate. Interestingly, it was found that the mutation R728A disrupted the interaction of Rab3-GAP with the GTPase-deficient Rab3A Q81L protein. This observation might indicate that Arg-728 is positioned, within the Rab3-Rab3-GAP complex, in the vicinity of Gln-81 and, therefore, near the catalytic site. During completion of this work, Albert et al. (20) reported the identification of arginine residues essential for the activity of Gyp1 and Gyp7, two yeast Yp/Rab-GAPs. Arg-458 in Gyp7 contributes a factor of ~1000 to the total 2 × 10⁵-fold stimulation of the GTPase reaction. Another arginine residue, Arg-615, accelerates the GTPase reaction by ~30-fold. Arg-615 might be analogous to Arg-903 in p120Ras-GAP or Lys-122 in p50Rho-GAP, which play a role in stabilizing the finger loop. Altogether, these observations suggest that Rab-GAPs also use an arginine finger mechanism to accelerate the reaction. However, the crystal structure of GppNHp-bound Rab3A (35) revealed that the γ-phosphate is buried in Rab3A, in contrast to Ras. The side chains of Phe-51 and Ser-31 seem to be detrimental to the insertion of an arginine residue into the catalytic site. Future structural studies of the Rab3-Rab3-GAP complex will have to determine whether Rab3-GAP can open the conformation of the catalytic site to introduce a catalytic residue or whether it accelerates GTP hydrolysis by reori-

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**Fig. 5. Effect of Ca²⁺ and calmodulin on Rab3-GAP activity.** A, increasing amounts of calmodulin were mixed with 1 μM Rab3A-[γ³²P]GTP and 50 mM Rab3-GAP in the presence of 100 μM free Ca²⁺. B, effect of Ca²⁺ ions (▲, 100 μM free Ca²⁺ concentration) or Ca²⁺/calmodulin (●, 50 μM calmodulin, 100 μM free Ca²⁺) on the interaction of Rab3A Q81L with Rab3-GAP (50 μM) measured by inhibition of GAP-stimulated Rab3A-[γ³²P]GTP hydrolysis. Reactions were terminated after 6 min at 25 °C. Data are expressed as percentages (mean ± S.D., n = 3) of the values determined in the absence of calmodulin (A) or Rab3A Q81L (B).

CaM in the filter assay did not change the activity of Rab3-GAP (Fig. 5A). *Kₐ* is not affected by the presence of Ca²⁺/CaM (not shown). Furthermore, Ca²⁺/CaM did not interfere with the binding of Rab3-GAP to Rab3A, as demonstrated by the fact that the inhibitory effect of GTP-loaded Rab3A Q81L on the activity of Rab3-GAP was not significantly modified by the presence of Ca²⁺/CaM (Fig. 5B). In the absence of CaM, we did not observe any effect of Ca²⁺ ions neither on the activity of Rab3-GAP nor on its interaction with Rab3 (Fig. 5B).

An Arginine Residue Is Essential for the Catalytic Activity of Rab3-GAP—The mechanism of GAP action on several GT-Pases, including Ras and Rho, has been elucidated recently. One important feature of this mechanism is the existence of a critical arginine residue, which is positioned in the active site, binds to the γ-phosphate group and neutralizes the developing charge during the reaction (24). In Ga subunits, an arginine residue provided by the GTPase itself plays a similar role. It seemed, therefore, possible that Rab-GAPs also use an “arginine finger” to accelerate the GTPase reaction. To identify putative catalytic arginine residues in Rab3-GAP, sequence homology searching was performed. As reported previously (14), no significant homology was found with any known protein, including the GAPs for other Rab proteins. However, several uncharacterized sequences were found in sequence data banks with similarity to human Rab3-GAP (Fig. 6). Within this group of sequences, several arginine residues appeared to be conserved. Arginines 619, 700, 728, and 753 belonging to the catalytic domain of Rab3-GAP defined by Fukui et al. (14) were mutated in alanine. The corresponding proteins were expressed in *E. coli*, purified, and tested in the filter assay. Rab3-GAP R619A, R700A, and R753A had a normal activity, but Rab3-GAP R728A was found to be inactive on Rab3A (Fig. 7).
Fig. 6. Alignment of sequences related to Rab3-GAP. The region of the catalytic domain (660–981) of human Rab3-GAP (GenBank™ accession number D31886) with the most significant homology to other sequences is shown. GenBank™ accession numbers are shown on the left. Sequences are from the following species: AAF51358, Drosophila; Z78542, Caenorhabditis elegans; AB010071, Arabidopsis thaliana; N41083, Brugia malayi microfilaria. An “insertion” of 34 residues was suppressed from AB010071 (between positions 53 and 54). Identical residues (in >50% of the sequences) are boxed in black, conservative substitutions are highlighted in gray. Arrows indicate conserved arginine residues. The alignment was done with the program “pileup” from the GCG package and the shading with “Boxshade.”

Fig. 7. Effect of arginine substitutions on the enzymatic activity of Rab3-GAP. A, SDS-polyacrylamide gel electrophoresis of purified His6-tagged wild type and mutated Rab3-GAP proteins used in this study. Gels were stained with Coomassie Blue. B, increasing amounts of Rab3-GAP (•, wild type (WT); ○, R619A; □, R700A; ◇, R728A; △, R753A) were incubated with 1 μM Rab3A-[γ-32P]GTP for 6 min at 25 °C. Data are expressed as the percentage of the radioactivity retained on nitrocellulose filters in the absence of Rab3-GAP. Each point is the mean of two experimental determinations.

ent intrinsic residues (Ser-31, Ser-53, and Gln-81) in a manner similar to the RGS proteins.

Rab3-GAP and Rabphilin binding to Rab3A are mutually exclusive. This finding, which confirms previous observations made with a brain extract enriched in Rab3-GAP activity (36), raises the question of the mechanisms that control the duration of the GTP signal. Due to the overlap between the binding domain of Rab3-GAP and of the effector, Rab3-GAP would be able to modulate the kinetics of GTP hydrolysis and the duration of the GTP signal only if the lifetime of the Rab3-effector complex were short. In the case of Ras, the lifetime of Ras-Raf1 complex was shown to be short (37). Hence, Ras-GAP may control the duration of the “on” state and thus the number of Raf1 molecules activated by Ras. In the case of ARF, the scenario differs. ARF:GTP forms a stoichiometric and probably stable complex with coatomer. However, ARF-GAP can interact with this complex and thus controls its duration (29). The competition between Rabphilin and Rab3-GAP for binding to Rab3 suggests a “Ras-like” catalytic mechanism, but further kinetic studies are required to establish this point.

Although Rab3-GAP accelerates the rate of GTP hydrolysis by several orders of magnitude, it has a very low affinity (~100 μM) for its substrate. The affinities of Gyp1 and Gyp7 for their substrates (K_m = 125–400 μM) are also very low (20); this is also the case of other GAPs for small G proteins. However, G proteins are associated with membranes, and the reaction thus takes place on the membrane and not in solution. Ras-GAP is located predominantly in the cytosol but translocates to the cell periphery upon tyrosine phosphorylation (38–40). The PH domain of GAP1m binds to phosphatidylinositol trisphosphate and mediates the recruitment of the protein to the plasma membrane upon epidermal growth factor-induced activation of phosphatidylinositol 3-kinase (41). Diacylglycerol enhances the activity of ARF-GAP, most likely by promoting the recruitment of the enzyme on the membrane, where the local concentration of the substrate is high (42). Thus, different mechanisms are used to drive the translocation of GAPs toward membrane compartments. Oishi et al. (43) reported that Rab3-GAP was enriched in the synaptic soluble fraction. Although the vast majority of Rab3-GAP was found in the cytosol, some immunoreactivity was found in the membrane fractions. Furthermore, the immunostaining of Rab3-GAP in primary cultured hippocampal neurons was not diffuse but enriched at synaptic contacts. It is noteworthy that Burstein et al. (34) found that a Rab3-GAP activity from rat brain was equally distributed between cytosolic and membrane fractions. Recruitment of Rab3-
GAP on membranes could thus account for the targeting of Rab3-GAP to nerve terminals and, most importantly, could facilitate the interaction with its substrates by increasing its local concentration. An attractive possibility is that the subcellular localization of Rab3-GAP is dependent on the noncatalytic subunit.

In agreement with Oishi et al. (43), we also observed that overexpression of wild type Rab3-GAP in PC12 cells had no detectable effect on the secretory activity (not shown). Thus, the amount of cellular Rab3-GAP is not limiting. Other factors probably control the activity of Rab3-GAP. Under the conditions used in this study (i.e. in the absence of phospholipids and of inosynpholipids of Rab3A), neither calcium ions nor calmodulin had any detectable effect on the activity of Rab3-GAP. Since a number of studies have highlighted the role of GTP hydrolysis by Rab3 in the secretory process, it will be of prime importance to characterize the parameters (like membrane recruitment) that control the activity of Rab3-GAP.

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