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Biochemical Characterization of Baculovirus-Expressed *rap*1A/ K*rev*-1 and Its Regulation by GTPase-Activating Proteins†

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Normal human *rap*1A and 35A *rap*1A (which encodes a protein with a Thr-35→Ala mutation) were cloned into a baculovirus transfer vector and expressed in Sf9 insect cells. The resulting proteins were purified, and their nucleotide binding, GTPase activities, and responsiveness to GTPase-activating proteins (GAPs) were characterized and compared with those of Rap1 purified from human neutrophils. Recombinant wild-type Rap1A bound GTPγS, GTP, and GDP with affinities similar to those observed for neutrophil Rap1 protein. The rate of exchange of GTP by Rap1 without Mg^{2+} was much slower than that by Ras. The basal GTPase activities by both recombinant proteins were lower than that observed with the neutrophil Rap1, but the GTPase activity of the neutrophil and wild-type recombinant Rap1 proteins could be stimulated to similar levels by Rap-GAP activity in neutrophil cytosol. In contrast to wild-type Rap1A, the GTPase activity of 35A Rap was unresponsive to Rap-GAP stimulation. Neither recombinant Rap1A nor neutrophil Rap1 protein GTPase activity could be stimulated by recombinant Ras-GAP at a concentration 25-fold higher than that required to hydrolyze 50% of H-Ras-bound GTP under similar conditions. These results suggest that the putative effector domains (amino acids 32 to 40) shared between Rap1 and Ras are functionally similar and interact with their respective GAPs. However, although Rap1 and Ras are identical in this region, secondary structure or additional regions must confer the ability to respond to GAPs.

ras gene products are membrane-associated proteins with molecular weights of approximately 21,000 that bind and hydrolyze GTP (for reviews, see references 2 and 8). The cellular function of these proteins has yet to be established. However, overexpression or mutation of *ras* causes transformation of cultured fibroblasts, and mutated Ras proteins have been detected in a number of human tumors. Transforming mutations decrease the intrinsic GTPase activity of Ras or decrease the affinity of the protein for guanine nucleotides. Both changes favor formation of the active, GTP-bound form of Ras in vivo (C.J.D., unpublished data). Introduction of additional mutations that prevent membrane localization of Ras blocks its ability to induce cellular transformation (51). Thus, analogous to the signal-transducing G proteins (13), *ras* gene products appear to be active in a GTP-bound state, are localized to the plasma membrane, and probably interact with other membrane-bound proteins.

Amino acid substitutions between residues 32 and 40 have been shown to destroy the transforming potential of *ras* (43, 52). Therefore, this region of Ras has been postulated to interact with an effector molecule of Ras in a biological pathway. While the intrinsic GTPase activity of Ras is low, it is stimulated 100-fold by a cytosolic protein (GTPase-activating protein; GAP) (46). The point mutations in the *ras* effector region that block the transforming potential of Ras also block the ability of GAP to stimulate GTP hydrolysis (1, 6, 27, 50). It therefore seems likely that GAP interacts with this site and may be an effector of Ras function. This notion is supported by the observation that although GAP does not stimulate the GTPase activity of transforming proteins containing Gly-12→Val and Gln-61→Lys mutations, there is still an interaction between GAP and the GTP-bound forms of these proteins (50).

Over the past 5 years, a 20- to 30-member superfamily of *ras*-related proteins has been discovered by protein purification and molecular cloning techniques. These proteins include the R-Ras (22), Ral (7), Rab (45), Rho (25), Rac (10), and Rap (19, 21, 34, 35) families. Unique GAPs have been identified for *ras* (1, 6, 12)-, *rhoA* (12)-, and *rap*1 (20, 49), and it is likely that each family interacts with specific GAPs. The *rap*1 gene products (Rap1A and Rap1B) are of particular interest because the putative effector and/or GAP interaction domain of Rap1 is identical to that of H-Ras, K-Ras, N-Ras, and R-Ras. High expression of *rap*1A mRNA has been correlated with reversion of viral K-*ras*-transformed mouse fibroblasts (and thus *rap*1A has also been called K*rev*-1 [21]). Therefore, although Rap GTPase activity is stimulated by its own GAP(s), it has been speculated that Rap might reverse cellular transformation by competing with Ras for Ras-GAP. Rap1 has been demonstrated to be a substrate for cyclic AMP-dependent protein kinase in vitro and in vivo (16, 18, 28; G.M.B. and L.A.Q., Biochem. J., in press). However, in vitro phosphorylation had no effect on the ability of purified Rap protein to bind or hydrolyze guanine nucleotides (16, 28; G.M.B. and L.A.Q., in press).

We used the baculovirus expression system (23, 45) to produce recombinant wild-type (WT) Rap1A and 35A Rap1A (which contains a Thr-35→Ala mutation) in the Sf9 insect cell line. These proteins were purified, and their nucleotide-binding properties were compared with each other and with those of Rap1 protein from human neutrophils. In addition, we determined whether alteration of the putative effector region of Rap blocks the ability of Rap to be

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regulated by Rap-GAP(s) and whether the GTPase activity of Rap can be stimulated by Ras-GAP.

MATERIALS AND METHODS

Materials. [α-^{32}P]GTP (800 Ci/mmol), [α-^{32}P]GDP (3,000 Ci/mmol), and [γ-^{32}P]GTP (10 to 50 or 6,000 Ci/mmol) were from Dupont, NEN Research Products. Purified recombinant Ras-GAP was prepared as already described (47). Rapl from human neutrophil membranes was purified as previously described (G.M.B. and L.A.Q., in press). The neutrophil protein was determined to be Rapl by N-terminal amino acid sequencing, reactivity with anti-Rap antibodies R52-1 and R64-1, and phosphorylation by cyclic AMP-dependent protein kinase (G.M.B. and L.A.Q., in press). Sf9 cells were from Max Summers (44) and were grown as previously described (33). All other materials were as reported elsewhere (4, 5; G.M.B. and L.A.Q., in press).

Molecular cloning and oligonucleotide-directed mutagenesis of the *rap*1A gene. A 35-amino-acid N-terminal sequence was obtained from a purified 22-kilodalton GTP-binding protein isolated from human neutrophil membranes (5). Two synthetic oligonucleotides corresponding to the amino acid sequence were used to screen a dimethyl formamide-differentiated human promyelocytic leukemia (HL60) cell λ gt11 cDNA library (37). A 1.3-kilobase *Eco*RI fragment was obtained from a clone that was positive with both probes and subcloned into M13mp18 for dideoxy sequencing (38) and oligonucleotide-directed mutagenesis (9, 53). The predicted amino acid sequence produced by the gene was identical to that subsequently reported for Rap1A (34), Krev-1 (21), and *smg*-p21 (19). The coding and noncoding DNA sequences were identical to those of K*rev*-1, except for a single-base change which did not alter the amino acid sequence.

An A→T base change was introduced at codon 6 to generate a *Hin*dIII site without altering the amino acid sequence. An A→G base change was subsequently made in codon 35 to create a Thr→Ala mutation. A 0.7-kilobase-pair *Hin*dIII-*Bgl*II fragment of 35A *rap*1A containing codons 6 to 184 and the 3′-untranslated region was swapped with codons 6 to 189 of H-*ras* in the pAT*ras* bacterial expression vector (9). Expression of *rap*1A was induced with isopropyl-β-D-thiogalactopyranoside in *Escherichia coli* PR13Q, with maximal protein yield at 6 to 8 h postinduction. The 0.7-kilobase-pair *Hin*dIII-*Bgl*II *rap*1A fragment was also subcloned into the *Kpn*I-*Bgl*II sites of the pAcC12 baculovirus vector polylinker. Complementary synthetic 18-base (CAAA TATGCGGGAATACA) and 26-base (AGCTTGTATTCC CGCATATTTGGTAC) oligonucleotides were annealed together to generate 5′ *Kpn*I and 3′ *Hin*dIII sticky ends to complete the *rap*1A sequence (codons 1 to 5) and to enable initiation of the coding sequence 52 base pairs downstream from the original polyhedron protein initiation site. pAcC12 was derived from pVL941 (24) by deleting the unique *Eco*RI site at 7.15 kilobases and introducing a polylinker sequence (GGTACCCGGGCTGCAGAATTCTAGATCTCGAGCT CCATGGTGGATCC) at the unique *Bam*HI insertion site. Generation and isolation of a recombinant virus expressing 35A *rap*1A was performed essentially as described by Summers and Smith (44). A *Hin*dIII-*Eco*RI fragment encoding WT *rap*1A residues 6 to 184 was also synthetically generated, as will be described elsewhere (K. Zhang and D. R. Lowy, unpublished data) and was introduced into the pAcC12 vector as described above. This construct was used for expression of WT *rap*1A.

Purification of WT and 35A Rap1A proteins. *Spodoptera frugiperda* (Sf9) cells were infected with a recombinant WT *rap*1A-carrying virus and cultured in suspension. At 3 days postinfection, cells were harvested and cell pellets were stored at −70°C. A 25-g cell pellet (from an approximately 2-liter cell culture) was disrupted by Dounce homogenization and two 10-s pulses of sonication in 5 volumes of 25 mM Tris (pH 8.0)–1 mM EDTA–1 mM dithiothreitol–5 mM $MgCl_2$ (TEDM) containing 20 mM NaCl, 50 U of aprotinin per ml, and 1 mM phenylmethylsulfonyl fluoride. Sodium cholate was added to 0.9% (wt/vol) in 2 volumes of homogenization buffer, and proteins were extracted for 60 min on ice. The cholate extract was centrifuged (130,000 × g, 80 min), and the supernatant was diluted with 3 volumes of TEDM containing 0.9% cholate (DEAE column buffer). The sample was loaded onto a DEAE-Sephacel column (600 ml) and washed once with column buffer, and proteins were eluted with a 1.2-liter linear 0 to 225 mM NaCl gradient. A single peak of GPTγS-binding activity was eluted at approximately 100 mM NaCl, which coincided with the major protein band of 22 kilodaltons. The peak fractions were pooled, concentrated with an Amicon PM10 membrane, and loaded onto a column (2.5 by 100 cm) of Ultragel AcA44, which was preequilibrated with TEDM containing 0.9% cholate and 100 mM NaCl. The peak fractions of GTPγS-binding protein eluted from this column were pooled, and the cholate concentration was adjusted to 0.2% with TEDM–100 mM NaCl before loading onto a 50-ml column of heptylamine-Sepharose. The column was washed with 2 volumes of TEDM containing 0.2% cholate and 100 mM NaCl, and a single peak of GTPγS-binding material was eluted in the middle of a 200-ml linear gradient of TEDM–0.2% cholate–250 mM NaCl to 1.2% cholate–50 mM NaCl.

35A Rap1A was similarly purified from a 500-ml suspension culture by DEAE-Sephacel (110-ml column) and heptylamine-Sepharose chromatographies.

Guanine nucleotide binding and GTPase activity. To determine the K_d value for GTPγS binding to Rap1A protein, 2 pmol of purified Rap1A was incubated with 25 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; pH 8.0)–1 mM dithiothreitol–0.09% lubrol PX and various concentrations of GTPγS (25,000 cpm/pmol) in 10 mM $MgCl_2$ or an excess of EDTA (0.25 mM $MgCl_2$, 4 mM EDTA). Incubations were for 5 min without or 120 min with Mg^{2+} (at which point equilibrium binding was achieved) and were terminated by addition of 2 ml of stop mixture (25 mM Tris hydrochloride [pH 8.0], 100 mM NaCl, 30 mM $MgCl_2$, 2 mM dithiothreitol, 1 mg of bovine serum albumin per ml). Nonspecific binding was determined by performing experiments with unlabeled GTPγS at a concentration 500 times the K_d; these values were then subtracted from the total binding. Binding to Rap1A was quantitated by vacuum filtration on BA-85 nitrocellulose filters and liquid scintillation counting (29). For nucleotide competition curves, GTPγS binding was performed at 50 mM, which was the K_d value for the nucleotide in $MgCl_2$. Rap1A (2 pmol) was incubated as described above with 50 nM GTPγS (200,000 cpm/pmol) in the indicated concentrations of unlabeled GTP or GDP, and the samples were processed as described previously. GTPase activity was determined as already described (36), by using 200 ng of nucleotide-binding activity per assay point over a 90-min time course.

Nucleotide off rates. α-^{32}P-labeled GTP or GDP (0.5 μM; 13,200 cpm/pmol) was incubated with Rap protein (100 nM) as described in the GAP assay. After 5 min at 30°C, $MgCl_2$ was added to a final concentration of 1 mM and the tubes were placed on ice. Samples (37.5 μl) were then prewarmed

to 30°C, and 62.5 μl of 25 mM HEPES (pH 7.5)–710 μM GTP–1.25 mg of bovine serum albumin per ml containing either 5 mM $MgCl_2$ or 16 mM EDTA was added. Samples were incubated for the indicated times, and reactions were stopped by transferring 15-μl samples into 2 ml of ice-cold stop mixture. The amount of the remaining Rap-bound nucleotide was then determined by filtration.

GAP assay. GAP activity was assessed by preequilibrating Rap1 or Ras protein with [γ-^{32}P]GTP and then quantitating the decrease in the radiolabeled protein-GTP complex following incubation with neutrophil cytosol or recombinant Ras-GAP. Cytosol from 6×10^7 cells was recovered in 50 ml following N_2 cavitation and centrifugation (3). Rap1 and Ras (125 nM) were preequilibrated with [γ-^{32}P]GTP by incubation (5 min, 30°C) in 25 mM HEPES (pH 8.0)–4.5 mM excess of EDTA–1 mM dithiothreitol–0.09% lubrol PX–1 μM [γ-^{32}P]GTP (10 to 50 Ci/mmol). A standard GAP assay was performed for 5 min at room temperature in a final volume of 50 μl containing 25 mM HEPES (pH 7.5), 5 mM $MgCl_2$, 1 mM EDTA, 50 μg of bovine serum albumin, 0.8 pmol of Rap1 protein (10^5 cpm of prebound [γ-^{32}P]GTP) plus GAP or a buffer blank. For studies with Ras-GAP, the concentrations of GTP and GTP-bound protein were decreased 30-fold and GTP of higher specific activity (6,000 Ci/mmol) was used. Reactions were terminated by addition of 2 ml of the ice-cold nucleotide-binding stop mixture. The amount of protein-bound GTP was determined by nitrocellulose filtration.

Miscellaneous. Cell lysate and cytosol protein concentrations were determined by the Pierce BCA assay. Purified protein concentrations were determined with amido black (41). Western blotting (immunoblotting) was performed as already described (15), with ^{125}I-labeled goat anti-rabbit immunoglobulin G as the second antibody. The primary antisera were raised against peptides representing Rap1A amino acids 118 to 129 (R52-1) or 127 to 137 (R64-1) essentially as already described (4) and did not cross-react with the low-molecular-weight GTP-binding proteins, ADP-ribosylation factor (ARF), H-Ras, Aplysia Rho, Rac, or neutrophil G_{24K}. There was no immunoreactivity of R64-1 with lysates of Sf9 cells infected with wild-type virus.

RESULTS

Purification and characterization of recombinant Rap1A protein. Expression of *rap1A* in isopropyl-β-D-thiogalactopyranoside-induced *E. coli* varied between 0 and 5% of total cell protein. This protein was insoluble, required extraction with 3 M guanidine hydrochloride, and bound guanine nucleotides poorly. The baculovirus expression system was therefore used to generate recombinant Rap1A protein. Both WT and Rap1A protein represented approximately 5 to 8% of total virus-infected Sf9 cell protein (Fig. 1, lanes 2 and 3). The identities of these proteins were confirmed with polyclonal antisera (R52-1 and R64-1) raised against peptides unique to the Rap1 sequence (lanes 4 to 7). Following extraction of total cell protein in 0.9% sodium cholate and sequential chromatography on DEAE-Sephacel, Ultragel AcA44, and heptylamine-Sepharose, recombinant WT Rap1A was purified to approximately 90% homogeneity with just one significant contaminant (Fig. 1, lane 9). A 4.5-mg quantity of GTPγS-binding activity was recovered from 19 mg of activity in the cholate extract, and approximately 30% of the purified protein bound guanine nucleotides. 35A Rap1A was partially purified by the same approach but without the AcA44 gel filtration step (Fig. 1, lane 3).

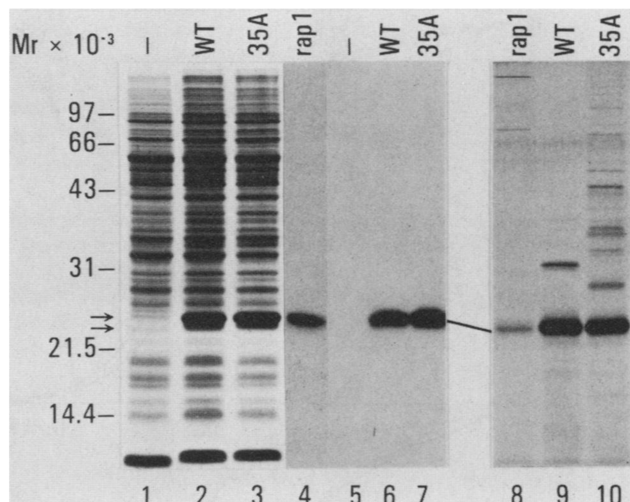

FIG. 1. Expression of Rap1A proteins in SF9 cells. Lanes 1 to 3 show Coomassie blue staining of total protein (50 μg) from Sf9 cells infected with recombinant baculovirus containing WT or 35A *rap1A*, or a clone which failed to translate as indicated. Lanes 4 to 7 show Western blots performed with the R64-1 anti-Rap1 peptide antibody on 75 μg of protein from the indicated cell lysates or 250 ng of purified neutrophil Rap1 protein. A similar result was obtained with antibody R52-1 (data not shown). Lanes 8 to 10 show silver staining of the purified recombinant Rap1A proteins (250 ng) or neutrophil Rap1 (80 ng). Both gels were 13% acrylamide.

The affinity of N-Ras for GTP has been shown to be lower with than without Mg^{2+} (14), and we have obtained similar results with Rap1 purified from human neutrophils (G.M.B. and L.A.Q., in press). Therefore, the affinities of the purified recombinant Rap1A proteins for guanine nucleotides were determined in 10 mM Mg^{2+} or an excess of EDTA (0.25 mM $MgCl_2$, 4 mM EDTA). The affinity of recombinant WT Rap1A for [^{35}S]GTPγS was similar to that of neutrophil Rap1 with (apparent K_d, 54 ± 4 nM) or without (apparent K_d, 141 ± 5 nM) Mg^{2+}. Apparent K_d values for GTP and GDP, determined from competition curves with 50 nM GTPγS in Mg^{2+}, were 170 ± 17 and 233 ± 22 nM, respectively. As observed with neutrophil Rap1, the affinity of WT Rap1A for GTP (apparent K_d, 105 ± 20 nM) was greater than that for GDP (497 ± 15 nM) with excess EDTA (Fig. 2C).

No significant differences in the off rates of GTP and GDP from WT Rap1A were observed in millimolar Mg^{2+}, and little nucleotide release occurred over a 100-min incubation period (Fig. 3A). Without Mg^{2+}, the rate of release of GDP was rapid (Fig. 3C). However, the dissociation rate of GTP was extremely slow, with a $t_{1/2}$ of 50 min. Similar results were obtained with purified neutrophil Rap1 (Table 1).

Stimulation of Rap GTPase activity by cytosolic GAP. The basal GTPase activity of the recombinant WT Rap1A protein was approximately 0.0005 min^{-1} compared with 0.01 min^{-1} for neutrophil Rap1 (Table 1). However, the rate of GTP hydrolysis by WT Rap1A was stimulated by neutrophil cytosol in a dose- and time-dependent manner (65 μg of cytosol protein hydrolyzed greater than 90% of the Rap1-bound GTP in 15 min (data not shown). A concentration of cytosol that promoted 50% hydrolysis of Rap1-bound GTP in a 5-min assay was routinely used to remain within the linear region of the activity curve, and this rate was similar to that achieved by neutrophil Rap1 with cytosol (Fig. 4A and B). The ability of cytosol to increase the rate of loss of protein-bound $^{32}PO_4^-$ was not due to enhanced nucleotide ex-

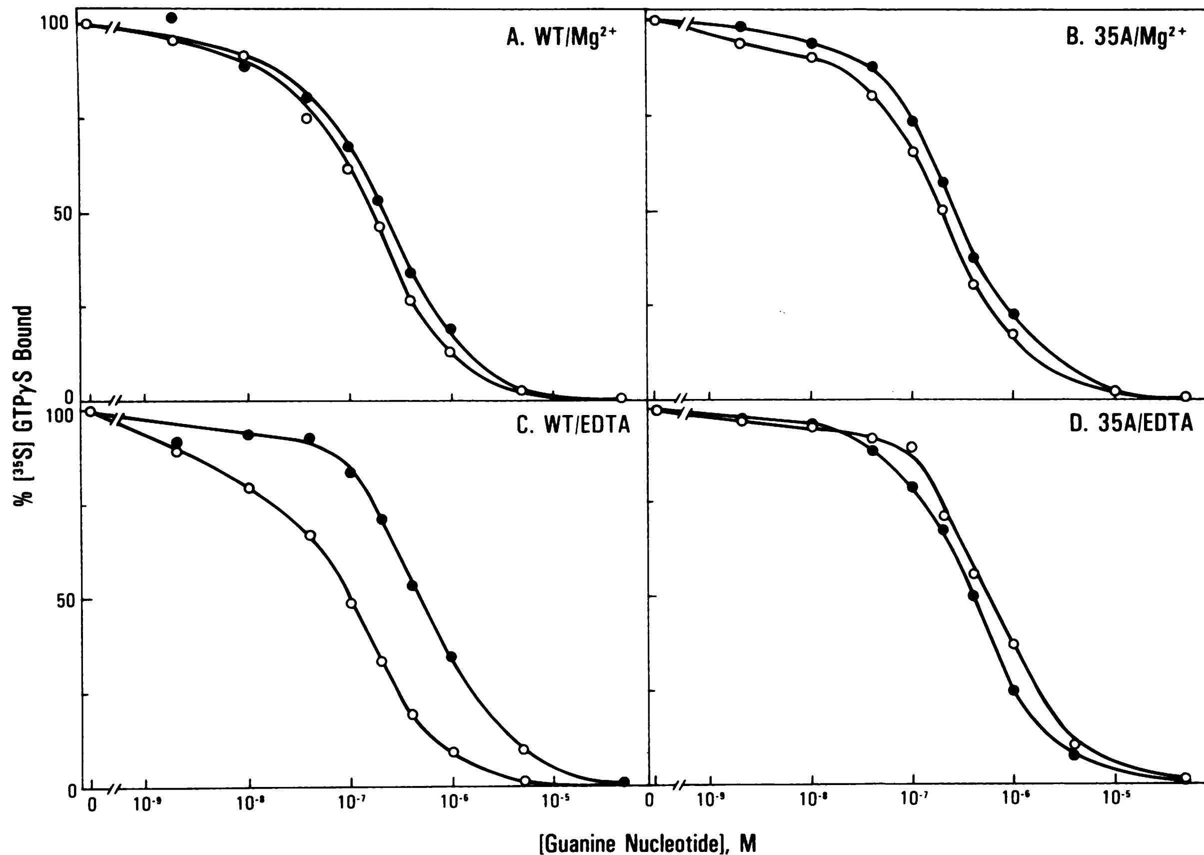

FIG. 2. Affinity of recombinant Rap1A proteins for GTP and GDP with and without Mg^{2+}. The abilities of GTP and GDP to compete with [^{35}S]GTPγS (50 nM) binding to recombinant WT and 35A Rap1A protein were assessed in 10 mM Mg^{2+} or an excess of EDTA. Incubations were for 120 min with or 5 min without Mg^{2+}. Competition curves are shown with open symbols for GTP and closed symbols for GDP. From 19,000 to 24,000 cpm was bound with no competing nucleotide. The data are representative of three experiments performed in duplicate.

change, as indicated by the retention of protein-bound [α-^{32}P]GTP (data not shown). This cytosolic activity was abolished by boiling (Fig. 4) but not by removal of low-molecular-weight material on Sephadex G25 (data not shown), suggesting the presence of a GTPase-activating protein for Rap (Rap-GAP) in neutrophil cytosol. GAP activity was confirmed by measuring the conversion of [α-^{32}P]GTP to [α-^{32}P]GDP by thin-layer chromatography with 0.75 M KH_2PO_4 (pH 3.3) as the solvent (data not shown).

Consequences of a 35A-encoding mutation in the putative effector domain of Rap1A. An Ala-35 substitution in the putative effector domain of Ras does not affect its nucleotide-binding properties in Mg^{2+} but does block the ability of GAP to stimulate GTP hydrolysis (6). Therefore, a Thr-35A→Ala substitution was introduced into Rap1A to determine whether this region is functionally analogous to the Ras effector domain.

The apparent affinities of 35A Rap1A for GTPγS and GDP were similar to those obtained for the WT protein, both with (65 ± 8 and 230 ± 15 nM, respectively) and without (156 ± 23 and 457 ± 102 nM, respectively) millimolar $MgCl_2$ (Fig. 2B and D; data not shown). With Mg^{2+}, the affinity of 35A Rap1A for GTP was similar to that of the WT protein, having a K_d value of 203 ± 26 nM (Fig. 2B). However, without Mg^{2+}, 35A *rap*1A had a much reduced affinity for GTP (560 ± 64 nM; Fig. 2D). Similarly to the analogous mutation in Ras (6), conversion of Thr-35 to Ala in *rap*1A blocked the

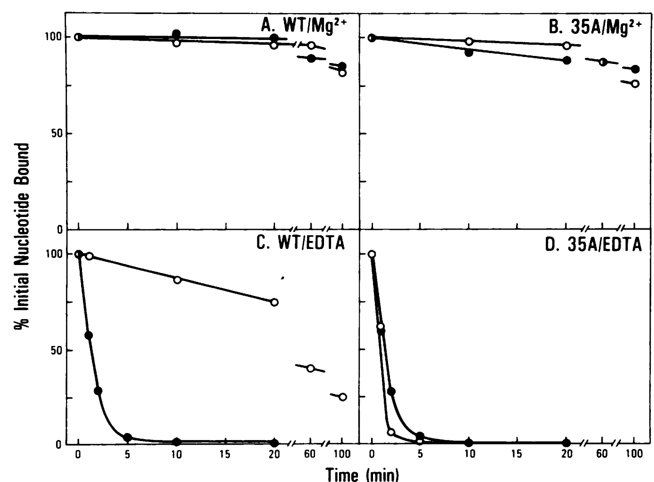

FIG. 3. Nucleotide exchange rates of recombinant Rap1A proteins with and without millimolar $MgCl_2$. Rap1A protein was prelabeled with [α-^{32}P]GTP (open symbols) or GDP (closed symbols) and then incubated with an excess of unlabeled GTP with or without Mg^{2+} as described in Materials and Methods, and the rate of loss of the prebound nucleotide was determined. Approximately 10,000 cpm was bound at time zero. The data represent two experiments performed in duplicate.

TABLE 1. Summary of the biochemical properties of human- and baculovirus-produced Rap proteins

Protein	K_d for nucleotide (nM)			GTP off rate without Mg^{2+} ($t_{1/2}$ [min])	GTPase activity (pmol/nmol per min)	Stimulation by Rap-GAP[a]
	GTPγS plus Mg^{2+}	GTP				
		Plus Mg^{2+}	Minus Mg^{2+}			
Neutrophil Rap1	40[b]	100[b]	20[b]	~45	10[b]	+
WT Rap1A	54	170	105	~50	0.5	+
35A Rap1A	65	203	560	1	0.5	−

[a] There was no stimulation by Ras-GAP.
[b] G.M.B. and L.A.Q., in press.

ability of the GTPase activity of the resulting protein to be stimulated by Rap-GAP (Fig. 4C).

No stimulation of Rap GTPase activity by Ras-GAP. Since the putative effector site of Ras (amino acid residues 32 to 40) is completely conserved in Rap1, it was of interest to determine whether Rap1 GTPase activity could be stimulated by Ras-GAP. Recombinant Ras-GAP, at a concentration of 370 ng/ml, stimulated hydrolysis of 50% of Ras-bound GTP during 5 min of incubation and hydrolyzed more than 90% of the bound GTP at a concentration of 1.85 μg/ml (Fig. 5). However, Ras-GAP was unable to stimulate the GTPase activity of recombinant or neutrophil Rap1 protein when incubated with concentrations of up to 10 μg/ml. A 25-μg sample of cytosol was used as a positive control.

DISCUSSION

We produced recombinant human Rap1A protein in Sf9 insect cells by using the baculovirus expression system. The biochemical properties of WT Rap1A and a putative effector site mutant, 35A Rap1A, were characterized and compared with those of human neutrophil Rap1. The level of expression of Rap1 was relatively high in human neutrophils (at least 0.2% of membrane protein). However, to obtain an abundant source of Rap1A that was readily separable from other Ras-related GTP-binding proteins (including the *rap*1B and *rap*2 gene products) for biochemical analysis, it was necessary to generate a source of recombinant protein. We found that expression of *rap*1A by using the baculovirus system was advantageous because, in contrast to bacterially produced protein, the Rap1A produced by Sf9 cells was both soluble and biochemically active. Overall, the similar biochemical properties of insect cell-expressed and neutrophil-derived proteins demonstrated the benefits of the baculovirus expression system for isolation of recombinant Rap1A protein.

An advantage of the baculovirus system is that insect cells perform many of the posttranslational modifications of proteins characteristic of mammalian cells (23). Sf9 cell Rap1A migrated as a doublet on polyacrylamide gels, and the lower band, which comigrated with neutrophil membrane Rap1, has been shown to be carboxyl methylated, farnesylated, and translocated to the plasma membrane, similar to the mammalian protein (J. E. Buss, L. A. Quilliam, K. Kato, P. J. Casey, P. A. Solski, G. Wong, R. Clark, F. McCormick, G. M. Bokoch, and C. J. Der, submitted for publication). Palmitoylation of H-Ras has also been described recently in Sf9 cells (31), making this system useful for production and purification of fully processed Ras and Ras-related proteins. Similar to Rap1 purified from human neutrophils, the baculovirus-expressed protein served as a substrate for cyclic AMP-dependent protein kinase in vitro, being phosphorylated with a stoichiometry of approximately one phosphate molecule per molecule of GTPγS bound (data

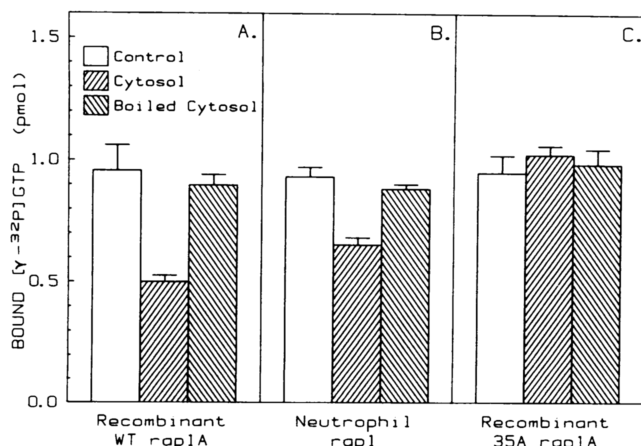

FIG. 4. Effect of neutrophil cytosol Rap-GAP on Rap1 protein GTPase activities. A 1-pmol sample of a Rap1-GTP complex was incubated with untreated or heat-denatured (3 min, 110°C) neutrophil cytosol (25 μg of protein) for 5 min as described in Materials and Methods, and the amount of remaining *Rap*-bound GTP was determined by nitrocellulose filtration. The data (mean ± standard deviation; n = 3) are representative of three to eight experiments.

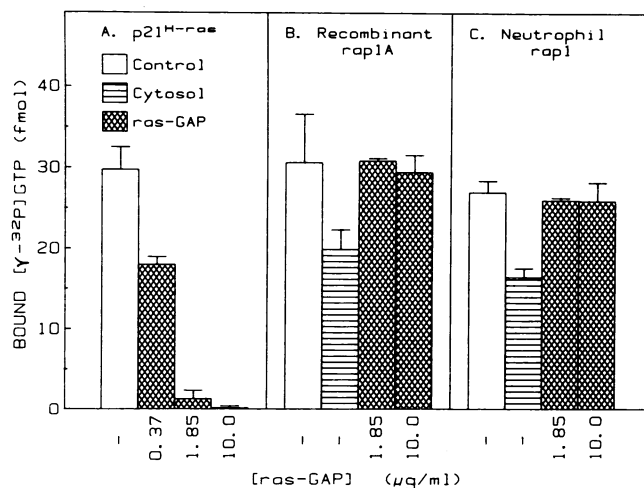

FIG. 5. Effect of Ras-GAP on the rates of GTP hydrolysis by H-Ras and Rap1. The indicated amounts of Rap1-GTP or Ras-GTP were incubated in the GAP assay with cytosol (25 μg of protein) or the indicated concentration of purified, recombinant Ras-GAP, and the extent of GTP hydrolysis was determined by filtration assay. The data (mean ± standard deviation; n = 3) are representative of at least three experiments.

not shown). Therefore, insect cell-produced Rap1A appears to be physically homologous to human cell-derived protein and capable of undergoing similar posttranslational modifications in vivo and in vitro.

Before using the recombinant Rap1A proteins in biochemical and biological assays, it was important to compare the intrinsic properties of these proteins with those of mammalian Rap1. The guanine nucleotide-binding properties of WT Rap1A were similar to those of recombinant Ras (14) and Rap1 purified from mammalian sources, although some minor differences were observed. Specifically, the affinities for GTP and GDP were 1.5- to 5-fold lower than those observed for neutrophil Rap1 (Table 1); however, the K_d value obtained for GTPγS binding to baculovirus-expressed Rap1A in Mg^{2+} was similar to that reported for Rap1 purified from several mammalian sources (19, 30; Table 1). As observed for Ras and neutrophil Rap1 (14; G.M.B. and L.A.Q., in press) without Mg^{2+}, the affinity of Rap1A for GTP was higher than that for GDP. Hall and Self (14) proposed that the rapid off rate of GDP from Ras and the higher affinity of the protein for GTP than for GDP at very low Mg^{2+} levels in vitro could be similar to that which occurs in vivo in response to a cell surface receptor-nucleotide exchange factor. This may also be true for the Rap1 protein. Since no release of [α-^{32}P]GTP was stimulated by neutrophil cytosol, such a factor is apparently not abundant in this fraction of the cell.

Although we cloned the *rap*1A gene from a human granulocytic cell library (37), we are unable to exclude the possibility that the Rap1 protein purified from human neutrophils is partially or wholly Rap1B (which is 95% homologous to Rap1A; 35) by N-terminal sequencing or cross-reactivity with R52-1 and R64-1 antisera. Thus, the minor differences between the properties of recombinant and neutrophil Rap1 proteins could be due to incorrect folding or posttranslational modification of the recombinant protein or differences between Rap1A and Rap1B. This latter explanation is suggested by the fact that *smg*-p21 purified from bovine brain and human platelets, which has biochemical properties similar to those of neutrophil Rap1, has recently been shown to be Rap1B (26).

We observed that 35A Rap1A had a decreased affinity for GTP without Mg^{2+}. The reason for this is not clear; however, it is of interest that the hydroxyl group of Thr-35 (which is the only amino acid residue in this domain of Rap that is conserved among all members of the Ras superfamily) has been implicated in interaction with Mg^{2+} in the GTP-binding domain of H-Ras and that the main chain amide is probably hydrogen bonded to an oxygen in the gamma phosphate of the GTP molecule (32). Therefore, the decreased affinity for GTP but not GTPγS or GDP might be due to altered interaction with the gamma phosphate of GTP.

The slow rate of release of GTP from Rap1 (recombinant or from human neutrophils) without Mg^{2+} contrasts with the relatively rapid dissociation rate of GTP from Ras (14, 17). Differences between the primary sequences of the nucleotide-binding domains of Rap and Ras occur at residues 11, 61, and 144. Although residues 11 and 144 vary among Ras-related GTP-binding proteins, the glutamine at position 61 is conserved in all but the *rap* and *rap*-related D*ras*3 (42) gene products (which possess a threonine at this position; 8, 39). It is therefore possible that Thr-61 is responsible for the slow GTP exchange rate of Rap1. Indeed, the dissociation rate of GTP from Glu-61→His Ras is strongly reduced in Mg^{2+} (17). Alternatively, the variable region between residues 63 and 96 might be responsible for the differences in nucleotide binding. Antibody Y13-259, which binds to a region of Ras encompassing residues 63 to 73 (43), can dramatically reduce the off rate of [^{3}H]GTP from Ras (1, 46). The divergence of this region in Rap1A could conceivably have a similar effect on the GTP off rate of this protein. The faster off rate of 35A Rap is most probably due to the lower affinity of this protein for GTP than that of WT Rap1A.

The observation that the 35A mutation in Rap1A prevented stimulation of GTPase activity by Rap-GAP suggests that this region performs a similar role in both Ras and Rap1. Whether this region is part of the GAP-binding site of Rap1 or merely undergoes a conformational change upon binding to GAP has not been established. However, the former is most likely, since Rap1A can bind recombinant Ras-GAP with high affinity (M. Frech, J. John, V. Pizon, P. Chardin, A. Tavitian, R. Clark, F. McCormick, and A. Wittinghofer, submitted for publication). Two forms of Rap-specific GAP have been reported (20), but it has not been demonstrated whether Rap1 can respond to Ras-GAP, as suggested by the shared effector domains. Our results demonstrate that even at concentrations much higher than those required to stimulate Ras, Ras-GAP cannot modulate the GTPase activity of Rap1.

Since Rap1 does not respond to Ras-GAP and Takai and co-workers (20, 49) have demonstrated that Ras GTPase activity is not stimulated by Rap-GAP(s), the primary sequence of residues 30 to 44 must not be sufficient to enable bound GAPs to stimulate the GTPase activity of Ras proteins. By using a *ras-rap* chimera, we have shown that the transforming activity of *ras* resides in the N-terminal half of the protein (J. E. Buss et al., submitted for publication). The regions flanking the putative effector-binding site of Ras and Rap that are unique to these proteins and are the regions of most divergence in the N termini of Ras and Rap could provide the specificity for GAPs to modulate the GTPase activities through the region of residues 30 to 40. This is consistent with the observation that a peptide corresponding to amino acid residues 17 to 32 of Ras can reduce Ras–Ras-GAP interaction (40). However, it has also recently been shown that purified Ras-GAP stimulates the GTPase activity of the R-ras gene product, the sequence of which diverges from H-Ras, K-Ras, and N-Ras in the regions flanking the putative effector domain (48). Thus, it seems more likely that the secondary structure of the effector region, alone or in conjunction with unique sites more distant in the primary structure (such as the region encompassing residue 61), dictates whether there is a productive interaction between these Ras-related proteins and their respective GAPs.

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