Identification of Distinct Cytoplasmic Targets for ras/R-ras and rho Regulatory Proteins*

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Michelle D. Garrett, Annette J. Self, Connie van Oers, and Alan Hall
From the Institute of Cancer Research, Chester Beatty Laboratories, Fulham Road, London SW3 6JB, Great Britain

The protein products of the mammalian ras genes, p21\textsuperscript{ras}, are regulatory guanine nucleotide binding proteins that are involved in the control of cell proliferation, though the exact biochemical processes regulated are unknown. Recently a cytoplasmic protein has been identified that interacts with and increases the GTPase activity of p21\textsuperscript{ras}. It has been shown that this GTPase-activating protein, or GAP, interacts with the effector domain of ras, leading us and others to propose that GAP may be the target for regulation by p21\textsuperscript{ras}. It has become apparent that ras is part of a much larger family of proteins, and at least 15 ras-related genes have now been identified in the mammalian genome. Each encodes a small (about 21 kDa) guanine nucleotide binding protein, but the functions of none of these regulatory molecules are known. We report here that mammalian cytoplasmic extracts contain GAP-like activity toward the products of two other ras-related genes, R-ras and rho. It appears that p23\textsuperscript{R-ras} interacts with the same 125-kDa GAP protein as p21\textsuperscript{ras} whereas p21\textsuperscript{rho} interacts with a distinct 29-kDa protein, rho GAP.

Amino acid substitutions in p21\textsuperscript{ras} have been detected in 20-40\% of some human malignancies, and the role of this protein in the control of cell proliferation has been the subject of much investigation (see Ref. 1 for review (2)). The p21\textsuperscript{ras} proteins are located on the cytoplasmic face of the plasma membrane, and they bind GTP/GDP and have an intrinsic GTPase activity suggesting that they behave similarly to regulatory G proteins (1). In agreement with this it has been shown that it is the GTP form of the protein that is active and that hydrolysis of GTP is not required for activity (3, 4). Ras genes have been detected in all eukaryotic species analyzed including yeast; mammalian genomes contain three ras genes, and Saccharomyces cerevisiae has two (RAS1 and RAS2). Detailed genetic and biochemical analysis in S. cerevisiae has shown that in this organism ras interacts with and controls the activity of adenylate cyclase and that at least one of the two ras genes is required for cell viability (5, 6). Attempts to find changes in adenylate cyclase activity in mammalian cells after introducing constitutively activated (oncogenic) ras genes have failed (7). Several groups have found evidence for changes in phospholipid turnover after transfection of ras genes into cells (8-10); however, the biochemical processes regulated by mammalian ras remain obscure.

Recently a cytoplasmic protein, GAP,\textsuperscript{1} has been detected that interacts with and increases the GTPase activity of normal p21\textsuperscript{ras} but has no effect on the GTPase activity of oncogenically activated proteins (4). Mutational analysis has revealed that GAP binds to a region of ras previously designated the effector site (amino acids 32-40) suggesting that GAP may indeed be the target for regulation by ras (11, 12). This 125-kDa protein has recently been purified (13), and the availability of GAP cDNA clones should help resolve if this protein is the target of ras control.

It is now clear that ras is part of a much larger superfamily of genes. Probes derived from ras genes have been used to identify at least 15 new cDNA and genomic clones, which after sequence analysis have been found to encode small (about 21 kDa) guanine nucleotide binding proteins. Five genes, raf, R-ras, rap1A, rap1B, and rap2, are most closely related to ras with 50\% amino acid homology (14-16). The rho family has around 30\% amino acid homology with ras though the three members of this family (A, B, C) are themselves around 95\% identical with each other (17, 18). As with the three ras genes, almost all the sequence divergence is located toward the carboxyl terminus. Finally, the rab family of genes has been described with around 30\% homology to ras (19). Within the four members (1-4) of this family there is around 40\% identity. In all these ras-related genes amino acids known to be essential for guanine nucleotide binding and membrane localization are highly conserved.

Unraveling the biochemical processes controlled by each of these regulatory proteins will be a difficult problem. In S. cerevisiae a genetic approach is possible, and it has been shown that deletion of one of the two RHO genes, RHO-1, is lethal (20). Alterations at the RHO loci in yeast have no effect on adenylate cyclase activity, and it is likely that these regulatory molecules control processes distinct from RAS. rab-1 appears to be the mammalian homologue of the yeast YPT gene which encodes a 23-kDa protein thought to be localized in the Golgi and involved in the secretory pathway (19, 21). In mammalian cells, the identification of GAP, through its effect on the GTPase activity of ras, suggests an approach to looking for targets of ras-related gene products.

We report here the biochemical analysis of the products of two ras-related genes, R-ras and rhoA, purified from Escherichia coli expression systems. The proteins, p23\textsuperscript{R-ras} and p21\textsuperscript{rho}, bind GTP/GDP and have an intrinsic GTPase activity. We have identified GAP-like activities for both R-ras and rho in addition to ras in cytoplasmic extracts of a variety of mammalian cells and Xenopus oocytes, but not in S. cerevisiae.

**EXPERIMENTAL PROCEDURES**

cDNA derived from the human rhoA gene (22) was expressed in E. coli in an identical way to that previously described for the ras proteins (23, 24). Briefly, a HindIII site was engineered immediately

\footnote{The abbreviations used are: GAP, GTPase-activating protein; FPLC, fast protein liquid chromatography; tryp promoter, tryptophan operon promoter from E. coli.}
upstream of the start codon for rhoA using site-directed mutagenesis. The cDNA was then inserted downstream of the tryp promoter (23). An additional rho-expressing plasmid was constructed with Gly-14 altered to Val-14, an equivalent change to the oncogenic activating mutation Val-12 in p21\textsuperscript{ras}. An expression plasmid containing the R-ras cDNA downstream of the tryp promoter was kindly provided by D. Lowe, Genentech (25). All three proteins were grown in E. coli overnight, and the following day the cells were washed, transferred to minimal medium at an 8-fold dilution, and grown for a further 4 h. Cells were harvested and normal rho, Val-14 rho, and normal R-ras proteins purified in Mg\textsuperscript{2+}-containing buffers to preserve bound nucleotide as previously described (24). GTP/GDP nucleotide exchange rates were determined as described previously (24). GTP hydrolysis rates were determined by first incubating purified protein (4 \mu M) with [\gamma-\textsuperscript{32P}]GTP (10 \mu M) (1 Ci/mmol, Amersham Corp.) in low Mg\textsuperscript{2+} using a 25-ml reaction volume containing 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.1 mM dithiothreitol for 10 min at 30 °C. The exchange reaction was stopped by placing the reaction on ice and adding MgCl\textsubscript{2} to a final concentration of 5 mM. The intrinsic GTPase rates were determined by incubating the [\gamma-\textsuperscript{32P}]GTP-loaded protein (0.4 \mu M) at 30 °C in 30 \mu l of buffer A (50 mM Tris-HCl, pH 7.5, 0.1 mM dithiothreitol, 5 mM MgCl\textsubscript{2}) containing bovine serum albumin (1 mg/ml) and cold GTP (1 \mu M). At various times, aliquots (5 \mu l) were removed, diluted with ice-cold buffer A (1 ml), and the amount of radioactivity still bound to the proteins was determined by Millipore filtration and scintillation counting. Cytoplasmic extracts from mammalian cells, Xenopus oocytes, or S. cerevisiae were prepared as described (11). The effect of these extracts on the GTPase rates as determined by addition of extract to the GTPase rate assays. The extracts had no effect on any of the nucleotide exchange rates. Preliminary characterization of GAP-like activity was carried out using FPLC chromatography. A human spleen extract (40 mg of protein) was applied to a 1-ml MonoQ HR ion-exchange column (Pharmacia LKB Biotechnology Inc.) in 20 mM Tris, pH 7.6, 10 mM NaCl, 0.1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride at 4°C. After washing, bound protein was eluted with a linear salt gradient (10-500 mM NaCl). Fractions (1 ml) were assayed for GAP activity toward ras, rho, and R-ras, and active fractions were pooled and concentrated in Centricon 10 tubes (Amicon) to 0.2 ml. The concentrate was then applied to a Superose 12 gel filtration column (31 \times 1 cm, Pharmacia LKB), and fractions (0.3 ml) were collected and assayed for GAP activity using rho, R-ras, and ras proteins.

**RESULTS AND DISCUSSION**

The yields of both R-ras and rho proteins from the E. coli expression vectors were around 0.2 mg/liter, around 20-fold less than with similar plasmid constructs containing ras cDNAs. DEAE ion exchange column chromatography followed by Sephadex G-75 gel filtration yielded p21\textsuperscript{ras} and p21\textsuperscript{ras} protein of around 50% purity with no contaminating guanine nucleotide binding or phosphatase activity. The purification of the proteins was followed by Western blot analysis using rabbit anti-peptide antibodies generated against amino acids 65–75 of rhoA (22) and 191–209 of R-ras (25).

The guanine nucleotide binding activities of the purified proteins were determined in high (5 mM) and low (0.5 \mu M) Mg\textsuperscript{2+}. We have previously reported a dramatic increase in the nucleotide off rate of p21\textsuperscript{ras} in low Mg\textsuperscript{2+} (24), and Fig. 1 shows an identical behavior of p21\textsuperscript{ras} and p23\textsuperscript{ras} proteins. For each protein the rate-limiting step for nucleotide exchange is the off rate (data not shown), and the half-lives for the pseudo-first order reactions in 5 mM Mg\textsuperscript{2+} for ras (24), normal rho (Fig. 1A), and normal R-ras (Fig. 1B) are 50, 70, and 40 min, respectively. In low Mg\textsuperscript{2+} all half-lives are less than 1 min. A similar effect has been observed for the yeast YPT gene product (26). It has been observed that the oncogenic mutation Val-12 when introduced into ras results in a small 2–3-fold decrease in the nucleotide exchange rate (27). Fig. 1A shows a similar effect on the exchange rate of p21\textsuperscript{ras} when a Val-14 substitution is introduced.

To determine the intrinsic GTPase activities, the proteins were preloaded with [\gamma-\textsuperscript{32P}]GTP and the rate of release of the \gamma-phosphate determined by measuring residual bound radioactivity in a filter binding assay. Fig. 2 shows the results obtained. In contrast to a previous report using a slightly modified p21\textsuperscript{ras} derived from an Aplysia californica cDNA (28) we found the half-life for GTP hydrolysis of human rhoA is 15 min (k = 0.046 min\textsuperscript{-1}), 2-fold faster than for normal ras. R-ras has a half-life for GTP hydrolysis similar to ras, around 30 min using this assay. Introduction of Val-14 into rhoA increases the half-life for GTP hydrolysis to greater than 100 min in an identical fashion to the Val-12 alteration in ras (1). It is interesting to note that codon 15 in rhoA is alanine whereas the corresponding position (codon 13) in ras is glycine (22). Alteration at codon 13 of ras have been shown to be oncogenic (29), and it has been speculated that the normal rho proteins may be biochemically more similar to oncogenic ras and have a low GTPase activity (18). However, the data shown here do not support this speculation, and indeed we have shown that substitution of alanine for glycine at codon 13 of ras is not an oncogenic mutation (data not shown).

A cytoplasmic protein capable of increasing the GTPase activity of normal but not oncogenic ras has been identified in Xenopus oocytes and a wide range of mammalian cells (4). This protein, GAP, has recently been purified from bovine brain and is an approximately 125-kDa protein that acts catalytically on normal ras to increase its GTPase activity (13). We wished to see if GAP-like activities against p23\textsuperscript{ras} and p21\textsuperscript{ras} could be detected in cell extracts. Fig. 2 shows the effect of a crude cytoplasmic extract of human spleen tissue on the GTPase activity of ras and the two ras-related proteins. A GAP-like activity is detected against all three proteins. Fig. 2C also shows that the mutant Val-14 p21\textsuperscript{ras} does not respond to this GAP activity. This is identical behavior to the effect

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**FIG. 1. On rates for guanine nucleotide exchange and the effect of Mg\textsuperscript{2+} concentration.** A, normal p21\textsuperscript{ras} (●) or mutant (Val-14) p21\textsuperscript{ras} (△) (0.5 \mu M) was preloaded with [\gamma-\textsuperscript{32P}]GTP (2.0 \mu M) as described under “Experimental Procedures” in 5 mM or 0.5 \mu M free Mg\textsuperscript{2+} as shown. B, normal p23\textsuperscript{ras} (●) was incubated as in A in 5 mM or 0.5 \mu M Mg\textsuperscript{2+}. Although on rates are shown the rate-determining step is the nucleotide off rate making the reactions pseudo-first order.
Distinct GTPase Activating Proteins Interact with ras and rho

![Graph](image)

**Fig. 2.** Intrinsic GTPase activities of three ras-related proteins and the effect of cytoplasmic extracts. A, normal (○), or mutant (Val-12, Δ, △) p21<sup>ras</sup> was preincubated with [γ-<sup>32</sup>P]GTP in low Mg<sup>2+</sup> as described under "Experimental Procedures." After addition of Mg<sup>2+</sup> (to 5 mM) aliquots were incubated at 30 °C, and remaining bound radioactivity was measured at time intervals. The assay was done in the absence (○, Δ) or presence (△) of human spleen tissue cytoplasmic extract. B, as in A but normal p23<sup>Ras</sup> was used. C, as in A but normal (○, △) or mutant Val-14 (△, Δ) p21<sup>ras</sup> was used.

![Graph](image)

**Fig. 3.** FPLC gel filtration chromatography of GAP activities present in human spleen extract. The extract (0.2 ml) was passed down a Superose 12 column (volume, 23 ml) and fractions collected (0.3 ml). GAP activity to p21<sup>ras</sup> (○), p23<sup>Ras</sup> (△), and p23<sup>Rho</sup> (△) was determined using 5 μl of each fraction for p21<sup>ras</sup> and p23<sup>Ras</sup> and 0.5 μl for p23<sup>Rho</sup>. Activity is given as percent of bound GTP hydrolyzed under the assay conditions. Molecular weights were determined by passing molecular weight markers through the column.

of a Val-12 oncogenic mutation in p21<sup>ras</sup> (Fig. 2A).

In an attempt to separate the various GAP activities, human spleen extract was first chromatographed using FPLC monoQ ion-exchange chromatography. All three activities co-eluted on application of a salt gradient at about 138 mM NaCl (results not shown). Fig. 3 shows the results obtained when this monoQ active eluate was subjected to gel filtration chromatography using an FPLC Superose 12 column. It can be seen that the GAP activity toward R-ras and ras co-eluted at around 100 kDa whereas the rho GAP activity eluted at around 29 kDa. The same molecular weight elution profile was obtained using extracts made from mouse brain and a rat fibroblast cell line. GAP activity for the three proteins was also detected in Xenopus oocytes but not in S. cerevisiae. On extended storage of spleen extracts the ras GAP activity is proteolytically degraded to an active 60-kDa species (data not shown). Under these conditions R-ras activity co-migrated at 60 kDa whereas the rho activity migrated at around 15-20 kDa.

We conclude that p23<sup>Ras</sup> probably interacts with the same 125-kDa GAP protein as p21<sup>ras</sup> (13). This is not entirely surprising; mutational analysis of p21<sup>ras</sup> has implicated amino acids 32-40 as the binding site for GAP (11, 12), and the sequence of R-ras in the corresponding region is identical. Interestingly, this region of ras has also been implicated as the effector domain (30), suggesting that GAP itself may be the target for regulation by p21<sup>ras</sup> (11, 12). Since the biological effects of ras and R-ras are demonstrably different (unlike ras, R-ras cannot cause cell transformation (31)), we are led to the conclusion that the interactions of p21<sup>ras</sup> and p23<sup>Ras</sup> with GAP have different biochemical consequences. It is worth noting that rap1A also has an identical effector region to ras and R-ras (16) and would be predicted to interact with ras GAP.

We have also shown that p21<sup>ras</sup> interacts with a distinct cytoplasmic GAP-like molecule, rho GAP, with an estimated molecular mass of 29 kDa. When amino acids 32-40 of ras are compared with the corresponding region of rho there are five differences (22). At least one of these, Asp-38 (ras)/Glu-40 (rho), is an alteration that is known to destroy the ability of ras, R-ras not to cause cell transformation (31), we are led to the conclusion that the interactions of p21<sup>ras</sup> and p23<sup>Ras</sup> with GAP have different biochemical consequences. It is worth noting that rap1A also has an identical effector region to ras and R-ras (16) and would be predicted to interact with ras GAP.

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**Note Added in Proof**—Using recombinant GAP protein obtained from an E. coli expression system (very kindly supplied by F. McCormick, Cetus Corp.) we have confirmed that both p21<sup>ras</sup> and p23<sup>Ras</sup> interact with the same GAP molecule.

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