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Identification of a nucleotide exchange-promoting activity for p21^{ras}

(GTP-binding protein/signal transduction)

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ABSTRACT **The biological activity of proteins encoded by the *ras* family of oncogenes is dependent on whether they are bound to GTP or GDP: the type of nucleotide bound is dependent on the rate of GTP hydrolysis (promoted by the GTPase-activating protein, GAP) and the rate of nucleotide exchange with cytosolic pools. A protein that stimulates the rate of exchange of guanine nucleotide on p21^{ras} has been identified and characterized in cytoplasmic extracts of human placenta. The exchange-promoting protein runs on a gel filtration column with an apparent relative molecular weight of about 60,000. It is sensitive to heat and to trypsin. The exchange-promoting protein acts reversibly and does not cause degradation of p21^{ras}. It is inactive towards the α subunit of a heterotrimeric GTP-binding protein ($G_{o\alpha}$) but acts on a large number of different mutant *ras* proteins, including transforming and effector mutants that are insensitive to the action of GAP. This protein, which we have termed REP (*ras* exchange-promoting), has the characteristics expected of a physiological activator of p21^{ras} in cellular growth-signal-transduction pathways.**

Members of the *ras* family of oncogenes encode M_r 21,000 proteins (p21) that bind and hydrolyze guanine nucleotides. The transforming potential of the *ras* protooncogenes can be activated by point mutations that either decrease the intrinsic GTPase activity of p21 or increase the rate with which p21 exchanges guanine nucleotides (1). Activated *ras* oncogenes have been found in up to 30% of human tumors (2). By analogy with other oncogene products, it has been speculated that p21 normally functions as a component of a growth-signal-transduction pathway. One would therefore expect it to interact with several distinct cellular proteins that act variously to impart signals to p21, to receive signals from p21, or to modulate signal transduction. A protein that activates the GTPase activity of p21 (GAP) has been identified (3); this may represent a downstream effector of p21 action (4, 5). The nature of the upstream elements of the pathway that control p21 activity remains unknown: by analogy with the heterotrimeric GTP-binding proteins (G proteins) (6) it might be expected that stimulation of the activity of p21 would involve an increase of the rate at which it exchanges guanine nucleotide with soluble pools. Here we report the identification of an activity in a human tissue extract that promotes the exchange of guanine nucleotides on p21. This factor, termed REP (*ras* exchange-promoting), has properties consistent with its being a physiological activator of the *ras* proteins.

MATERIALS AND METHODS

Purification and Assay of Exchange Activity. Two normal human placentas were homogenized in a domestic blender with 1 liter of ice-cold 50 mM potassium phosphate buffer (pH 7.5) containing 1 mM EGTA, 0.3 M sucrose, 1 mM dithiothreitol (DTT), 10 μg of soybean trypsin inhibitor per ml, 10 μg of leupeptin per ml, 10 μg of aprotinin per ml, and 10 mM benzamidine. Particulate matter was pelleted by centrifugation at 50,000 × *g* for 60 min at 4°C. The supernatant was applied to a column of DEAE-Sephacel (5 × 50 cm), which was washed with 1 liter of potassium phosphate buffer, pH 7.5/1 mM DTT and then eluted with a linear gradient from 0 to 1 M NaCl. Fractions were dialyzed against 50 mM potassium phosphate, pH 7.5/5 mM $MgCl_2$/1 mM DTT in a BRL microdialysis system overnight at 4°C. Assays for GAP activity were performed on these samples as described (4), with the addition of 1 mM unlabeled GTP and GDP during the incubation, which was for 20 min at 37°C. The buffer was standardized in all cases to 50 mM potassium phosphate, pH 7.5/5 mM $MgCl_2$. The activity is presented as the percentage of GTP in total nucleotide bound to p21. Exchange activity was measured by determining the total amount of ^{32}P label in the washed immunoprecipitates. Fractions 60–75, which contained the highest amount of apparent exchange activity, were pooled and diluted with 5 volumes of 50 mM potassium phosphate, pH 7.5/1 mM DTT and then loaded onto a column of Matrex gel Green A (Amicon; 2.5 × 30 cm). This was washed with 500 ml of the same buffer and bound material was eluted with a linear gradient from 0 to 1 M NaCl. Samples were dialyzed and assayed as before. Fractions 85–115 were pooled, reduced in volume to 20 ml with an Amicon ultrafiltration unit (YM30 filter), and then loaded onto an AcA 34 gel filtration column (2.5 × 100 cm) run in 50 mM potassium phosphate, pH 7.5/5 mM $MgCl_2$/1 mM DTT. Fractions were assayed directly.

Iodination of p21. Fifty micrograms of purified bacterially expressed p21$^{c\text{-}Ha\text{-}ras}$ was iodinated with 0.1 mCi of ^{125}I in the presence of chloramine T (0.1 mg/ml) for 1 min. Iodinated p21 was separated from free iodine on a Sephadex G-25 column. Immunoprecipitations of 1-μg aliquots of iodinated p21 in the presence of various amounts of DEAE-Sephacel-purified REP were performed using the same protocol as for the exchange reaction (above) but without the [α-^{32}P]GTP. The amount of label in the immunocomplexes was determined and is expressed as a percentage of the amount brought down in the absence of REP.

Other Methods. $G_{o\alpha}$ was a gift from Eva Neer (Brigham and Women's Hospital, Boston); nucleotide exchange reactions were performed on it as for p21 except that the G protein was separated from unbound nucleotide at the end of the incubation by application to a nitrocellulose filter. All *ras* mutants were obtained from Frank McCormick (Cetus) except numbers 1741, 1742, 1743, 1617, 1701, and 1703, obtained from Berthe Willumsen (University of Copenhagen) and E119, A116, P61, K61, and T59, obtained from Channing Der (La Jolla Cancer Research Foundation, La Jolla, CA).

RESULTS

Detection of p21-Directed Nucleotide-Exchange Activity in a Tissue Extract. Both the GTPase-stimulating activity GAP

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Abbreviations: DTT, dithiothreitol; GAP, GTPase-activating protein; G protein, heterotrimeric GTP-binding protein; REP, *ras* exchange-promoting.

and a putative guanine nucleotide exchange-promoting activity might be detected in an assay that monitors the fate of [α-^{32}P]GTP bound to p21: interaction with GAP would result in p21 carrying [α-^{32}P]GDP, while interaction with an exchange protein would cause the release of radiolabel and its replacement by soluble, unlabeled nucleotides.

A homogenate was prepared from human placentas in a hypotonic buffer. After clarification, the homogenate was applied to a column of DEAE-Sephacel, which was then washed and eluted with a gradient of NaCl from 0 to 1 M. After prolonged dialysis of the eluted fractions against a Mg^{2+}-containing buffer, they were assayed for their ability to promote either the hydrolysis or the dissociation of guanine nucleotides bound to p21. Purified bacterially expressed $p21^{c\text{-}Ha\text{-}ras}$ was prelabeled by loading with [α-^{32}P]GTP, excess unlabeled GTP and GDP were added, and then dialyzed column fractions were added to aliquots of this mixture. Fig. 1*A* shows the amount of guanine nucleotide remaining bound to p21 at the end of a 20-min incubation with column fractions. Also shown is the proportion of GTP relative to total GTP and GDP on p21. From the latter measurement it can be concluded that GAP was eluted from the ion-exchange column at a salt concentration of about 50 mM. On the other hand, the total amount of radiolabeled nucleotide bound to p21 showed a marked reduction when fractions that were eluted from the column around 100 mM salt were assayed.

The ability of these latter fractions to promote the dissociation of nucleotide from p21 suggested the presence of a protein that specifically increases the rate at which p21 exchanges guanine nucleotide with the soluble nucleotide pool. We termed this activity REP to signify its *ras* exchange-promoting function. In order to investigate this activity further, fractions from the DEAE-Sephacel column showing the greatest exchange-promoting activity were pooled and applied to a dye column containing Matregel Green A. The column was washed and bound material was eluted with a linear salt gradient (0–1 M NaCl). The eluted fractions were dialyzed to remove salt and then assayed, as before, for their ability to promote nucleotide dissociation from p21 that had been labeled with [α-^{32}P]GTP (Fig. 1*B*). A broad peak of nucleotide exchange-promoting activity was eluted from the dye column at about 300 mM NaCl. To further analyze this activity, the active fractions from the Matregel Green A column were reduced in volume and applied to an AcA 34 gel filtration column. Fractions eluted from this column were assayed once again for their ability to stimulate nucleotide exchange from p21 (Fig. 1*C*). A peak of activity was eluted at a volume corresponding to a predicted molecular weight of about 60,000.

Characterization of the Nucleotide Exchange-Promoting Activity. The partially purified activity appeared to be capable of stimulating the rate at which nucleotide exchanged between p21 and the soluble pool. A number of experiments were carried out to establish whether this activity was likely to be of any significance *in vivo*. First, fractions from the DEAE column containing peak amounts of nucleotide exchange activity were subjected to treatments designed to inactivate proteins. As shown in Table 1, incubation of these fractions at 60°C for 10 min decreased their REP activity from 160% above basal to only 35% above basal under the assay conditions described. Similarly, trypsin treatment caused a decrease to 24% above basal from 135% for mock-trypsinized controls. It therefore appears that the activity in question is proteinaceous and unlikely to be a low molecular weight compound such as EDTA or ATP, which are known to alter p21 nucleotide exchange rates through their ability to chelate Mg^{2+} (7).

Other possible explanations of the observed activity included the presence of a protease that causes guanine nucleotide release by degrading p21. Fig. 2*A* shows the effect of

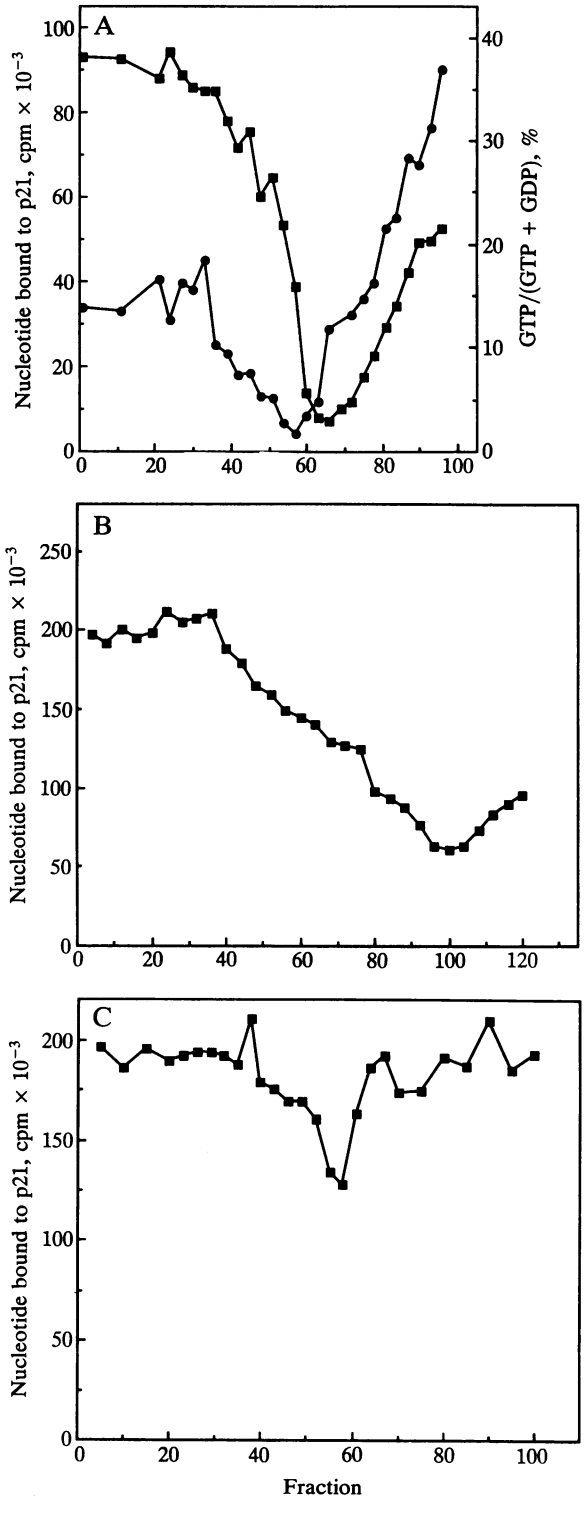

FIG. 1. Partial purification of a nucleotide exchange factor for p21. (*A*) GAP activity (●) and apparent exchange activity (■) in fractions from a DEAE-Sephacel column loaded with a cytosolic extract of human placentas. (*B*) Exchange activity in fractions from a Matregel Green A dye column loaded with the peak of exchange activity from the DEAE column. (*C*) Exchange activity in fractions from an AcA 34 sizing column loaded with the peak of exchange activity from the dye column.

increasing concentrations of exchange activity-containing fractions from the DEAE column on the ability of the anti-p21 monoclonal antibody Y13-259 to immunoprecipitate ^{125}I-labeled p21 following incubation under the conditions used in the exchange assays. The amount of immunoprecipitated p21

Table 1. Effect of heat treatment and proteolysis on REP

Treatment	$t_{1/2}$ for nucleotide on p21, min
No REP	15.0
REP	5.8
REP heated at 60°C, 10 min	11.1
REP, mock-trypsinized	6.4
REP, trypsinized	12.1

Assays were performed as in Fig. 1*A*. To trypsinize REP, trypsin (10 mg/ml) was added for 20 min at 37°C, followed by soybean trypsin inhibitor (20 mg/ml). For the mock-trypsinized sample the order of addition of trypsin and trypsin inhibitor was reversed.

was not substantially decreased by incubation with these fractions, nor was its molecular weight in any way altered as measured by SDS/polyacrylamide gel electrophoresis (Fig. 2*A Inset*). These data indicate that the activity causes nucleotide to dissociate from p21 without altering its gross structure or the efficiency of its immunoprecipitation in the assay.

Another characteristic expected of a physiologically significant nucleotide exchange protein is that its action should be reversible. Thus, following an interaction with the REP protein, p21 should once again be able to bind nucleotide as efficiently as previously. Table 2 shows the results of an experiment designed to test whether the exchange activity identified here meets this criterion. After incubation with REP-containing fractions from the DEAE column, p21 was recovered by immunoprecipitation with monoclonal antibody and washing of the immunocomplex. Measurement of subsequent [α-^{32}P]GTP binding to the immunocomplex demonstrated that the prior exposure of p21 to the exchange activity did not alter its ability to bind nucleotide. This measurement was not effected by the presence in the immunocomplex of p21 deriving from the placental homogenate; analysis of fractions from the ion-exchange column indicated that <5% of the p21 in the immunocomplexes could be of placental origin (data not shown). These data confirmed that the observed REP activity did not result from an irreversible inactivation or denaturation of p21.

Table 2. Reversibility of the action of REP on p21

Treatment	Nucleotide recovered, cpm × 10^{-3}	
	p21 labeled before treatment	p21 labeled after immunoprecipitation
None	930	1560
+ REP	82	1610

p21 was labeled with [α-^{32}P]GTP before REP treatment as described in Fig. 1 or in the immunocomplex after washing with the same labeling mixture; in this case the pellets were then washed a further four times before counting. The REP used was from DEAE-Sephacel column chromatography of placental homogenate. Incubation with p21 was for 20 min at 37°C.

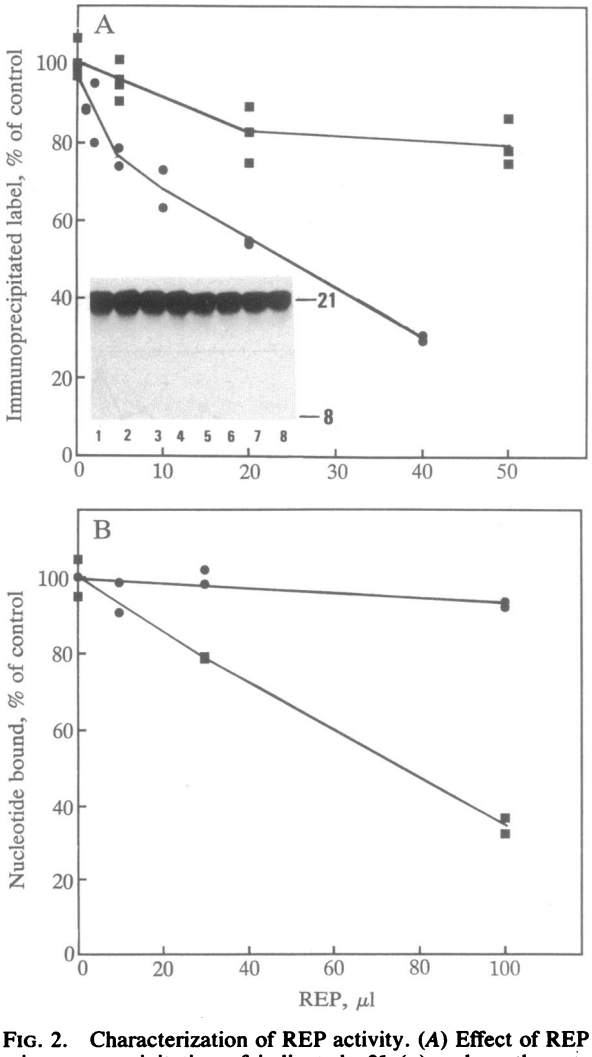

FIG. 2. Characterization of REP activity. (*A*) Effect of REP on the immunoprecipitation of iodinated p21 (■) and on the rate of exchange of labeled guanine nucleotide from p21 (●). (*Inset*) Autoradiograph of SDS/polyacrylamide gel of immunoprecipitated iodinated p21 with molecular weight standards. (M_r × 10^{-3} at right.) Lanes: 1 and 2, no REP; 3 and 4, plus 5 μl of REP; 5 and 6, plus 20 μl of REP; 7 and 8, plus 50 μl of REP. (*B*) Effect of REP on the rate of exchange of nucleotide from p21 (■) and from $G_{o\alpha}$ (●).

A further indication of apparent physiological interaction between the REP protein and p21 derived from comparison of the effects of the REP activity on p21 and on another guanine nucleotide-binding protein, $G_{o\alpha}$. The latter, in association with β and γ subunits, is presumed to participate in a signal-transducing pathway that is distinct from the one in which p21 functions (6). Like p21, it is thought to cycle between active and inactive signaling states through the binding and hydrolysis of GTP. Fig. 2*B* shows the extent of nucleotide exchange observed from $p21^{c\text{-}Ha\text{-}ras}$ with increasing volume of exchange activity-containing fractions from the DEAE column under the same assay conditions. Also shown is the effect of these fractions on the exchange of nucleotide from $G_{o\alpha}$. While the rate at which nucleotide exchanges from p21 increased by up to nearly 5-fold, the rate of exchange of guanine nucleotide from the G protein was not affected by these fractions. This further suggests a specific interaction between REP and p21.

The time course with which the exchange activity acts upon $p21^{c\text{-}Ha\text{-}ras}$ is shown in Fig. 3. The unstimulated off rate demonstrates an exponential decay with a half-life of 20 min at 37°C for [α-^{32}P]GTP-labeled p21. The REP-stimulated off rate also follows exponential decay kinetics, in this case with a half-life of 2.6 min. This represents a stimulation of the exchange reaction of nearly 8-fold. For [α-^{32}P]GDP-labeled p21 the unstimulated exchange reaction has a half-life of 14 min, whereas the exchange activity-stimulated reaction had a half-life of 2.4 min, giving a stimulation of 5.4-fold. That the exchange activity is effective on GDP- as well as GTP-labeled p21 indicates that it is unlikely to be caused by GAP itself, since this protein is believed not to interact with GDP-bound p21, at least as far as competition for GTPase-activating activity is concerned (8). Further indications that the REP and GAP activities reside in different proteins comes from the previously described DEAE chromatography of placental homogenate, in which the two activities did not comigrate (Fig. 1*A*) and from the observation that purified GAP itself did not appear to alter the rate of p21 nucleotide exchange significantly.

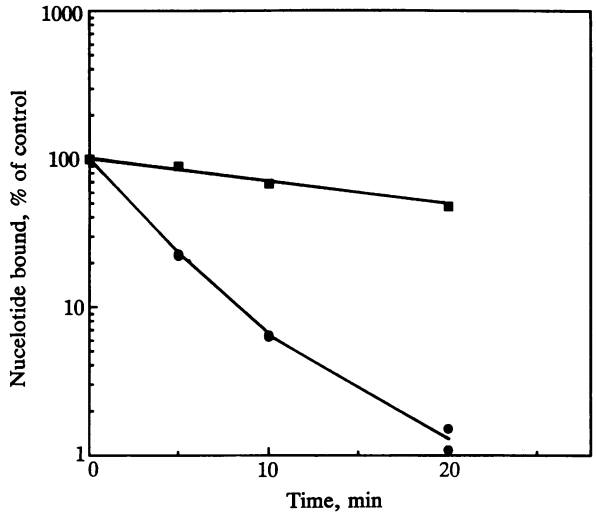

FIG. 3. Time course of the effect of REP on the exchange of nucleotide from p21. [α-^{32}P]GTP-labeled p21$^{c\text{-}Ha\text{-}ras}$ was treated with REP (●) or with control buffer (■) for the indicated times at 37°C in the presence of 1 mM unlabeled GTP and GDP prior to immunoprecipitation of p21 and determination of label in the immunocomplex by scintillation counting. The REP used was purified using a DEAE-Sephacel column.

Interaction of the Exchange-Promoting Protein with Mutant *ras* p21 Proteins. To further investigate the distinctions between the REP activity and GAP, a number of mutant *ras* proteins were assayed for their ability to interact with the two activities (Table 3). The mutant *ras* proteins were expressed in *Escherichia coli* and purified as described (9). They were labeled with [α-^{32}P]GTP and then either were treated with GAP purified from human placenta (10) and assayed for the rate of hydrolysis of GTP or were treated with REP activity-containing fractions from the DEAE column and assayed for the rate of nucleotide exchange. Of the mutant proteins assayed, several were fully resistant to GAP action, particularly those with amino acid alterations in the "effector domain" (residues 35–40; ref. 11) and in the phosphoryl binding regions (residues 12, 59, and 61). However, several of these mutant proteins were responsive to the REP activity, albe it in some cases less so than the wild-type p21. Notable exceptions among these GAP-resistant mutant proteins are those having amino acid substitutions at position 59 (mutant T59; threonine instead of wild-type alanine) and position 61 (mutant K61; lysine instead of wild-type glutamine); these showed greatly reduced sensitivity to the REP activity. We also note that these two mutants both have greatly altered basal exchange rates relative to wild-type p21: K61 exchanges very slowly, whereas T59 exchanges very rapidly.

Other mutant proteins that showed altered responsiveness to the exchange activity are the deletion mutants 1617 (lacking residues 102–108) and 1701 (lacking residues 166–183) (11). Mutant 1617 showed reduced sensitivity to GAP. On the other hand, mutant proteins having smaller deletions within this area (mutants 1742 and 1743) showed normal sensitivity to exchange activity and also normal sensitivity to GAP. It is possible that the conformation of 1617 is significantly altered by the large deletion within the core of the protein that appears to effect at least two apparently independent functions. Mutant 1701, which is deleted at the carboxyl terminus but still carries the last four amino acids that determine membrane binding, appears to interact normally with GAP and have a normal basal exchange rate but is no longer stimulatable by REP. It is possible that this mutation removes a specific site of interaction between p21 and an exchange-promoting protein.

DISCUSSION

A number of criteria indicate that the p21-directed guanine nucleotide exchange activity described in this report, which we term REP, is distinct from the activity associated with GAP. (*i*) REP and GAP do not copurify on ion-exchange column chromatography (Fig. 1*A*). (*ii*) REP has a relative molecular weight of about 60,000 whereas that of GAP is

Table 3. Effects of REP and GAP on *ras* p21 mutants

p21*	Nucleotide exchange		GTP hydrolysis	
	Basal $t_{1/2}$, min	Stimulation by REP, fold decrease in $t_{1/2}$	Basal $t_{1/2}$, min	Stimulation by GAP, fold decrease in $t_{1/2}$
c-Ha-*ras* mutants				
Wild type	22.3	15.6	85.6	21.1
1741 (92LIR96)	20.9	14.1	72.1	18.9
1742 (100LIR104)	25.6	17.2	69.5	16.2
1743 (106LIR109)	23.1	13.2	77.1	17.3
1617 (101PDQ109)	24.9	1.4	97.8	2.7
1701 (165PDQ184)	19.3	1.9	102.4	14.7
1703 (96LIR104)	26.7	11.0	67.0	7.8
E119	13.6	4.7	129.6	10.5
A116	12.5	9.7	134.3	12.9
P61	31.2	6.0	167.2	1.3
K61	56.8	2.3	175.9	0.9
T59	1.7	1.4	104.7	1.0
D12	25.1	8.3	215.1	1.1
A35, L36	12.3	6.4	163.0	1.4
A38	21.8	5.9	145.7	0.9
N-*ras* mutants				
Wild type	29.0	17.8	60.3	22.6
V12	23.7	8.2	188.4	1.2
K61	73.5	1.9	171.5	0.7
R117	12.4	8.3	149.6	5.1

GAP was purified from placenta (10); 0.5 μg of p21 was used for each determination.
*Point mutants are designated by the one-letter symbol(s) representing the amino acid that replaces the wild-type residue, followed by the position number. Deletion mutants are described in parentheses.

120,000. (*iii*) Purified GAP does not possess nucleotide exchange activity (12). (*iv*) The spectrum of p21 mutants that REP fails to interact with is different from those that are resistant to GAP (Table 3). These observations make it very unlikely that REP is GAP, although the possibility that it is a modified form of GAP that has radically altered activity cannot be completely eliminated until the structure of the REP protein has been determined.

The REP activity described here appears to be associated with a cytosolic protein; no exchange activity can be found in membrane preparations washed with 0.5 M NaCl. However, we have preliminary evidence that the treatment of washed placental membranes with low concentrations of trypsin causes the release of a nucleotide exchange-promoting activity. It is possible, therefore, that the exchange activity described here is derived from a larger membrane protein that is inactive in broken cells but is activated and released from the membrane by proteolysis during the preparation of placental homogenates. Proteolytic activation of enzymes is well documented (13). Whether such a proteolytic activation of REP would have any physiological significance is unclear.

REP acts *in vitro* to stimulate the rate at which p21 will exchange either GTP for GDP or GDP for GTP. An insight into the possible physiological significance of this comes from examination of the free nucleotide levels and the p21-associated nucleotides within the cell. In normal cells, >95% of the p21 is associated with GDP (14). Moreover, in such cells the concentration of GTP in the cytosol is about 20 times that of GDP. Consequently, undirected stimulation of nucleotide exchange on p21 within the cell would almost always lead to the removal of GDP and its replacement by GTP. p21 would thus become activated as a result of its interaction with REP. In this sense, REP shows the characteristics expected of a positive regulator of p21. It could be the mammalian analogue of CDC25, the *Saccharomyces cerevisiae* protein that appears to promote nucleotide exchange on $p29^{RAS}$ and to be responsible for activating it in the yeast system (15, 16). REP may also resemble the receptor molecules that control the nucleotide exchange rates of the trimeric G proteins (6). If REP is indeed the physiological activator of p21, then it in turn is likely to be regulated; the ultimate origins of the signals controlling p21 activity thus remain unknown.

The REP activity described here is associated with defined column fractions but hardly can be ascribed to a discrete molecular species on the basis of currently available evidence. We note that a carboxyl-terminal deletion of p21 strongly affects p21–REP interaction while leaving other activities of p21 unaffected. Since this domain varies substantially between the four cellular *ras* p21 proteins (1), it is possible that REP represents a class of related proteins, each of which interacts with a distinct p21. In this fashion, diverse incoming excitatory signals could be funneled via the common effector domains of the *ras* proteins to a single downstream target.

After completion of this work, two reports appeared describing activities that stimulate guanine nucleotide exchange on p21 (17, 18). The relationship of these other activities with the one described herein remains to be clarified.

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