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REGULATION OF THE GTPase ACTIVITY OF THE RAS-RELATED RAP2 PROTEIN

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SUMMARY : The small GTP-binding protein rap2A exhibits a high level of identity with rap1 and ras proteins (60% and 46%, respectively). Nevertheless, its intrinsic GTPase activity is not stimulated by ras-GAP, and unlike the rap1A protein, it cannot compete with ras proteins for their interaction with ras-GAP. In addition, rap1-GAPm that is highly active on the GTPase activity of the rap1A product, also stimulates the GTPase activity of the rap2A protein but with a 30-40-fold lower efficiency. An activity that greatly stimulated the GTPase activity of the rap2 protein (rap2-GAP) was found in bovine brain cytosol and purified. However, it copurified with the cytosolic form of rap1-GAP and was more efficient at stimulating the GTPase activity of the rap1 protein; this 55 kD polypeptide, that is recognized by an antibody raised against rap1-GAPm, likely represents a degraded and soluble form of the full size 89 kD molecule. In bovine brain membranes, a weak GAP activity toward the rap2A protein was also detected; however, it was also attribuable to the membrane-associated rap1-GAPm. Thus, it appears that a single rap-GAP protein, complete or degraded, is able to stimulate the GTPase activity of both rap1 and rap2 proteins.

The superfamily of ras-related proteins comprises a large number of 20-30 kD GTP-binding proteins that are involved in the control of a wide variety of essential cellular functions such as growth, differentiation, cytoskeletal organization and intracellular vesicle transport (1). These proteins cycle between an "inactive" GDP-bound form and an "active" GTP-bound form that exhibit important conformational differences (2, 3). Two types of regulatory proteins may modulate the relative levels of both forms : guanine nucleotide exchange factors which stimulate the release of GDP bound to ras-related proteins and therefore promote its replacement by GTP, and GTPase activating proteins (GAPs) which accelerate the rate of GTP hydrolysis (2, 3).

The abbreviations used are : GAP : GTPase activating protein; EDTA : ethylene glycol-bis(β-aminoethyl ether); DTT : dithiothreitol; SDS: sodium dodecyl sulfate.

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The first GAP activity that was identified stimulated the GTPase activity of ras proteins (ras-GAP) by several orders of magnitude (4). It may be considered as a negative regulator of ras activity since it promotes the return of ras proteins to the inactive GDP-bound form, however, numerous lines of evidence suggest that it may also function as a downstream effector (5, 6). More recently, it has been found that neurofibromin, the protein product of the neurofibromatosis type 1 gene NF1, exhibits significant sequence homology with the ras-GAP protein and also stimulates the GTPase activity of ras proteins (7, 8).

Amongst ras-related proteins, the rap proteins (rap1A, rap1B, rap2A, rap2B) are the most closely related to ras proteins: they share approximately 50% identity and have the same effector region (region 32-42) through which ras proteins are thought to exert their biological effects (9-12). This had prompted speculations that rap proteins could antagonize the effects of ras proteins, possibly by competing for a common effector, and some studies suggested that this could actually be the case. M. Noda's group showed that the phenotype of K-ras transformed NIH 3T3 cells could be reverted by overexpression of the Krev-1 cDNA encoding the rap1A protein (13). Other workers have since demonstrated that rap1A and rap1B proteins (that exhibit 90% identity) could compete efficiently with the c-H-ras protein for its interaction with ras-GAP in vitro, but that ras-GAP is incapable of stimulating the GTPase activity of rap1 proteins (14). The rap2A protein is 61% identical to the rap1A product and exhibits a similar "effector" region, except for the substitution of a phenylalanine for a serine at position 39 (9). It was therefore conceivable that the rap2A protein may similarly antagonize the action of ras proteins. However, we have demonstrated that overexpression of the rap2A protein exhibits neither growth-promoting nor growth-inhibitory effects and that it does not interfere with ras-induced transformation (15). Thus, in spite of their great similarity, the rap1A and rap2A proteins seem to have distinct physiological properties.

A GAP activity specific for the rap1A and rap1B proteins (rap1-GAP) has been identified in the cytosol of bovine brain (16) and in both membranes and cytosol from HL-60 cells (17). The cytosol appeared to contain the major part of the rap1-GAP activity (about 90 %) and only approximately 10% of the activity was recovered from membranes. The membrane-associated rap1-GAP (rap1-GAPm) was purified to homogeneity (17), and its cDNA cloned (18); no significant homology to any known sequences was detected. Here, we have studied the regulation of the GTPase activity of the rap2A protein by ras-GAP, rap1-GAPm and extracts from bovine brain. We show that the rap2 protein is unsensitive to ras-GAP but that its GTPase activity is stimulated by rap1-GAPm. In addition, we have identified in bovine brain cytosol a factor that efficiently stimulates the GTPase activity of the rap2A protein: this GAP activity co-purified with the cytosolic rap1-GAP and likely represents a degradation product of the complete rap1-GAPm.

MATERIALS AND METHODS

Materials and chemicals : recombinant rap1A and rap2A proteins (respectively produced in baculovirus infected insect cells and *E. coli*) as well as platelet membrane rap1B protein were purified as described (19-21). Recombinant human c-H-ras protein and recombinant ras-GAP334 (the C-terminal 334 amino acids of ras-GAP)(22) were generous gifts from Dr A. Wittinghofer.

GAP assay : stimulation of the GTPase activity of the rap2 protein was measured by two assay methods using either [γ-^{32}P]GTP or [α-^{32}P]GTP.
Method 1: the rap2 protein was first loaded with [γ-^{32}P]GTP at 37°C for 15 min in a 100 µl reaction mixture containing 25 mM Tris-HCl pH 7.5, 0.8 mM EDTA, 2.5 µM rap2 protein and 5 µM [γ-^{32}P]GTP (Amersham, 4000cpm/pmol). A 10 µl aliquot from this reaction was added to a 180 µl mixture containing $MgCl_2$ at a final concentration of 10 mM and extract. Portions were removed after various times of incubation at 37°C ; protein-associated nucleotide was determined as previously described (19). GTPase activity was calculated from the decrease in the radioactivity of the protein-GTP complex following incubation (15 minutes unless otherwise stated). No GTP binding to the cytosolic bovine extract was detected in the absence of added rap2 protein. This method was used for routine determinations.
Method 2: in order to directly assess the effect of bovine brain cytosol on guanine nucleotides bound to the rap2 protein, GAP assays were performed by an alternative method. The rap2 protein was loaded with [α-^{32}P]GTP (Amersham, 2400 cpm/pmol) as described above with [γ-^{32}P]GTP, and excess unbound nucleotide was removed by gel filtration through a Sephadex G-25 fine column. The rap2 protein-[α-^{32}P]GTP complex (2.5 µM) was then incubated at 37°C in the presence of 10 mM $MgCl_2$ with 100 µl of bovine brain cytosol or buffer. 5 µl portions were removed at the indicated times and the nucleotide content was analyzed by thin-layer chromatography on polyethyleneimine cellulose sheets (19).
GAP assays with the rap1 protein were performed as previously described (17).

Purification of cytosolic rap2-GAP : All steps were performed at 4°C. Bovine brain cytosol (11 g of protein) prepared as described (16) from two bovine brains (1 kg of tissue) was incubated batchwise with 400 ml of Q Sepharose Fast Flow resin (Pharmacia-LKB) for 2 hours and then washed on a Buchner funnel with 2 liters of buffer A (25 mM Hepes pH 7.5, 1 mM EDTA, 1 mM DTT). The resin was poured into a 5x20 cm column and washed with an additional 1 liter of buffer A. Elution was achieved with a 2-liter linear 0-0.25 M NaCl gradient, at a flow rate of 80 ml/h and fractions of 8 ml each were collected. Two symmetrical peaks of GAP activity were eluted at 50 and 130 mM NaCl respectively (see figure 3). The first peak contained approximately 20% of the recovered activity and was further partially purified by successive chromatography on hydroxylapatite resin (HA Ultrogel) and gel filtration through Ultrogel AcA34. The rap2-GAP activity of the second peak was purified to near homogeneity as follows: fractions containing the bulk of the activity (165-205 in figure 3) were pooled (500 mg of protein) and concentrated to 50 ml by ultrafiltration on an Amicon YM 30 membrane. This material was applied to a 2.5x20 cm column of hydroxylapatite, equilibrated in 10 mM sodium phosphate buffer pH 6.8. After washing, elution was performed with a 700-ml linear gradient of 0-200 mM sodium phosphate buffer pH 6.8; the rap2-GAP activity appeared in fractions containing 25-50 mM sodium phosphate. The extract was concentrated by ultrafiltration and applied to a 2.5x100 cm Ultrogel AcA34 gel filtration column

developed with buffer B (25 mM Tris-HCl pH 7.5, 50 mM NaCl, 1 mM DTT) at a flow rate of 15 ml/h. The peak of GAP activity was concentrated by ultrafiltration and further purified on a 5 ml EconoPac Blue cartridge (Bio-Rad), equilibrated with 25 mM Tris-HCl pH 7.5. The column was washed with 25 ml of buffer and eluted with a 25 ml gradient of 0-1 M NaCl : a single peak of activity appeared between 0.3 and 0.45 M NaCl. The pooled fractions were dialysed and finally applied to a Mono Q HR5/5 column equilibrated with 25 mM Tris-HCl pH 7.5. After the column was washed with 10 ml of the same buffer, elution was performed with a 10-ml linear gradient of 0 to 1 M NaCl in the same buffer at a flow rate of 0.5 ml/min.

Other procedures : Protein concentrations were determined according to Bradford using bovine serum albumin as a standard (23). Polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE) was performed essentially as described by Laemmli (24). Gels were stained with Coomassie brilliant blue or analysed by Western blotting with polyclonal rabbit antibodies elicited against residues 121-137 of the rap1 protein (25), or purified recombinant rap2 (26) and rap1-GAPm (18) proteins.

RESULTS AND DISCUSSION

Detection of rap2-GAP activity

The presence of rap2-GAP activity was determined in bovine brain cytosol by two alternative methods. Purified recombinant rap2 protein was loaded with [γ-^{32}P]GTP and the decrease in the radiolabeled GTP-protein complex was determined following incubation with bovine brain extracts. Figure 1A shows that the extract

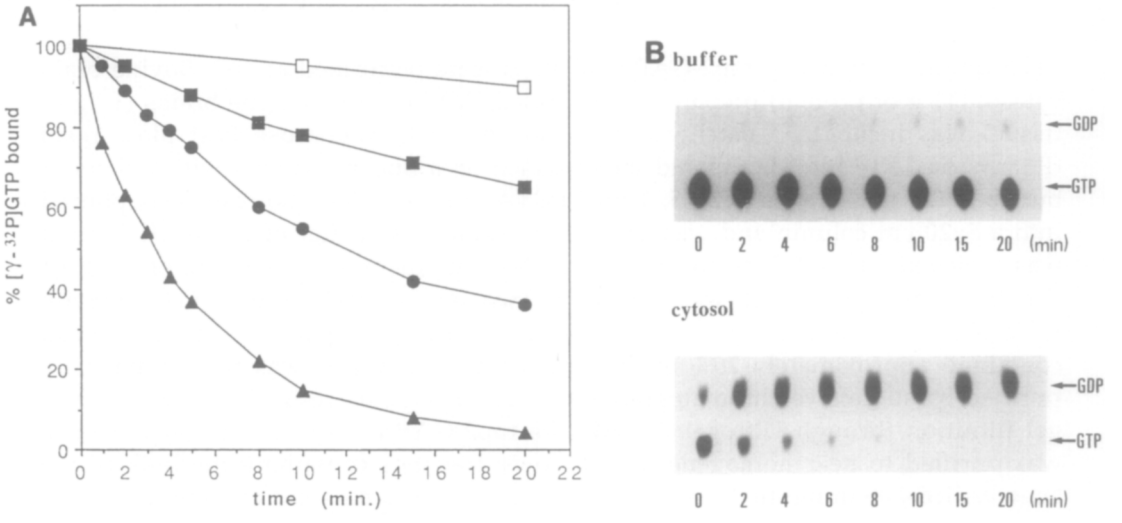

Figure 1. Detection of rap2-GAP activity in bovine brain cytosol
(A) The rap2-GAP activity in bovine brain cytosol was measured by method 1 as described in "Materials and Methods". Open squares : buffer control; closed squares : 10 µl of cytosol; closed circles : 50 µl of cytosol extract; closed triangles : 100 µl of
(B) The hydrolysis of GTP loaded onto the rap2 protein was measured by method 2 with buffer only or 100 µl of the same cytosol extract as in (A).
The data are reprentative of at least two experiments.

stimulates the GTPase activity of the rap2 protein in a time- and dose-dependent manner. Alternatively, the preformed rap2-[α-^{32}P]GTP complex was mixed with cytosol and guanine nucleotides bound to the rap2 protein after the incubation were analysed by thin layer chromatography. Figure 1B shows that cytosol promoted a rapid conversion of GTP bound to the rap2 protein to GDP. This demonstrates that the decrease in radioactivity in the first assay was indeed due to GTP hydrolysis and not to an exchange for non radioactive nucleotide or to protein degradation. In effect, the rate of GTP dissociation from the rap2 protein in the presence of $MgCl_2$ is extremely slow and negligible during the time of the assay (19). Rap2-GAP activity was destroyed after boiling brain cytosol for 10 min and was reversibly inhibited by high concentrations of NaCl (data not shown).

Effect of ras-GAP and rap1-GAPm on the GTPase activity of the rap2 protein

As brain cytosol contains high levels of ras-GAP, we tested whether this molecule was able to stimulate the GTPase activity of the rap2 protein. Figure 2A shows that recombinant ras-GAP-334 could efficiently stimulate the GTPase activity of ras-p21 but that a similar or a 10-fold higher (not shown) amount had no effect on the GTPase activity of the rap2 protein, indicating that rap2-GAP is distinct from ras-GAP. Previous reports have demonstrated that the rap1A protein is able to compete with ras proteins for their interaction with ras-GAP, although binding of the rap1A protein to ras-GAP cannot stimulate its GTPase activity (14). As shown in figure 2A,

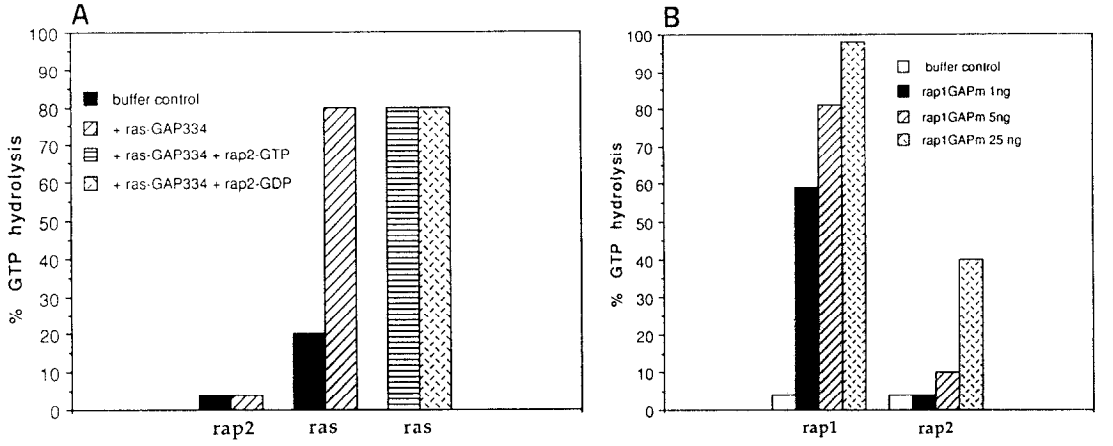

Figure 2. Interaction of the rap2 protein with ras-GAP 334 and rap1-GAPm

(A) 500 nM ras or rap2 proteins were preloaded with [γ-^{32}P]GTP and incubated for 15 min. at 37°C with buffer or 80 nM ras-GAP334. When indicated, 12.5 μM rap2-GTP or rap2-GDP, isolated from unbound nucleotides by filtration through a Sephadex G-25 column, were added to the [γ-^{32}P]GTP-ras complex prior to incubation with 80 nM ras-GAP334. GTP hydrolysis was measured by filtration through nitrocellulose filters. The data are representative of at least two experiments.

(B) Rap1 or rap2 proteins that had been previously bound to [γ-^{32}P]GTP were incubated for 15 min. at 37°C with the indicated amounts of rap1-GAPm protein. GTP hydrolysis was assessed as above.

such is not the case for the rap2 protein since the stimulation of the GTPase activity of the c-H-ras protein (500 nM in the assay) by ras-GAP could not be inhibited by rap2-GTP or rap2-GDP at concentrations as high as 12.5 µM. We can therefore conclude that the rap2 protein is unable to interact with the catalytic domain of ras-GAP.

A GAP activity specific to the rap1 proteins has been purified from membranes of HL-60 cells and bovine brain (rap1-GAPm) (17, 18); we compared its activity on rap1 and rap2 proteins by assessing the amount of rap1-GAPm necessary to obtain similar levels of GTP hydrolysis by both rap1 and rap2 proteins (figure 2B). Although rap1-GAPm stimulated the GTPase activity of the rap2 protein, it was 30-40-fold more potent at stimulating the GTPase activity of the rap1 protein. The GAP activity that we uncovered in bovine brain cytosol may therefore play an important role in regulating of the GTPase activity of the rap2 protein; we sought to purify and characterize this rap2-GAP.

Purification of rap2-GAP

Upon fractionation of bovine brain cytosol on a Q Sepharose column, two peaks of rap2-GAP activity were eluted by the NaCl gradient (figure 3). The first peak represented about 20% of the total activity and was 10 to 20-fold more efficient at stimulating the GTPase activity of the rap1 than the rap2 protein. It was further partially purified by chromatography on hydroxylapatite and gel filtration through Ultrogel AcA34 : the rap1-GAP and rap2-GAP activities co-eluted at each step and

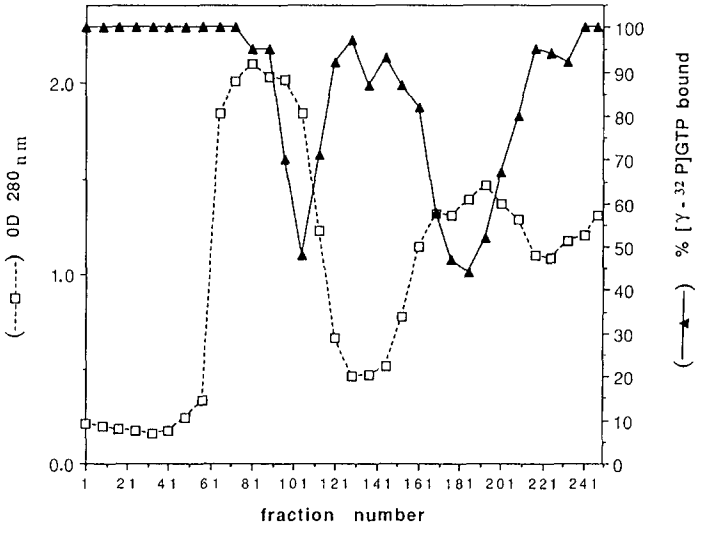

Figure 3. Q Sepharose chromatography of bovine brain cytosol
The cytosol from two bovine brains was fractionnated on a Q Sepharose column as described in "Materials and Methods". Rap2-GAP activity was measured on 50-µl aliquots.

the activity remained more efficient on the rap1 protein. Therefore, no GAP activity specific to the rap2 protein could be isolated from this first peak.

As the second peak from the Q Sepharose column represented the major part of the rap2-GAP activity, we purified it to near homogeneity as described in methods through successive chromatographies on hydroxylapatite, Ultrogel AcA 34, Affigel blue and HPLC Mono Q columns. From the gel filtration chromatography on Ultrogel AcA34, we estimated that the rap2-GAP activity was carried by a protein of molecular weight 200 kDa (data not shown). At each step, we observed that the rap1-GAP and rap2-GAP activities co-eluted. During the last step of purification on a Mono Q HR5/5 column, the rap2-GAP activity eluted as a single peak (figure 4A); the rap1-GAP activity was found in the same fractions. SDS-PAGE analysis revealed the presence of a major polypeptide doublet of molecular mass of 55 kDa in those fractions containing rap1- and rap2-GAP activities (figure 4B). Partial proteolytic digestion of both bands from the 55kD doublet performed with V8 protease from *S. aureus* revealed similar degradation patterns (data not shown), suggesting that they represent slightly different forms of the same polypeptide.

The cytosolic rap-GAP stimulates the GTPase activity of both rap1 and rap2 proteins and likely represents a degraded form of rap1-GAPm

We compared the activity of our purified preparation toward the c-H-ras, rap1A, rap1B and rap2A proteins. Figure 5A shows that it had no effect on the GTPase activity of the c-H-ras protein, whereas it strongly stimulated the GTP hydrolysis of

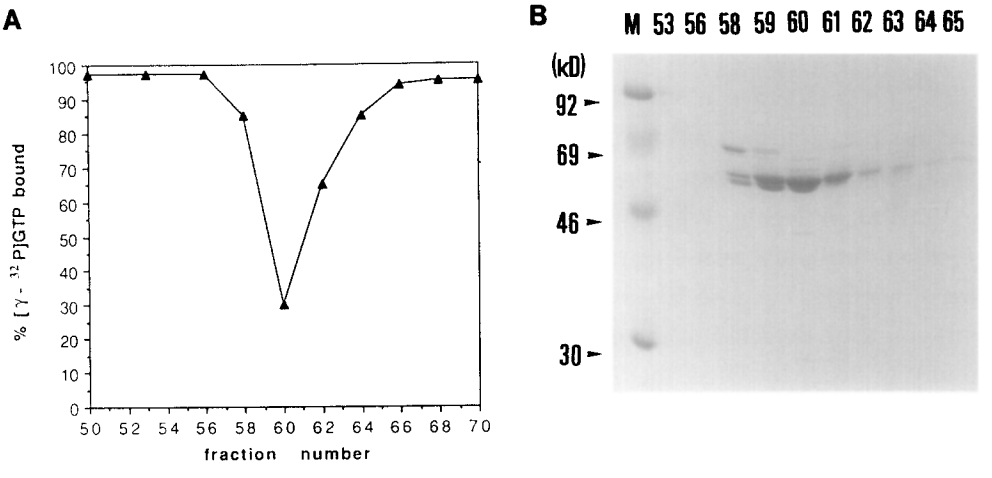

Figure 4. Final purification of rap2-GAP by HPLC MonoQ chromatography
(A) Active fractions eluted from the Affigel blue column were further purified by HPLC on a MonoQ column as described in Methods. 5 µl of each fraction was assayed by method 1.
(B) 20 µl of the indicated fractions was analysed by SDS-PAGE and proteins were stained with Coomassie brilliant blue. The molecular weight of standard proteins (M) is indicated on the left.

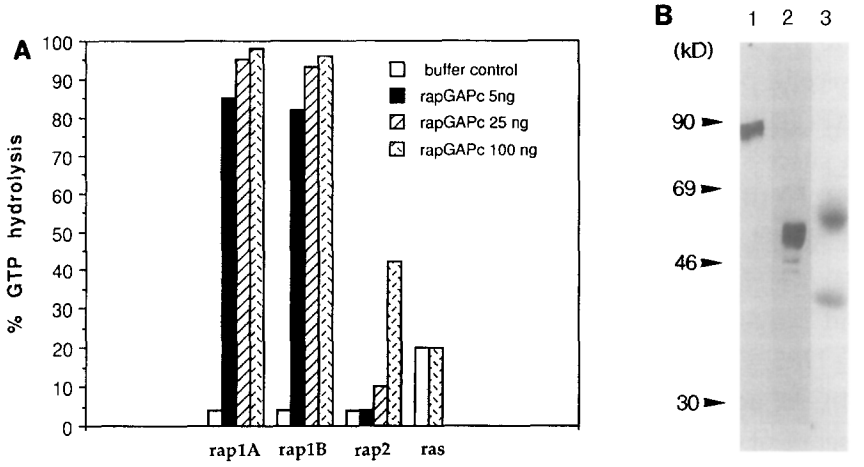

Figure 5. The cytosolic rap-GAP stimulates the GTPase activity of both rap1 and rap2 proteins and is likely to be a degraded form of rap1-GAPm
(A) The indicated amounts of purified cytosolic rap-GAPc were assessed for their ability to stimulate the GTPase activity of rap1A, rap1B, rap2 and c-H-ras proteins preloaded with [γ-^{32}P]GTP. GTP hydrolysis was measured after 10 min. incubation at 37°C by method 1.
(B) 25 ng recombinant rap1-GAPm (lane 1), 300 ng purified cytosolic rap-GAPc (fraction from the Mono Q column, lane 2) and partially purified rap2-GAP activity from the first peak of the Q Sepharose column in figure 4 (lane 3) were subjected to SDS-PAGE and analysed by Western blotting with a 1:1000 dilution of anti rap1-GAPm antiserum. The migration of molecular weight markers is shown.

all three rap proteins tested. Since this single protein seems to carry GAP activity towards both rap1 and rap2 proteins, we shall thereafter refer to it as rap-GAPc. Rap-GAPc was similarly effective against recombinant rap1A protein extracted from insect cells and rap1B protein purified from human platelets; in contrast, it was 25- to 50-fold less effective at stimulating the GTPase activity of the rap2A protein.
Since our preparation was so much more active on rap1 proteins than on the rap2A protein, we sought to determine whether it was related to the previously identified rap1-GAPm molecule. Western blot analysis of our purified preparation, using a polyclonal antibody raised against recombinant rap1-GAPm (18), revealed a broad band of Mr 55 kD (figure 5B, lane 2) ; a small amount of full size rap1-GAPm (89 kD form) could be detected in the extract upon overexposure of the same blot (data not shown). In order to detect the 55kD rap-GAPc with this antiserum, it was necessary to load 200-500 ng of purified protein on the gel, as was also the case for an C-terminal truncated form of rap1-GAPm (P. Polakis, unpublished observation), whereas as little as 25 ng full length rap1-GAPm yielded a strong immunological reaction (figure 5B, lane 1). Taken together with the facts that much of the reactivity to this antibody is localized on the C-terminal portion of rap1-GAPm and that C-terminally-truncated forms of rap1-GAPm retain GAP activity (P. Polakis, unpublished observation), these data suggests that the cytosolic 55 kD rap-GAPc that

we have purified represents an N-terminal fragment resulting from partial proteolysis of the 89kD rap1-GAPm. Our results are consistent with the recently published data from Nice et al (27) who have also purified from bovine brain cytosol a rap1-GAP protein of 55 kD that contains common sequences with the full length rap1-GAPm. As described higher in the text, a minor portion of rap2-GAP activity was eluted from the initial Q Sepharose column at a lower salt concentration, and co-fractionnated upon further purification with rap1-GAP activity. Analysis of this partially purified material by Western blotting revealed two bands of Mr 57 kDa and 40 kDa that reacted with the polyclonal anti-rap1-GAPm antibody (figure 5B, lane 3), indicating that the GAP activity of this extract was probably also attributable to partially degraded forms of rap1-GAPm. A weak rap2-GAP activity was also detected in extracts from bovine brain membranes (data not shown); however, since it was much more effective on the rap1 as compared with the rap2 protein, this activity was also attribuable to the rap1-GAPm molecule.
Taken together, our results demonstrate that a single cytosolic GAP protein, which is a degraded form from the membrane-associated rap1-GAPm, can stimulate the GTPase activity of both rap1 and rap2 proteins. It remains possible that a GAP activity specific for the rap2 protein is also present in bovine brain but could not be isolated by our current methods.

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