Purification and *N*-terminal sequence of the p21^{rho} GTPase-activating protein, rho GAP

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Eukaryotic cells contain numerous small-molecular-mass GTP-binding proteins, but the processes that they regulate are not known. Different members of this protein family appear to be associated with specific GTPase-activating proteins (GAPs), and we have previously reported the identification of a cytoplasmic GAP (rho GAP) that stimulates the GTPase activity of p21^{rho} but not of other small-molecular-mass GTP-binding proteins. We have now purified rho GAP 2000-fold from human spleen tissue using f.p.l.c. Electrotransfer of this 27.5 kDa protein on to an Immobilon-P transfer membrane followed by reconstitution of its enzymic activity confirmed its identity. Rho GAP was subjected to *N*-terminal sequence analysis and 15 amino acids were obtained. The sequence showed 53 % identity with a region present in IRA1, a protein which stimulates the GTPase activity of RAS proteins in *Saccharomyces cerevisiae*. These results suggest that there is a family of sequence-related GAP proteins, which to date includes ras GAP and its yeast counterparts IRA1 and IRA2, rho GAP and the Neurofibromatosis gene product NF1.

INTRODUCTION

The superfamily of ras-related small-molecular-mass GTP-binding proteins currently comprises around 30 members [1]. Most are expressed ubiquitously, they all bind GTP/GDP and have an intrinsic GTPase activity, and the ras proteins themselves at least are located at the cytoplasmic face of the plasma membrane [1,2]. The signal transduction pathways controlled by ras and the other related proteins still, however, remain obscure. The discovery of a cytoplasmic GTPase-activating protein (GAP), which stimulates the intrinsic GTPase activity of p21^{ras}, has provided a new approach to the study of ras function [3]. Although GAP was originally identified as a negative regulator of ras, mutational analysis has suggested that GAP also contributes to the downstream signals elicited by ras [4–6]. In support of this, it has since been found that ras and GAP together can affect the opening of K⁺ channels in atrial cells [7]. In addition, several groups have reported that in response to growth factors, e.g. platelet-derived growth factor (PDGF), GAP becomes phosphorylated on tyrosine and complexes to at least three other proteins, including the activated PDGF receptor itself [8–11]. Clearly GAP is intimately involved in the signal transduction pathways controlling growth, and its function is being intensely investigated.

Although the ras proteins have been studied in the greatest detail, the kinds of biological processes regulated by other members of the superfamily of small GTP-binding proteins are now beginning to be revealed [1]. The members of the rho sub-group each have around 30 % amino acid identity with ras. To date, this sub-group comprises seven proteins in mammalian cells: rho A, B and C, rac 1 and 2, TC10 and G25K [1,12–14]. In *Saccharomyces cerevisiae*, three rho-like genes, *RHO1*, *RHO2* and *CDC42*, have been identified, and genetic analysis suggests that CDC42 (the yeast homologue of G25K) is involved in the organization of cell polarity in budding yeast [15,16].

The human *rhoA* gene has been expressed in *Escherichia coli* and the purified recombinant protein (p21^{rho}) has a similar guanine nucleotide exchange rate and intrinsic GTPase activity to p21^{ras} [17]. We have identified a distinct 29 kDa protein, rho GAP, which stimulates the intrinsic GTPase activity of p21^{rho} [17]. An amino acid substitution of glycine by valine at codon 14 in rhoA lowers the intrinsic GTPase activity of p21^{rho} and blocks the stimulation of rho GTPase activity by rho GAP, an effect similar to that caused by the oncogenic mutations in codon 12 in ras [17]. Two other proteins that interact with rho have been identified by others; one, GDI, inhibits the dissociation of GDP from p21^{rhoB}, and a second, GDS1/2, stimulates the dissociation of GDP from p21^{rhoA} and p21^{rhoB} [18,19].

The rho proteins are substrates for the bacterial enzyme C₃ ADP-ribosyltransferase from *Clostridium botulinum* [20]. Micro-injection of this enzyme into mammalian cells causes the disruption of actin filaments followed by the rounding up of cells, and we have shown that this is a consequence of the inactivation of endogenous rho after ADP-ribosylation [21,22]. Micro-injection of constitutively activated [Val¹⁴]p21^{rho} into mammalian cells, on the other hand, induces dramatic changes in cell morphology, consistent with a reorganization of the actin cytoskeleton [22]. These experiments point to a role for p21^{rho} in the control of cytoskeletal organization and, by analogy with p21^{rho}, we would expect rho GAP to play an important regulatory role in this process. As a first step in analysing its biochemical function, we report in this paper the purification of rho GAP from human spleen tissue and its *N*-terminal sequence.

EXPERIMENTAL

Materials

Human spleens were obtained and stored as described by Major *et al.* [23] [γ -³²P]GTP (30 Ci/mmol) was purchased from

Abbreviations used: GAP, GTPase-activating protein; QSFF, Q-Sepharose Fast-Flow; DTT, dithiothreitol; PMSF, phenylmethanesulphonyl fluoride; PDGF, platelet-derived growth factor.

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NEN-DuPont U.K. Recombinant p21^{rhoA} was expressed in *E. coli* and purified according to [17].

Purification of rho GAP

All manipulations were carried out at 4 °C unless otherwise stated. Whole tissue extracts were prepared from 420 g (wet weight) of spleen in buffer A [50 mM-Tris/HCl, pH 8.3 at 22 °C, 0.5 mM-EDTA, 1 mM-dithiothreitol (DTT)] by the method of Pegg *et al.* [24], and rho GAP activity was precipitated from the extract with ammonium sulphate (final concentration 50%, w/v). The precipitate was collected by centrifugation at 143 000 *g*_{max} for 40 min, then redissolved in 1200 ml of buffer A. After dialysis for 18 h against three lots of buffer A (total volume 81 litres), the extract was clarified by centrifugation (as above) for 60 min, and the supernatant was collected.

Using an f.p.l.c. system (Pharmacia LKB Biotechnology), the clarified dialysate was loaded on to a water-jacketed XK 50/30 column packed with 300 ml of Q-Sepharose Fast-Flow (QSFF) anion-exchange resin (Pharmacia) pre-equilibrated with 3 litres of buffer A, with the column cooled to 5 °C. The column was washed with 400 ml of buffer A and elution was performed as follows. (1) Buffer A (1200 ml) adjusted to pH 7.13 (at 22 °C) was applied and the eluate was collected in 8 × 150 ml fractions. (2) Then 850 ml of buffer A adjusted to pH 7.85 (at 22 °C) and 50 mM-NaCl was applied, and the eluate was collected as one 250 ml fraction, followed by 4 × 150 ml fractions. (3) Stepwise elution was carried out using buffer A, pH 7.85 (at 22 °C), containing higher concentrations of NaCl (see Fig. 1). Upon elution, leupeptin, pepstatin and phenylmethanesulphonyl fluoride (PMSF), to final concentrations of 0.5 µg/ml, 0.7 µg/ml and 0.2 µM respectively, were added to each fraction. Two peaks, A and B, of rho GAP activity were detected in fractions eluted with 100 mM- and 175 mM-NaCl respectively.

Fractions from peak B (610 ml) were pooled and dialysed overnight against 4 litres of buffer B (50 mM-Tris/HCl, pH 8.3, 0.5 mM-EDTA, 1 mM-DTT, 0.2 mM-PMSF, 0.5 µg of leupeptin/ml, 0.7 µg of pepstatin/ml), with two further changes of 4 litres of buffer B. After dialysis, the sample was applied to a column (5 cm × 5.1 cm) of matrix gel Green A resin (Amicon) pre-equilibrated with buffer B. The column was washed with buffer B until the *A*₂₁₄ returned to baseline. The protein was eluted with a 1500 ml linear gradient of NaCl (0–0.5 M) in buffer B and 50 ml fractions were collected. Active fractions (5–18) were pooled and ammonium sulphate was added to a final concentration of 80% (w/v). After 30 min the precipitate was collected by centrifugation at 25 000 *g*_{max} for 60 min and then redissolved in 90 ml of buffer C (10 mM-potassium phosphate, pH 6.8, 0.1 mM-DTT, 0.1 mM-PMSF) and dialysed overnight against 4 litres of buffer C, with one change of 4 litres of buffer C.

The dialysate was loaded on to a DNA-grade Bio-Gel column (Bio-Rad Laboratories; 1.5 cm × 12 cm) pre-equilibrated with buffer C, and the column was washed until the *A*₂₁₄ returned to baseline. Elution was performed with a 60 ml linear gradient of 10–58 mM-potassium phosphate, followed by a 450 ml linear gradient of 58–130 mM-potassium phosphate, and 9 ml fractions were collected. An f.p.l.c. system was used to achieve the gradients of potassium phosphate by mixing buffer C and buffer D (250 mM-potassium phosphate, pH 6.8 at 4 °C, 0.1 mM-DTT, 0.1 mM-PMSF). Fractions containing rho GAP activity (13–22) were pooled and dialysed overnight against 4 litres of buffer E (10 mM-Mes, pH 6.0, 10 mM-NaCl, 0.5 mM-EDTA, 0.1 mM-DTT, 0.1 mM-PMSF) with one further 4 litre change of the same buffer.

The dialysate was applied to a MonoS HR5/5 column (1 ml; Pharmacia) pre-equilibrated with buffer E and the column was washed with 10 ml of the same buffer. Elution was performed

with a 20 ml linear gradient of NaCl (10–350 mM) in buffer E, and 0.5 ml fractions were collected. Rho GAP activity was eluted as two peaks between fractions 11 and 19 (see Fig. 2).

Attempts to further purify peak A from the first QSFF anion-exchange column, using similar procedures, failed. On the Bio-Gel and MonoS columns, rho GAP activity coeluted with total protein, and no significant purification was achieved.

Reconstitution of rho GAP activity from Western blots

This procedure was adapted from a method described by Szewczyk & Summers [25]. All reagents were high quality; in particular, SDS used in the sample buffer and the Triton X-100 used in the elution buffer were Surfact-Amps-purified detergent solutions purchased from Pierce & Warriner (U.K.). Acetone was AristaR grade from BDH, and BSA and SDS used in the elution buffer were Ultrapure grade from Gibco-BRL.

Samples of the MonoS column fractions containing rho GAP activity were boiled for 5 min in sample buffer [80 mM-Tris/HCl, pH 7.8, 0.1 mM-DTT, 10% (v/v) glycerol, 2% (v/v) SDS, 0.1% Bromophenol Blue] and applied to a polyacrylamide gel (4% stacking gel and 15% separating gel; 1.5 mm thickness) and electrophoresis was carried out using the discontinuous buffer system [26]. Electrophoretic transfer of the protein from the gel on to an Immobilon-P transfer membrane (Millipore) was carried out according to Matsudaira [27] for 90 min at 0.5 A at 4 °C, and the proteins were visualized with Coomassie Blue [27] and destained with 50% methanol.

The lanes containing electrophoresed protein were dissected into fragments, placed in Microfuge tubes (1.5 ml) and washed once with methanol (1 ml) and three times with water (1 ml). Elution buffer (100 µl) containing 50 mM-Tris/HCl, pH 9.0, 2% (w/v) SDS, 1% (v/v) Triton X-100 and 1 mg of BSA/ml was added, and each sample was incubated on a shaking platform at room temperature for 1 h. The samples were then centrifuged for 10 min in a Microfuge, and acetone (800 µl) was added to each supernatant. Protein in the samples was allowed to precipitate for 1 h in a solid-CO₂/ethanol bath and the precipitate was collected by centrifugation for 20 min in a Microfuge. The protein precipitate was left to air-dry and was then redissolved in 5 µl of 5 M-guanidine hydrochloride for 1 h. Finally, 245 µl of renaturation buffer (20 mM-Tris/HCl, pH 7.5, 0.1 mM-DTT) was added, and the samples were incubated for at least 24 h at 4 °C before being tested for GAP activity.

N-Terminal sequence analysis

Samples (50 µl) of fractions 13 and 18 eluted from the MonoS column containing rho GAP activity (peak B) were boiled for 5 min in sample buffer and loaded on to a polyacrylamide gel (4% stacking gel, 15% separating gel; 0.5 mm thickness). Electrophoresis was carried out using the discontinuous buffer system [26], with the addition of 0.1 mM-thioglyoxylate to the anode compartment. Electrophoretic transfer of protein from the gel on to an Immobilon-P transfer membrane was carried out for 10 min at 0.5 A at 4 °C as described by Matsudaira [27]. Proteins were visualized with Coomassie Blue, destained and washed according to Matsudaira [27], except that the destaining solution did not contain acetic acid. The single band identified as containing rho GAP activity in fraction 13 and the higher-molecular-mass band from fraction 18 containing rho GAP activity were excised from the membrane using scalpels. Sequencing of these bands was performed on an Applied Biosystems model 477A Pulse Liquid Sequencer equipped with a microreaction cartridge and using rapid chemistry and analysis cycles (N. Totty & R. Mattaliano, unpublished work).

Other assays and procedures

The molecular mass of rho GAP was estimated by Superose gel filtration (Pharmacia) in buffer F (50 mM-Tris/HCl, pH 7.6, 0.5 mM-EDTA, 160 mM-NaCl, 0.1 mM-DTT, 0.1 mM-PMSF), using thyroglobulin (670 kDa), γ -globulin (158 kDa), BSA (67 kDa), ovalbumin (44 kDa), chymotrypsin (25 kDa) and ribonuclease A (13.7 kDa) as the calibrating protein standards. GAP assays were carried out as previously reported [18]. One unit of GAP activity is defined as the amount necessary to decrease the percentage of bound GTP on p21^{rho} from 75% (background intrinsic rate) to 30% after 5 min of incubation at 30 °C. For each assay the protein sample was diluted until 1 unit of GAP activity was detected. Protein concentrations were estimated by the Bradford method [28] using Bio-Rad dye reagent, with BSA as the protein standard.

RESULTS AND DISCUSSION

GAP activity towards p21^{rho} was purified as described in the Experimental section to achieve a degree of purification suitable for N-terminal sequencing of the rho GAP protein. Fig. 1 shows the elution profile of rho GAP activity after QSFF anion-

Fig. 1. QSFF anion-exchange column chromatography

Spleen extract was applied to a QSFF column and washed with 50 mM-NaCl in buffer A. Arrows indicate the NaCl concentration at the start of each step of batch elution: 1, 100 mM; 2, 175 mM; 3, 250 mM; 4, 500 mM; 5, 1000 mM, in buffer A at pH 7.85. ●, Rho GAP activity; ○, protein present in each fraction.

Table 1. Purification of rho GAP from human spleen tissue

After the 50% ammonium sulphate substrate step, the results represent the purification of peak B only. The MonoS data represent the sum total of peak B1 and peak B2.

Step	Protein (mg)	Activity (munits)	Specific activity (munits/mg)	Purification (fold)	Yield (%)
Extract	32820	5807	0.177	—	100
Ammonium sulphate (50%)	10200	5200	0.51	2.88	89.5
QSFF (peak B)	616	3850	6.25	35.3	66.3
Green A	123	3000	24.39	137.8	51.6
Bio-Gel	10.2	1275	124.6	704	22.0
MonoS (peaks B1 and B2)	1.895	675	356.2	2012	11.6

Fig. 2. MonoS column chromatography

(a) Elution profile of rho GAP activity from the final purification column. The absorbance at 214 nm is also shown (—). (b) SDS/PAGE of 10 μ l aliquots of MonoS fractions. Protein was visualized with Coomassie Blue. The protein markers were BSA (69 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (21.5 kDa) and lysozyme (14.3 kDa). Bands exhibiting GAP activity after reconstitution of all proteins visualized in lanes 13 and 18 are shown.

exchange column chromatography. The GAP activity separated into two peaks eluting at 100 mM- (peak A) and 175 mM-NaCl (peak B). Although peak A bound to and was eluted from the second column (matrex gel Green A) in a similar fashion to peak B, we were unable to achieve further significant purification of peak A by subsequent Bio-Gel, MonoS and MonoQ column chromatography. We observed for each of these columns that rho GAP activity was eluted across a broad range of fractions which encompassed most of the eluted protein. The reasons for this are unclear at the present time.

In contrast, the rho GAP activity present in peak B could be further purified, as described in the Experimental section. This activity gave an estimated molecular mass of between 27 and 32 kDa by gel filtration chromatography. A combination of matrex gel Green A, Bio-Gel and MonoS column chromatography yielded a final preparation that was 2012-fold enriched in rho GAP activity (Table 1). Two peaks of rho GAP activity (B1 and B2) were observed after elution from the final MonoS column, at 118 and 153 mM-NaCl (Fig. 2a). SDS/PAGE of MonoS fractions revealed only one significant band in the correct molecular mass range for peak B1, at 27.5 kDa, but several bands in the same region for peak B2 (Fig. 2b). Reconstitution assays were performed on 20 μ l samples of these MonoS peaks (fraction 13 for B1 and fraction 18 for B2) after SDS/PAGE and electrotransfer on to an Immobilon-P transfer membrane. In our experiments we have repeatedly detected between 4 and 10% recovery of the rho GAP activity originally loaded on to the SDS/polyacrylamide gel after reconstitution from the Immobilon-P transfer membrane. This high level of recovery was dependent on the use of only high grade chemicals and, in particular, the use of AristaR grade acetone was essential.

Rho GAP		Y D D F L K S T Q K S P A T A
IRA1	1530	I D D F L K Y I I K N P I L A
IRA2	1676	A D K F L R Y T I E H P Q L S
Ras GAP	720	Y S E F K E L I L Q K E L H V

Fig. 3. *N*-Terminal amino acid sequence of rho GAP compared with IRA1, IRA2 and ras GAP

Numbers indicate the position from the *N*-terminus. The IRA1 and IRA2 sequences were taken from [29]; the ras GAP sequence was taken from [30].

In this way we confirmed that the single band seen at 27.5 kDa in peak B1 had rho GAP activity. Rho GAP activity was detected in two bands (25.7 and 26.9 kDa) of peak B2 (Fig. 2*b*). We think it likely that rho GAP activity detected in peak B2 represents degradation products of the 27.5 kDa protein of peak B1.

Attempts to obtain an *N*-terminal sequence from the 26.9 kDa band in peak B2 were unsuccessful, suggesting that the protein may have become blocked at the *N*-terminus by chemical modification (the 25.7 kDa band was not tested). *N*-Terminal sequencing of the 27.5 kDa band of peak B1, however, was successful, and a stretch of 15 amino acids was obtained before the signal became too weak to interpret (see Fig. 3). This sequence was not identical to any sequences in the Swiss-Prot Protein Database (release 14), but did exhibit 53% identity to a region of the IRA1 protein, as shown in Fig. 3 [29]. IRA1 and IRA2 appear to negatively regulate the yeast RAS proteins of *S. cerevisiae* by stimulation of their intrinsic GTPase activities [29,30]. The sequence similarity of rho GAP to IRA1 is found at the beginning of a region of the IRA1 protein that has identity with the catalytic domain of mammalian ras GAP [31]. Further protein sequencing of rho GAP will be required to define the exact relationship between this and other GAP-like proteins.

Two GAP activities (GAP 1 and GAP 2) towards rap1A, another member of the ras superfamily, have been detected [32]. It may be that there is a family of sequence-related GAP-like proteins which interact with distinct members of the ras superfamily. In addition, it has recently been reported that the product of the Neurofibromatosis type 1 (NF1) gene has identity with the catalytic domain of mammalian ras GAP and IRA1 [33]. Although rho GAP clearly stimulates the intrinsic GTPase activity of p21^{rho} [17], it is not known if it also performs other roles in the cell. In the case of ras GAP, there is evidence that this protein is involved along with ras in downstream signalling and, in addition, ras GAP has been shown to bind to the activated PDGF receptor [7–11]. These observations point towards a complicated regulatory role for GAP proteins, in addition to their ability to down-regulate small GTP-binding proteins. We expect that further work with p21^{rho} and rho GAP will help to establish in more detail the processes regulated by these proteins.

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