Characterization of human platelet GTPase activating protein for the \textit{Ral} GTP-binding protein

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Abstract

\textit{RalA}, a \textit{ras} \textit{p21} related 27 kDa GTP-binding protein, was expressed as a fusion protein in \textit{Escherichia coli} and purified to homogeneity using an immunoaffinity column. The purified protein was capable of binding and hydrolyzing GTP. Addition of platelet cytosolic or detergent solubilized particulate proteins stimulated the intrinsic GTPase activity of \textit{ralA} by at least six-fold with maximal effect observed at pH 6.5. Addition of platelet proteins denatured by boiling had no effect on \textit{ralA} GTPase activity. Analysis of GTPase reaction products by thin layer chromatography demonstrated that in samples containing \textit{ralA}, 78.5 ± 6.3\% of the radioactivity was recovered in the GTP form while samples containing \textit{ralA} plus platelet cytosol or particulate proteins, only 7.5 ± 0.2\% and 9.0 ± 1.4\% of the radioactivity was in the GTP form respectively. The GTPase activating protein(s) in the cytosolic and particulate fraction was further characterized by measuring GAP activity in proteins eluted from gel slices after sodium dodecyl sulfate polyacrylamide gel electrophoresis. The \textit{ralA} GTPase activating protein present in the cytosol and particulate fractions was recovered in a single gel slice of identical apparent molecular weight. The molecular mass of the \textit{ral} specific GTPase activating protein was estimated to be 34 ± 2 kDa. This protein did not stimulate the intrinsic GTPase activity of \textit{ras} \textit{p21}, \textit{G25K/CDC42Hs} or \textit{rab3A} GTP-binding proteins. Results demonstrate that in human platelets, the activity/function of \textit{ral}-related GTP-binding protein(s) is under the regulation of a specific GTPase activating protein of molecular mass of 34 ± 2 kDa that is distributed equally in the cytosol and particulate fraction.

\textit{Keywords}: \textit{Ral}; \textit{Ras}-related; GTP-binding protein; GTPase activating protein; GAP; (Human platelet)

1. Introduction

The existence of a large number of GTP-binding proteins of molecular mass between 20–27 kDa and with varying degree of homology to the \textit{ras} \textit{p21} proteins has been established in the eukaryotic cell [1–4]. As is the case for the heterotrimeric G-proteins, \textit{ras} \textit{p21} and \textit{ras}-related proteins exist in the GTP-bound form upon activation and are converted to the inactive GDP-bound form by the intrinsic GTPase activity associated with these proteins [5,6]. In comparison to the heterotrimeric G-proteins, \textit{ras} \textit{p21} proteins have a lower intrinsic GTPase activity [5]. However, it has been shown that the GTPase activity of \textit{ras} \textit{p21} can be stimulated by a cytoplasmic protein termed, GAP [7]. The effector region of \textit{ras} \textit{p21} interacts with the GTPase activating protein suggesting an important role for GAP in \textit{ras} \textit{p21} regulated signal transduction pathways [8]. The \textit{ras} \textit{p21} specific GAP has been purified from bovine brain cytosol and shown to consist of a single polypeptide chain of molecular mass of 125 kDa [9]. The gene coding for \textit{ras}-GAP has been cloned and sequenced from a bovine brain cDNA library [10] and the C-terminal region of GAP is proposed to be necessary for its catalytic activity [11]. Insight into the structure of \textit{ras}-GAP has also led to the identification of another protein termed, neurofibromin, as an activator of the intrinsic GTPase activity of \textit{ras} \textit{p21} [12,13].
Studies on the regulation of GTPase activity of ras p21 suggested that function of ras-related GTP-binding proteins may also be controlled in a similar fashion. This has led to the identification and characterization of GAPs for some ras-related GTP-binding proteins. Thus, specific GAPs have been purified for the rap1A [14,15], rho [16] and G25K/CDC42Hs [17] proteins. In case of the ras-related GTP-binding protein coded for by the ral gene, a GAP activity has been detected in rat brain and mouse testis cytosol [18]. However, the identity of this protein has remained elusive.

The purpose of the current study was to establish the identity of ral-GAP. The ral gene codes for a GTP-binding protein of predicted molecular mass of 23.5 kDa that was originally cloned and sequenced from a simian B-cell cDNA library and shown to be ~ 50% homologous to the ras p21 protein [19]. Later, the ral gene was cloned and sequenced from a human placental cDNA library [20]. Now, two related ral genes, ralA and ralB, that are ~ 85% identical and differ essentially in their C-terminus region have been cloned and sequenced from a human pheochromocytoma [21], marine ray electric lobe [22] and rat liver [23] cDNA libraries. Previously, we have demonstrated the existence of a family of 23–27 kDa GTP-binding proteins in platelet and other tissues that can be specifically labeled by incubation of [32P]GTP with nitrocellulose blots containing polypeptides separated using SDS-PAGE [24]. Using antibodies raised against recombinant ralA, we have also shown that part of the platelet 27 kDa GTP-binding protein is coded for by the ral-related gene [25]. In addition, determination of the amino acid sequence of peptide fragments generated after digestion of a 28 kDa platelet GTP-binding protein has also confirmed the presence of ral gene product in human platelets [20]. However, the physiological function and biochemical regulation of ral protein in platelet has not been defined. The present report describes expression of ralA cDNA in E. coli, purification of recombinant protein to homogeneity and identification of a ral specific GTPase activating protein in the human platelet cytosol and particulate fractions. An abstract of this work has been published [26].

2. Materials and methods

2.1. Materials

The IBI pFLAG-1 Biosystem used for the expression and purification of ralA fusion protein from E. coli was obtained from InterSciences Inc. (Toronto, Ont., Canada). Restriction enzymes and other molecular biology reagents were from Pharmacia Canada Ltd. (Montreal, Que., Canada). DH5α strain of E. coli, ampicillin, IPTG and streptomycin sulfate were obtained from GIBCO-BRL (Oakville, Ont., Canada). Media for bacterial culture was from Difco Laboratories Ltd. (Detroit, MI, USA). Nitrocellulose membrane (0.2 μm pore), reagents and prestandard protein markers for SDS-PAGE were from Bio-Rad Laboratories (Canada) (Mississauga, Ont., Canada). Schleicher and Schuell nitrocellulose membrane discs (0.45 μm pore) and poly(ethylene)imine (PEI) cellulose thin layer chromatography (TLC) sheets were obtained from Mandel Scientific Ltd. (Toronto, Ont., Canada). CHAPS and Tris were obtained from Boehringer Mannheim (Laval, Que., Canada). AMPPNP, adenosine-5’-triphosphate (ATP), guanosine-5’-triphosphate (GTP), Hepes, Mes, Pipes, BSA, DTT, EDTA, PMSF and low molecular weight protein markers for SDS-PAGE were obtained from Sigma Chemical Co. (St. Louis, MO, USA). [γ-32P]GTP (6000 Ci/mmol) was from Dupont Canada (Mississauga, Ont., Canada). [α-32P]GTP (3000 Ci/mmol) was obtained from ICN Radiochemicals (Irvine, CA, USA). Liquid scintillation cocktail was from Beckman Instruments (Canada) (Mississauga, Ont., Canada). All other reagents used were of analytical grade.

2.2. RalA expression and purification

RalA was expressed as a fusion protein linked to the C-terminal end of flag peptide of the expression vector, pFLAG-1. For in frame cloning, an ~ 900 base pair HindIII cDNA fragment containing the complete coding sequence for rala [20] was inserted into the unique HindIII multiple cloning site of the pFLAG-1 vector and used to transform competent DH5α E. coli cells. Bacteria were spread on Luria-Bertani (LB)-agar plates containing 50 μg/ml ampicillin and allowed to grow overnight at 37°C. Several colonies were randomly picked and for preparing plasmid DNA, 2 ml bacterial cultures were grown overnight in LB medium containing 50 μg/ml ampicillin. Since rala gene has a single EcoRI site approximately 50 base pairs downstream of the initiation codon [19] and the pFLAG-1 vector also contains a unique EcoRI site in the multiple cloning site, HindIII or EcoRI digestion was used to establish the presence and orientation respectively of the rala insert in plasmid DNA preparations. Four bacterial colonies were positive for the insert with three containing the rala cDNA in the correct orientation. To detect expression of the rala fusion protein, colonies containing the insert were cultured overnight in LB medium containing 50 μg/ml ampicillin followed by a further 7 h in the absence or presence of IPTG. The bacterial proteins were separated by SDS-PAGE, the gel stained with Coomassie Blue or electroblotted to nitrocellulose and blot incubated with [α-32P]GTP as described below.

To purify the rala fusion protein, 2 l culture of a positive bacterial colony was grown overnight at 37°C in LB medium containing 50 μg/ml ampicillin followed by a further 7 h in the presence of 1.5 mM IPTG in the same culture medium. The bacteria were harvested by centrifugation and pellet resuspended in 50 ml of phosphate-buffered saline (0.01 M NaH2PO4, pH 8.4, 0.15 M NaCl

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and 1 mM PMSF) which also contained 0.25 mg/ml lysozyme. The bacterial suspension was lysed by freeze-thaw (3 times in liquid N₂) and by incubation at 37°C for 30 min. The lysate was centrifuged at 25 000 × g for 45 min at 4°C. To reduce viscosity and precipitate DNA, enough streptomycin sulfate was added to the supernatant to achieve a final concentration of 2.5% (w/v). After stirring at 4°C for 30 min, the suspension was centrifuged at 10 000 × g for 10 min at 4°C. For purification of the ralA fusion protein, supernatant was passed through an affinity column (provided with the pFLAG-1 vector). After washing the column three times with phosphate-buffered saline (0.01 M NaH₂PO₄, pH 7.4, 0.15 M NaCl and 1 mM PMSF), ralA fusion protein was eluted from the immobilized peptide (flag peptide) with 0.1 M glycine (pH 3.0). The fractions containing ralA were immediately neutralized by the addition of 1 M Tris and stored at −70°C until further use.

2.3. Preparation of platelet particulate and cytosolic fraction

Human platelets were obtained from the local Red Cross. To remove contaminating red blood cells, the platelet concentrate was centrifuged at 160 × g for 30 s and the pellet discarded. The supernatant was further centrifuged at 2000 × g for 15 min and the platelet pellet washed three times in a medium containing 13 mM trisodium citrate, 5 mM dextrose and 135 mM NaCl (pH 6.5). The final platelet pellet was resuspended in a buffer containing 10 mM Tris-HCl, pH 7.5, 5 mM EDTA and 1 mM PMSF (buffer A). The platelets were lysed by freeze-thaw (three times in liquid N₂) and the cytosolic and particulate fractions prepared by centrifugation at 100 000 × g for 1 h at 4°C. The particulate pellet was resuspended in buffer A and centrifuged at 100 000 × g for 1 h at 4°C. The cytosolic and particulate fractions were stored at −70°C until further use.

2.4. Solubilization of platelet particulate proteins

The platelet particulate fraction obtained in Section 2.3 was stirred for 1 h at 4°C in buffer A which also contained 1% CHAPS. The suspension was centrifuged at 100 000 × g for 1 h at 4°C and the supernatant containing solubilized proteins was stored at −70°C until further use.

2.5. Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Samples containing protein were precipitated by the addition of trichloroacetic acid to a final concentration of 10% (w/v). Precipitated proteins were collected by centrifugation and solubilized in 50 μl of electrophoresis sample buffer [27]. After neutralization of residual acid by the addition of 1 N NaOH, samples were heated for 3 min at 100°C and analyzed by SDS-PAGE with 13% (w/v) acrylamide in the separating gel [24,27].

2.6. Detection of proteins capable of binding [α-32P]GTP on nitrocellulose blots

The procedure has been described in detail elsewhere [24] and was used with minor modification. Briefly, polypeptides separated using SDS-PAGE were electrobotted to nitrocellulose using a transfer buffer containing 0.05% SDS [24,28] and the blot incubated for 30 min at 20°C in a buffer containing 50 mM Tris-HCl, pH 7.5, 0.3% (w/v) Tween 20 (buffer B) and 5% milk powder. At the end of this period, the blot was washed in buffer B (three times for 5 min each) and incubated for a further 30 min at 20°C in buffer B that also contained 1.0 μCi/ml [α-32P]GTP (final concn. 1 nM), 2 μM ATP and 4 μM MgCl₂. After washing three times in the same buffer without GTP, blots were air-dried and bound 32P detected by autoradiography (12–24 h at −70°C, using a Cronex intensifying screen, Picker International, Brampton, Ont., Canada).

2.7. GTPase assay

The GTPase activity was assayed by measuring loss of 32P from ralA that had been preloaded with [γ-32P]GTP. The procedure is a modification of the method described in [7]. Briefly, in the first step of the assay, ralA fusion protein (~2 μg) was incubated in a total volume of 100 μl for 15 min at room temperature in buffer containing 20 mM Tris-HCl, pH 7.5, 1 mM DTT, 0.1 mM AMPPNP, 2 mM EDTA, 40 mg/ml BSA and 0.5 mM [γ-32P]GTP (100 Ci/mmol). In the second step, an aliquot (15 ng) of [γ-32P]GTP loaded ralA was added to a mixture (total vol = 50 μl) containing 20 mM Tris-HCl, pH 7.5, 100 mM NaCl and 10 mM MgCl₂ and incubated for 15 min at room temperature. The reaction was terminated by the addition of 1 ml of ice-cold stopping buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl and 10 mM MgCl₂) and contents quickly filtered through nitrocellulose discs using the Millipore filtration apparatus. After washing five times (2 ml/wash) with stopping buffer, 10 ml of scintillation fluid was added and radioactivity associated with filters quantitated using a Beckman 5801 liquid scintillation counter.

To analyze products of the GTPase assay by thin layer chromatography, after filtration and washing, nitrocellulose filters were suspended in 500 μl of 0.1 N HCl to extract the guanine nucleotides. The sample was adjusted to pH 7.2 by the addition of 130 μl of 0.5 M Tris [29]. After making the extract 1 mM in GTP and GDP, an aliquot was spotted on poly(ethylene)imine cellulose sheets and chromatographed using 1 M KH₂PO₄ [29]. The GTP and GDP spots were visualized using autoradiography (12–24 h at −70°C, using a Cronex intensifying screen,
Picker International, Brampton, Ont., Canada). To determine radioactivity associated with GTP and GDP, the appropriate spot was cut out and quantitated using liquid scintillation counting as described above.

3. Results

3.1. Expression of ralA in E. coli and purification of recombinant protein

The cDNA containing the complete coding sequence for human ralA was subcloned into the pFlag-1 vector and the fusion protein expressed in DH5α strain of E. coli. Four clones were positive for the ralA cDNA with three containing the insert in the correct orientation. Upon addition of IPTG to the culture medium, bacterial colonies containing the ralA cDNA in the correct orientation expressed a fusion protein of predicted molecular mass of 30 kDa. The ralA fusion protein expressed in E. coli bound GTP when nitrocellulose blots containing polypeptides separated by SDS-PAGE were incubated with [α-32P]GTP.

The ralA fusion protein was purified to homogeneity in a single step using an immunoaffinity column that contained covalently bound antibody against the flag peptide (Fig. 1A, lanes 2 and 3). The average yield of the fusion protein was ~0.5 mg from an overnight 2 l bacterial

2.8. Detection of GAP activity

To establish presence of ralA specific GAP in human platelets, the GTPase activity was measured as described above except that in the second step of the assay, platelet cytosolic or detergent solubilized particulate proteins were added to the reaction mixture.

To determine optimal pH for GAP activity, platelets were lysed by freeze-thaw in buffer containing 10 mM Mes (pH 5.5) or 10 mM Mes (pH 6.0) or 10 mM Pipes (pH 6.5) or 10 mM Hepes (pH 7.0) or 10 mM Tris-HCl (pH 8.0) plus 5 mM EDTA and 1.0 mM PMSF. The cytosolic fraction was prepared as described above and used for measuring GAP activity. The GTPase assay was carried out as in Section 2.7 except that buffer used in the GAP assay was identical to that used for lysing the platelets.

2.9. Detection of GAP activity after SDS-PAGE

To obtain an estimate of the molecular mass of platelet ral-GAP, 1 vol. of 5 x Laemmli's sample buffer [27] was added to 4 vol. of solution containing cytosolic or detergent solubilized particulate proteins. The sample was heated at 56℃ for 3 min and the cytosolic and particulate proteins separated using SDS-PAGE in alternate lanes in duplicate along with molecular weight markers. One part of the gel containing platelet proteins and molecular weight markers was stained using Coomassie Blue and the second equivalent part was equilibrated by shaking for 15 min at room temperature in a buffer containing 10 mM Tris-HCl, pH 7.5, and 1 mM DTT. This gel portion was cut into slices from the top to the dye front followed by a cut in the middle of each slice to obtain the cytosolic and particulate protein sample. Individual gel slices were incubated in 150 μl of equilibration buffer at 37℃ for 1 h with mixing every 10 min. At the end of this incubation period, an aliquot of the supernatant from each gel piece was assayed for the presence of ral-GAP activity using the GTPase assay described above.

2.10. Protein assay

Samples containing proteins were precipitated by the addition of trichloroacetic acid to a final concentration of 10% (w/v). After solubilizing the pellet, protein was determined by the method of Lowry et al. [30] using a protein standard solution that contained 5% (w/v) human albumin and 3% (w/v) human globulin.
culture. However, the purified ralA fusion protein was cleaved at the C-terminus (~20 amino acids truncated) as it demonstrated faster mobility on SDS-PAGE analysis compared to the protein present in bacteria that were denatured immediately by the addition of Laemmli's sample buffer (Fig. 1A, lanes 2 and 3). Further support for this comes from studies on binding of [α-32P]GTP on nitrocellulose blots containing polypeptides separated by SDS-PAGE. As shown in Fig. 1B, the ralA fusion protein present in bacteria that were denatured immediately by the addition of Laemmli's sample buffer (lane 2) had a mobility on SDS-PAGE slower than that of the purified fusion protein (lane 3). In addition, purified ralA fusion protein was retained when passed for the second time through the pFLAG immunoaffinity column, confirming that the protein was cleaved at the C-terminus (results not shown). Variations in bacterial growth conditions, inclusion of a wide variety of proteolytic inhibitors during lysis of bacteria and purification steps did not protect the fusion protein from partial degradation.

3.2. Detection of GTPase activating protein

The ralA fusion protein purified from E. coli was preloaded with [γ-32P]GTP to establish the presence in human platelets of a protein(s) that could stimulate the intrinsic GTPase activity of ralA. Results presented in Fig. 2 demonstrate that addition of platelet cytosolic or detergent solubilized particulate proteins to the assay stimulated the GTPase activity of ralA resulting in at least a six-fold increase in the rate of GTP hydrolysis. The cytosolic fraction contained higher ral-GAP activity (Fig. 2). When cytosolic or particulate proteins were first denatured by boiling and the supernatant added to the assay, no increase in the GTPase activity of ralA was observed (Fig. 3) suggesting that a protein(s) was responsible for GAP activity. When ralA preloaded with [α-32P]GTP was used to measure GTPase activity, addition of cytosolic or particulate proteins to the reaction mixture did not cause a decrease in the amount of radioactivity associated with ralA at the conclusion of the assay (results not shown). These results confirm that the addition of platelet proteins to the GTPase assay did not promote an exchange or removal of GTP bound to ralA.

To obtain additional support for the observation that protein(s) present in the cytosolic and particulate fraction increased the intrinsic GTPase activity of ralA, fusion protein was preloaded with [α-32P]GTP and products of the GTPase assay were analyzed by thin layer chromatography.

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1 2 3
GDP →
GTP →
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Fig. 4. Analysis of GTPase assay products by thin layer chromatography. ralA protein was preloaded with [α-32P]GTP and used in the GAP assay as described in Section 2. After filtration, guanine nucleotides trapped on nitrocellulose filters were eluted using 0.1 N HCl. The pH of the eluant was adjusted to 7.2 by the addition of 0.5 M Tris and after the addition of 1 mM GTP and 1 mM GDP, an aliquot was spotted on poly(ethylene)imine cellulose sheets and chromatographed using 1 M KH₂PO₄. The GTP and GDP spots were visualized using autoradiography. The figure above represents, ralA alone (lane 1), ralA plus 50 µg of cytosolic (lane 2) and ralA plus 50 µg of detergent solubilized particulate (lane 3) proteins.

Fig. 2. Detection of ral-GAP activity in human platelets. Cytosolic and detergent solubilized particulate protein fractions were prepared as described in Section 2. To measure GAP activity, 50 µg of cytosolic or detergent solubilized particulate proteins were added to mixture containing 15 ng ralA loaded with [γ-32P]GTP. After 15 min incubation, the mixture was passed through a nitrocellulose disc and radioactivity associated with the filter was quantitated as described in Section 2. Data represent the average ± S.D. of at least three independent experiments.

Fig. 3. Effect of protein denaturation on ral-GAP activity. Human platelet cytosolic and particulate proteins were heated at 100°C for 5 min. The denatured proteins were pelleted by centrifugation and supernatant added to GTPase assay. The GAP activity was measured as described in Section 2.
3.4. Characterization of platelet cytosolic and particulate GAP(s)

Initial studies to determine the size of ral-GAP by molecular exclusion chromatography (AcA34) suggested that the GAP activity was associated with a protein of molecular mass in excess of 400,000 Da. This is similar to the results obtained for ral-GAP present in rat brain and mouse testis cytosolic fraction [18]. Attempts to dissociate the ral-GAP complex using high salt or urea were unsuccessful. Thus, to further characterize the cytosolic and particulate protein(s) responsible for stimulating the GTPase activity of ralA, platelet proteins from these fractions were separated using SDS-PAGE as described in Section 2. The presence of GAP activity in gel slices was measured by addition of proteins eluted from the gel to the GTPase assay. As shown in Fig. 6, ral-GAP activity in the cytosolic and particulate fractions was recovered in a single gel slice of identical apparent molecular weight. By comparing mobility of standard proteins on SDS-PAGE to that of the GTPase activating activity, the molecular mass of ral-GAP was determined to be 34 ± 2 kDa (n = 5). An identical molecular mass for ral-GAP was obtained when platelets were lysed by the addition of Laemmli’s sample buffer, the proteins separated using SDS-PAGE and GAP activity in gel pieces measured. This suggests that ral-GAP present in the cytosolic and particulate fraction was not proteolytically degraded. The specificity of ral-GAP was
confirmed by the fact that this protein did not stimulate the intrinsic GTPase rate of ras p21, G25K/CDC42Hs or the rab3A proteins (results not shown).

4. Discussion

In order to investigate the regulation of GTPase activity of ral by the GTPase activating protein, we have expressed the human ralA protein in E. coli and purified the recombinant protein to homogeneity. Although the purified fusion protein was proteolytically cleaved at the C-terminus, it was able to bind and hydrolyze GTP. The basal GTPase rate for ralA obtained in the current study is lower than that reported for this protein by Frech et al. [31]. The reason for this discrepancy is not clear. It could be due to differences in the assay conditions used in the two studies. However, using the ralA fusion protein produced in the current study we have established the existence of ral specific GAP in human platelets. Even though in platelets the ral-related GTP-binding protein(s) are firmly associated with the particulate fraction [24,25], GAP activity for ral was detected in approximately equal amounts in the cytosolic and particulate fraction. This type of subcellular distribution has been reported for GAP for rap1 [15], rho [16], G25K/CDC42Hs [17] and rab3A [32]. The intrinsic GTPase activity of ralA was stimulated at least six-fold by ral-GAP. This degree of stimulation is of the same magnitude as that reported for GAPS for some of the other ras-related GTP-binding proteins. However, it is much lower than the 10^3-fold stimulation of the basal GTPase activity of ras p21 by ras-GAP [33].

When ral-GAP was analyzed by molecular exclusion chromatography, a molecular mass in excess of 400000 Da was obtained. This is in agreement with the value obtained for ras-GAP from rat brain and mouse testis cytosol [18] suggesting that platelet ral-GAP may be similar to that present in other tissues. However, to date no protein of molecular mass in the range of 400 kDa has been implicated in the regulation of the GTPase activity of ras-related proteins suggesting that ral-GAP may exist in a complex with itself and/or other protein(s) in the platelet. Thus, to further characterize platelet ral-GAP, we determined GAP activity in proteins eluted from gel slices after their separation by SDS-PAGE. The results demonstrated that the cytosolic and particulate ral-GAP activity was recovered in a gel slice of identical apparent molecular weight. The molecular mass of ral-GAP was determined to be 34 ± 2 kDa. This is clearly different from the ras p21 GAP which has a molecular mass of 125 kDa [9]. Furthermore, the ral-GAP detected in proteins eluted from the gel did not stimulate the intrinsic GTPase activity of ras p21. This is despite the fact that of all the newly identified ras p21 related GTP-binding proteins, the ras proteins share the greatest degree (~ 50%) of homology with ras p21 [21].

GAPs have been purified from a variety of tissues and cell sources for some of the low molecular mass GTP-binding proteins. Thus, rap1-GAP is a 85–95 kDa [14] and a 55 kDa [15] protein, G25K/CDC42Hs-GAP is a 25 kDa protein [17], rho-GAP is a 27.5 to 28 kDa [16] protein and rab3A-GAP has molecular mass of 295 kDa [32]. The molecular mass of 34 kDa reported for ral-GAP in the present study is clearly different from the GAPS for rap1 and rab3A proteins. In addition, we have reported in the present study that ral-GAP does not stimulate the intrinsic GTPase activity of the rab3A protein. The molecular mass of the G25K/CDC42Hs-GAP and rho-GAP are in the range of that for ral-GAP. However, ral-GAP did not stimulate the intrinsic GTPase activity of the G25K/CDC42Hs protein. In addition, the gene for CDC42Hs-GAP has been recently cloned and sequenced and shown to code for a protein of 54 kDa and the earlier form of G25K/CDC42Hs-GAP is considered to be a proteolytic fragment [34]. Furthermore, it has been shown that G25K/CDC42Hs-GAP can stimulate the GTPase activity of rho [17]. Thus, it is likely that G25K/CDC42Hs-GAP belongs to the rho subfamily of GTP-binding proteins and it is unlikely that ral-GAP is identical to the G25K/CDC42Hs or rho GTPase activating proteins.

Thus, the present study represents the initial report establishing molecular mass of the ral specific GTPase activating protein. Purification and partial/full sequencing of ral-GAP should help define, its homology to other known GAPS, its structural features and its role in the regulation of the function of ral-related proteins in platelet signalling pathways.

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References


