Identification of multiple *ral* gene products in human platelets that account for some but not all of the platelet G_n-proteins

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Polyclonal antibodies raised against specific recombinant low molecular mass GTP-binding proteins were tested for their ability to recognize partially purified human platelet membrane G_n-proteins (i.e. proteins that bind [α-^{32}P]GTP on nitrocellulose blots of SDS/polyacrylamide gels). An antiserum against simian *ral*A protein recognized a 27 kDa human platelet protein with the same apparent molecular mass as the major platelet G_n-protein (G_n27). In further analysis by two-dimensional polyacrylamide gel electrophoresis, the isoelectric focusing step permitted resolution of 12 major G_n-protein forms, seven of 27 kDa (G_n27a–g), one of 26 kDa (G_n26) and four of 24 kDa (G_n24a–d). The *ral*A antibody reacted strongly with the five most basic G_n27 species (a–e), weakly with G_n26 and not at all with G_n27f, G_n27g or G_n24a–d. We conclude that *ral* gene products account for some but probably not for all of the platelet G_n-proteins.

G_n-protein; GTP-binding protein; Gene product, *ral*; (Platelet)

1. INTRODUCTION

We have identified a family of GTP-binding proteins that can be specifically labelled by [α-^{32}P]GTP on nitrocellulose blots of membrane proteins that have been separated by SDS/polyacrylamide gel electrophoresis [1]. These proteins, which we have termed G_n [1], are most abundant in the platelet and brain. In the platelet, the G_n-proteins appeared to consist of a major 27 kDa form (G_n27) and lesser amounts of lower molecular mass species. In rat brain, two membrane-bound forms (G_n27 and G_n26) and a soluble form (G_n26) were detected. More recently, G_n-proteins have also been shown to be present in membranes from NIH3T3 cells [2], neutrophils [3], Swiss 3T3 cells [4] and in plant hypocotyl [5]. The function of G_n-proteins is not known, but it has been suggested that they could play a role in pertussis toxin-insensitive signal transduction [1,6], in the GTP[γS]-dependent activation of platelet cytosolic phospholipase C [7] and in membrane fusion [8]. Recently, evidence has been presented that G_n-proteins are associated with the fMLP receptor in neutrophils [9].

Many other low molecular mass GTP-binding proteins have also been identified in mammalian cells. Initially, these included the 21 kDa cellular products of the three closely related and highly conserved *ras* genes (c-H-*ras*, c-K-*ras*, and c-N-*ras*) [10], a 25 kDa protein now termed G25K [11] that has been purified from placenta [12], platelets [12] and bovine brain [13] and a 21 kDa protein (ARF) that stimulates cholera toxin-dependent ADP ribosylation [14]. Use of oligonucleotide probes coding for conserved regions of *ras* p21 proteins has now demonstrated the existence of several *ras*-related gene families (e.g. *rho, ral, rab, rap*) that can potentially code for other low molecular mass GTP-binding proteins [15]. For some of these *ras*-related genes, the corresponding protein products have now been purified and identified. Thus, a 20 kDa brain protein is coded for by one of the *rho* genes [16] and a 25 kDa brain protein by the *rab*3 gene [17]. The *rho* proteins are substrates for ADP-ribosylation by botulinum exoenzyme C3 [18,19]. In addition, a 21 kDa protein purified from bovine brain is coded for by the c-K-*ras* gene [20] and a protein (termed smg p21) purified from bovine brain [21] and human platelets [22] is coded for by the *rap*1A gene [23,24]. The protein products of some of the other *ras*-related genes (e.g. *ral*) have not yet been identified in mammalian cells.

Previously, we have shown that platelet G_n-proteins are distinct from *ras* p21 proteins, G25K, the platelet substrates of botulinum ADP-ribosyltransferase C3 and ARF [25]. In the present study we have tested the ability of antibodies raised against several specific recombinant *ras*-related proteins to recognize platelet G_n-proteins and have used two-dimensional polyacrylamide-gel electrophoresis [26] to resolve dif-

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Abbreviations: G_n-proteins, 23–27 kDa proteins detected by binding of [α-^{32}P]GTP to nitrocellulose blots [1]

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ferent G_n-proteins more completely than in earlier studies. The results indicate that the G_n-proteins are heterogeneous and that some but probably not all forms of G_n27 are *ral* gene products.

2. EXPERIMENTAL

2.1. *Materials*

[α-^{32}P]GTP (~3700 Ci/mmol) was from ICN Radiochemicals (Irvine, CA, USA). Bovine hemoglobin, protein A and low M_r protein standards for use as molecular mass standards on SDS/polyacrylamide gel electrophoresis were from Sigma Chemical Co. (St. Louis, MO, USA). Nitrocellulose membrane (0.20 μm pore), ampholytes for isoelectric focusing (Bio-Lytes 3/10) and prestained protein markers for use on SDS/polyacrylamide gels were obtained from Bio-Rad Laboratories (Canada) (Mississauga, Ont., Canada). Na^{125}I was from Chedoke-McMaster Hospitals (Hamilton, Ont., Canada) and ^{125}I-labelled protein A was prepared according to [27]. Rabbit antisera to recombinant *rab* proteins and a rabbit polyclonal antibody to a *rap*1A peptide were generously provided by N. Touchot and A. Zahraoui and by J. de Gunzburg, respectively (INSERM U-248, Faculté de Médicine Lariboisière-Saint-Louis, Paris).

2.2. *Expression of ralA protein in E. coli and antibody production*

Simian *ral*A cDNA was expressed in *E. coli* and the *ral*A protein purified as described elsewhere [28]. Rabbits were immunized with 100 μg of recombinant *ral*A protein, the injection was repeated twice more at 3 week intervals and serum was collected 4 days after the final injection. *Ral*A protein was coupled to CNBr-activated Sepharose and affinity-purified anti-*ral*A antibody was prepared.

2.3. *Electrophoresis of G_n-proteins*

Analysis of partially purified G_n-proteins by one-dimensional SDS/polyacrylamide gel electrophoresis was carried out as described elsewhere [1]. For analysis of G_n-proteins by two-dimensional polyacrylamide gel electrophoresis, the method of O'Farrell [26] was used as described in [29], but with the following modifications. The isoelectric focusing gels contained 2% (w/v) Bio-Lyte 3/10 and electrophoresis was carried out at room temperature for 16 h at 400 V and 2 h at 800 V, using 0.01 M H_3PO_4 in the lower reservoir (anode) and 0.1 M NaOH in the upper reservoir (cathode). After isoelectric focusing was complete, gels were extruded from the tubes and subjected to SDS/polyacrylamide gel electrophoresis in the second dimension [29]. When necessary, gels were silver-stained by the method of Morrissey [30].

2.4. *Nitrocellulose blotting and detection of proteins binding [α-^{32}P]GTP or antibody*

For transfer of protein on to nitrocellulose after SDS/polyacrylamide gel electrophoresis, gels were incubated for 30 min in a transfer buffer containing 0.05% (w/v) SDS before electroblotting [1]. The detection of G_n-proteins on nitrocellulose blots using [α-^{32}P]GTP has been fully described elsewhere [1]. To probe blots with antibody, the nitrocellulose sheets were shaken for 3 h at room temperature in a solution containing 10 mM Tris-HCl, pH 7.5, and 0.154 M NaCl (buffer A) to which 1% (w/v) bovine hemoglobin had been added. The nitrocellulose blots were then incubated for 3 h in buffer A containing 1% bovine hemoglobin and antibody (at a 1:250 dilution). After washing three times (10 min each) with buffer A, the blots were incubated for a further 1 h in buffer A containing 1% bovine hemoglobin and ^{125}I-labelled protein A (0.2 μCi/ml). After further washing of the blots in buffer A (five times for 5 min each), bound ^{125}I-labelled protein A was detected by autoradiography for 12–18 h at −50°C, using a Cronex intensifying screen (Picker International, Brampton, Ont., Canada).

2.5. *Protein assays*

Protein was determined by the method of Bradford [31], using a standard solution that contained 5% (w/v) human albumin and 3% (w/v) human globulin.

3. RESULTS

Partially purified platelet G_n-proteins were used in the studies described in this paper (fig.1). In brief, membranes prepared from outdated human platelets were extracted with a buffer containing 20 mM Tris-HCl, pH 8.0, 1 mM DTT, 1 mM EDTA, 0.1 mM PMSF and 1% (w/v) sodium deoxycholate (buffer A). The solubilized proteins were collected by centrifugation and applied to a DEAE-Sephacel column. After washing, the bound proteins were eluted from the anion exchange column using a linear gradient of 0–250 mM NaCl in buffer A. Fractions containing G_n-proteins were pooled, concentrated and further fractionated by molecular exclusion column chromatography (AcA44) using buffer A that now also contained 100 mM NaCl.

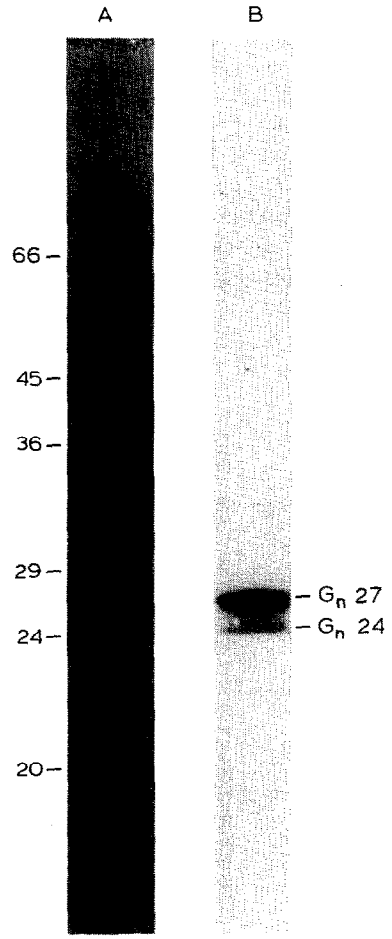

Fig.1. Partially purified G_n-proteins from human platelet membranes. Protein (10 μg) pooled from the low molecular mass fractions after AcA44 column chromatography was electrophoresed in duplicate. One part of the gel was silver-stained and the other blotted on to nitrocellulose. (A) silver-stained gel, (B) autoradiograph of the blot after probing with [α-^{32}P]GTP. The molecular masses of protein standards are shown on the left.

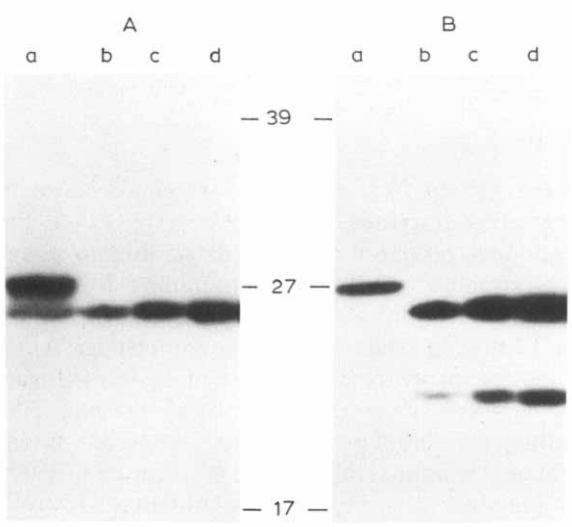

Fig.2. Analysis of G_n-proteins and recombinant *ral*A protein by one-dimensional SDS/polyacrylamide gel electrophoresis. Partially purified G_n-proteins (a, 50 μg) and varying amounts of purified recombinant *ral*A protein (b, 0.1 μg; c, 0.2 μg; d, 0.3 μg) were electrophoresed and blotted on to nitrocellulose. The autoradiographs above show: A, [α-^{32}P]GTP-binding proteins and B, proteins that bound *ral*A antibody, as detected by ^{125}I-labelled protein A. The positions of prestained molecular mass marker proteins used to align the autogradiographs are shown in the centre of the figure.

The G_n-proteins eluting in the low molecular mass fractions were pooled. Full details of the purification of G_n-proteins will be given elsewhere (Bhullar and Haslam, in preparation).

Samples of partially purified G_n-proteins and of recombinant *ral*A protein were electrophoresed in parallel on one-dimensional SDS/polyacrylamide gels and then blotted on to nitrocellulose sheets, which were probed with [α-^{32}P]GTP (fig.2A) or a rabbit polyclonal antibody raised against recombinant simian *ral*A protein (fig.2B). On incubation of the nitrocellulose blot with [α-^{32}P]GTP, G_n-proteins including G_n27 and G_n24 were detected (fig.2A, lane a), as previously described in experiments with platelet membranes [1]. The recombinant *ral*A protein also bound [α-^{32}P]GTP, but the GTP-binding component migrated with an apparent molecular mass of 24 kDa (fig.2A, lanes b–d). When an identical blot was incubated with the antibody against the *ral*A protein (fig.2B), only one immunoreactive band was observed in the lane containing partially purified G_n-proteins (lane a). This band had an electrophoretic mobility identical to that of the G_n27 protein (fig.2A, lane a). The finding that G_n24 did not bind antibody supports our previous evidence [1] that this protein is not a proteolytic fragment of G_n27. The antibody also detected two immunoreactive species with molecular masses of 24 kDa and 21 kDa in the samples containing recombinant *ral*A protein (fig.2B, lanes b–d). However, the 21 kDa species did not bind [α-^{32}P]GTP on nitrocellulose blots (fig.2A, lanes b–d) and is likely to be a proteolytic fragment of the *ral*A protein. The antibody used in this study did not inhibit GTP-binding by G_n27 on nitrocellulose blots (not shown). When antisera raised against certain other low molecular mass GTP-binding proteins (*rab*1, *rab*2,

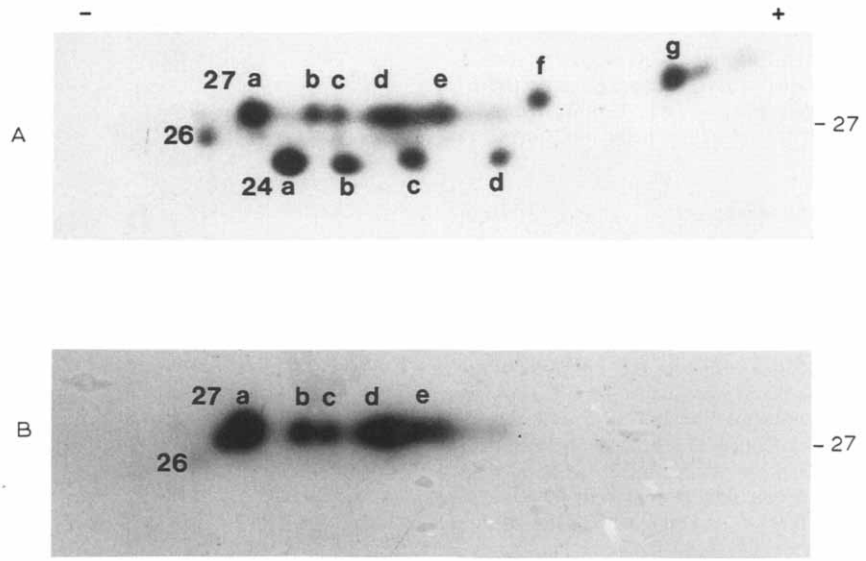

Fig.3. Analysis of G_n-proteins by two-dimensional polyacrylamide gel electrophoresis. Partially purified G_n-proteins (50 μg) were subjected to isoelectric focusing with the protein applied to the high pH (cathode) end of the gel. SDS/polyacrylamide gel electrophoresis in the second dimension was carried out using 13% (w/v) acrylamide. Two identical gels were then blotted on to nitrocellulose. To detect G_n-proteins and proteins recognized by the *ral*A antibody, one blot (A) was treated with [α-^{32}P]GTP and another (B) with *ral*A antibody followed by ^{125}I-labelled protein A. Autoradiographs are shown. The major proteins detected with [α-^{32}P]GTP are labelled G_n27a–g, G_n26 and G_n24a–d. The orientation of the cathode (-) and anode (+) during isoelectric focussing is indicated, along with the mobility of a prestained 27 kDa protein during electrophoresis in the second dimension.

*rab*4, *rab*5, *rab*6 and *rap*1A) were used, only the *rap*1A antibody recognized a protein present in the preparation of partially purified platelet G_n-proteins (results not shown). However, this protein had an apparent molecular mass of 21 kDa and did not bind [α-^{32}P]GTP on nitrocellulose blots under the conditions used [1]. The presence of the *rap*1A gene product in platelets has been documented by others [22,24].

The above results suggested that G_n27 might be a *ral* gene product. However, after one-dimensional SDS/polyacrylamide gel electrophoresis, the ratio of [α-^{32}P]GTP binding to antibody labelling was much higher in G_n27 than in the pure 24 kDa *ral*A protein (fig.2). This raised the possibility that G_n27 contained components that did not react with the *ral*A antibody. To check this, the G_n-proteins were analysed by two-dimensional polyacrylamide gel electrophoresis. When a blot containing G_n-proteins separated by this method was incubated with [α-^{32}P]GTP, a total of twelve distinct major GTP-binding species and other minor GTP-binding components were detected (fig.3A). The former comprised seven species with molecular masses of 27 kDa (G_n27a-g), one with a molecular mass of 26 kDa (G_n26) and four with molecular masses of 24 kDa (G_n24a-d) (fig.3A). However, when an identical blot was probed with the polyclonal antibody to *ral*A protein, only five highly immunoreactive G_n species (G_n27a-e) were detected (fig.3B), though G_n26 bound antibody weakly. This antibody did not recognize G_n27f, G_n27g or any of the G_n24 proteins (fig.3B).

4. DISCUSSION

G_n27 is the major platelet G_n-protein that can be specifically labelled by [α-^{32}P]GTP on nitrocellulose blots of membrane proteins that have been separated by SDS/polyacrylamide gel electrophoresis [1]. Although many new *ras*-related genes have been identified recently [15], the gene coding for the G_n27 protein had not yet been identified. Thus, we sought to determine whether or not any of the newly identified *ras*-related genes code for G_n-proteins by using antibodies raised against specific recombinant GTP-binding proteins. Although the initial experiments suggested that G_n27 might consist solely of *ral* gene products, the results obtained by two-dimensional polyacrylamide-gel electrophoresis show that this may not be the case. Thus, G_n27 proved to be highly heterogeneous, consisting of at least seven polypeptides with widely different charges of which the anti-*ral*A antibody recognized only five. This suggests that only the latter G_n27 species are coded for by genes closely related to *ral*A, which was originally identified in a simian B-cell cDNA library [32]. Recently, cDNAs corresponding to two related *ral* genes, *ral*A and *ral*B, have been cloned from a human pheochromocytoma library [33]. Simian and human *ral*A proteins appear to differ in only one amino acid residue. Moreover, since the predicted amino acid sequences of *ral*A and *ral*B proteins are about 85% identical and differ mainly in their C-terminal halves [33], we would expect that the polyclonal antibody raised against the simian *ral*A protein would be likely to recognize both human *ral* proteins. Thus, the 27 kDa and 24 kDa G_n-proteins that are not recognized by *ral*A antibody may be the products of genes other than *ral*A or *ral*B, and perhaps of genes that have not yet been identified.

The reason for the discrepancy between the calculated molecular mass of the *ral*A protein (23.5 kDa) [32] and that determined for the G_n27 proteins (27 kDa) [1] is not known, but could be accounted for by post-translational modifications [34]. Although recombinant *ral*A migrated on SDS/polyacrylamide gels with an apparent molecular mass of 24 kDa, there is evidence for proteolytic loss of some amino acid residues from this protein (Chardin, unpublished results). Post-translational modifications, including phosphorylation, could also account for some of the G_n-protein heterogeneity detected by isoelectric focusing, including the faint satellite spots (such as those observed close to G_n27g in fig.3A). Most of the heterogeneity observed was, however, highly reproducible and is more likely to represent the presence of G_n species generated by differential splicing from the transcripts of a small number of distinct genes, including *ral*A and *ral*B.

After the present work was completed, Polakis et al. [35] reported peptide sequences identical to those found in human *ral*A in a 28 kDa human platelet GTP-binding protein isolated from preparative SDS/polyacrylamide gels. This finding is in accord with our immunological evidence that platelets contain *ral* proteins, but it is also clear from our results that multiple GTP-binding polypeptides, including some that may not be closely related to *ral* gene products, are present in the 27-28 kDa range after one-dimensional SDS/polyacrylamide gel electrophoresis of partially purified platelet G_n-proteins. Our results also provide further evidence that the G_n-proteins, as defined by the methodology we have used [1], represent a distinct group of high-affinity GTP-binding proteins. Thus, *rap*1A protein, though present at high concentrations in platelets [22,24], was not readily detected on nitrocellulose blots. Similarly, platelet *ras* proteins [25] and G25K [35] were not easily detected by this technique. It remains to be determined whether the remarkable ability of the different G_n-proteins to bind [^{32}P]GTP on nitrocellulose blots, which presumably has a common structural basis, implies similarities in the functions or regulation of these proteins.

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