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Association of a 24-kDa GTP-binding protein, G_n24, with human platelet α-granule membranes

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Human platelets were disrupted using nitrogen cavitation and fractionated on sucrose density gradients to permit isolation of α-granules, the major secretory granule of platelets. Membrane proteins prepared from intact α-granules by alkali extraction were seperated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose and the blot probed for the presence of GTP-binding proteins using [α-^{32}P]GTP. Two low molecular mass GTP-binding proteins with molecular mass of 27 and 24 kDa, respectively, were identified on the α-granule membrane. In contrast to the 27-kDa protein which was present in significant amounts in the plasma membrane-enriched fraction, the 24-kDa protein showed a preferential association with the α-granule membrane. On immunoblotting with specific antiserum, the 24-kDa GTP-binding protein was found to be distinct from *rab*3A. To the best of our knowledge, the present report represents the first identification of low molecular mass GTP-binding proteins associated with a platelet secretory granule.

G_n-protein; GTP-binding protein; Platelet; α-Granule

1. INTRODUCTION

More than thirty mammalian GTP-binding proteins with molecular mass between 17–27 kDa have been identified in the past 5 years [2–4]. A specific function for any of the low molecular mass GTP-binding proteins remains to be elucidated. However, based on functional studies in temperature-sensitive yeast mutants [5–7] as well as subcellular localization studies in mammalian systems [8–12], there is reason to anticipate a role for some of these low molecular mass GTP-binding proteins in mediating the known GTP dependency of several intracellular membrane trafficking processes [13–16], including secretion by exocytosis [6,12,17]. The *rab* subfamily of *ras*-like GTP-binding proteins has received specific attention in this regard [18].

The subject of low molecular mass GTP-binding proteins in platelets has been recently reviewed [19]. While at least 15 such proteins have been identified in the platelet to date, little is known about their subcellular localization and even less about their possible function.

Platelets contain three recognized populations of secretory granules, namely, α-granules, dense granules and lysosomes, with α-granules being the most abundant [20]. The aim of the present work was to determine the presence of low molecular mass GTP-binding proteins in association with the platelet α-granule. We report the detection of a 24-kDa GTP-binding protein, termed G_n24, that demonstrates a preferential association with the α-granule membrane but is not recognized by antiserum raised against the *rab*3A protein. Possible identity with a previously reported platelet 24-kDa GTP-binding protein, capable of interaction with the GDP dissociation inhibitor protein for brain *smq* p25A [21], is discussed.

Abbreviations: GTPγS, guanosine 5'-[γ-thio]triphosphate; G_n-proteins, 23–27-kDa proteins detected by binding of [α-^{32}P]GTP to nitrocellulose blots [1].

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2. EXPERIMENTAL

2.1. *Materials*

[α-^{32}P]GTP was from ICN Radiochemicals (Irvine, CA, USA). Tris, ATP (disodium salt) and chymotrypsin were obtained from Boehringer Mannheim Canada Ltd. (Dorval, Que., Canada). Bovine serum albumin, 2-deoxy-D-glucose, EDTA (sodium salt), Triton X-100, cytochrome *c* type III, protein standard solution, *p*-nitrophenyl-2-acetamido-2-deoxy-β-D-glucopyranoside and low M_r protein standards for SDS-polyacrylamide gels were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Rotenone and phenylmethylsulfonyl fluoride (PMSF) were from Aldrich (Milwaukee, WI, USA). Nitrocellulose membrane (0.2 μm pore) and prestained protein markers for use on SDS-polyacrylamide gels were obtained from Bio-Rad Laboratories (Mississauga, Ont., Canada). Radioimmunoassay kit for β-thromboglobulin was from Amersham (Oakville, Ont., Canada). ^{125}I-labelled Concanavalin A was purchased from New England Nuclear (Montreal, Que., Canada). All other reagents used were of analytical grade.

2.2. *Platelet isolation*

Two hundred ml of blood was collected from healthy volunteer donors into 0.15 volumes of ACD anticoagulant and centrifuged at 280×*g* for 15 min at room temperature to produce platelet-rich plasma (PRP). The PRP was adjusted to 5 mM EDTA and centrifuged at 2500×*g* for 15 min at 4°C to yield a platelet pellet. The platelets were

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washed by resuspension and centrifugation at 1700×g for 10 min at 4°C in Ca^{2+}-free Tyrode's solution (137 mM NaCl, 2.7 mM KCl, 12 mM $NaHCO_3$, 0.42 mM NaH_2PO_4, 5.5 mM glucose, 2 mM $MgCl_2$) that also contained 10 mM PIPES (pH 6.5), 1 mM EDTA and BSA (3.5 mg/ml). The final platelet pellet was resuspended in 15 ml of this same buffer.

2.3. *Platelet sub-fractionation and isolation of α-granules*

Human platelet sub-fractionation was carried out using minor modifications to a protocol previously used for pig platelet [22]. The platelet suspension was adjusted to 5 mM in 2-deoxy-D-glucose and 20 μM rotenone (from 10 mM stock in DMSO) and incubated at 37°C for 40 min. Chymotrypsin (0.1 mg/ml) and ATP (final concn. 5 mM) were added and the incubation continued for a further 5 min at room temperature. Chymotrypsin was neutralized by the addition of a large molar excess of PMSF (1 mM) and platelets collected by centrifugation at 1700×g for 10 min at 4°C. The platelets were resuspended in a buffer (TRB) containing 10 mM Tris-HCl (pH 7.4), 250 mM sucrose, 1 mM EDTA and 0.15 mM PMSF, added to a pre-cooled nitrogen bomb (Parr Instruments Co., Moline, IL) and disrupted by three 10 min cycles of nitrogen cavitation at 1200 psi. The resulting homogenate was centrifuged at 1000×g for 20 min at 4°C to remove intact or incompletely lysed platelets and then re-centrifuged at 32 000×g for 30 min. The pellet obtained was resuspended in 3 ml of ice-cold TRB and layered onto a 30 ml discontinuous sucrose gradient (1.2–2.0 M sucrose in TRB, 0.2 M steps) and centrifuged at 135 900×g for 3 h at 4°C. Fractions were harvested from the sucrose gradient in 1-ml aliquots, pooled according to visible bands (see Fig. 1), slowly diluted into TRB to minimize granule lysis, and centrifuged at 32 000×g for 30 min. Pelleted fractions were resuspended in TRB for further analysis.

2.4. *Preparation of α-granule membranes*

The pellet corresponding to the α-granule-rich fraction (i.e. fraction III = B4 + B5, Fig. 1) was resuspended in 1 ml of 0.1 M Na_2CO_3 solution, incubated for 15 min at 4°C and centrifuged at 100 000×g for 30 min. The resulting pellet, designated 'α-granule membranes', was resuspended in TRB for further analysis.

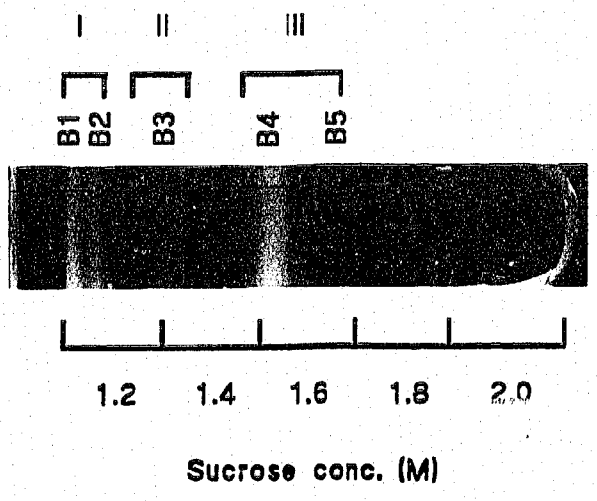


Fig. 1. Photograph of a typical sucrose density gradient platelet sub-fractionation. Platelets were isolated, subjected to ATP-depletion with 2-D-deoxyglucose and rotenone followed by limited proteolysis with chymotrypsin in the presence of ATP, as described in section 2. The platelets were disrupted by nitrogen cavitation and separated on a 30 ml discontinous sucrose gradient (1.2–2.0 M sucrose, in 0.2 M steps). The gradient was collected in 1 ml fractions and the bands pooled into three larger fractions as indicated (I–III).

2.5. *Marker assays*

Radiolabelled Concanavalin A was used to identify plasma membrane during the platelet sub-fractionation [23]. In brief, prior to the nitrogen cavitation step, intact platelets (4×10^9/ml) were incubated for 5 min at 37°C in TRB containing 210 nCi/ml of ^{125}I-labelled Concanavalin A. At the end of this incubation, the platelets were washed twice in ice-cold TRB and collected by centrifugation at 1700×g for 10 min at 4°C. The platelets were then resuspended in TRB for nitrogen cavitation and sub-fractionated as described above. ^{125}I associated with the various fractions was determined using an LKB Wallac universal gamma counter.

Cytochrome *c* oxidase was used as a marker for mitochondria [24] and *N*-acetyl glucosaminidase as a marker for lysosome [25]. β-thromboglobulin, an α-granule specific polypeptide [26], was measured using a commercially available radioimmunoassay kit. Briefly, for β-thromboglobulin measurement, the subcellular fractions were solubilized in 1% Triton X-100 (15 min at room temperature), diluted to 0.1% Triton X-100 with TRB, and further diluted as required with TRB containing 0.1% Triton X-100 for use in the radioimmunoassay. Human β-thromboglobulin standards were similarily prepared in TRB containing 0.1% Triton X-100. The RIA procedure described by the manifacturer was modified to assay twice the usual number of samples by decreasing the volumes two-fold. An additional $(NH_4)_2SO_4$ wash was also included [27].

2.6. *SDS-polyacrylamide gel electrophoresis and blotting of proteins*

For SDS-polyacrylamide gel electrophoresis, one volume of 5× concentrated Laemmli's sample buffer [28] was added to four volumes of solution containing proteins. The samples were heated at 100°C for 3 min and analyzed by SDS-polyacrylamide gel electrophoresis using 13% (w/v) acrylamide in the separating gel [28]. The resolved polypeptides were then transferred onto nitrocellulose using the procedure described in [29] except that the concentration of SDS used in the transfer buffer was 0.05%.

2.7. *Detection of G_n-proteins on nitrocellulose blots*

The procedure for the detection of G_n-proteins upon incubation of nitrocellulose blots with [α-^{32}P]GTP has been described in detail elsewhere [1]. Bound ^{32}P was detected by autoradiography (12–72 h at −50°C using a Cronex intensifying screen) and quantitated by counting Cerenkov radiation.

2.9. *Immunoblotting*

The detection of *rab*3A in the various platelet subcellular fractions and bovine brain membrane was carried out using antiserum raised against the recombinant *rab*3A protein. The method used for immunodetection has been described previously [30].

2.10. *Miscellaneous*

Protein quantitation was according to the method of Lowry et al. [31] using a protein standard solution that contained 5% (w/v) human albumin and 3% (w/v) human globulin.

3. RESULTS

To study α-granules for the presence of low molecular mass GTP-binding proteins we sub-fractionated human platelets by applying, with minor modifications, a method we had previously successfully applied to pig platelets [22]. To improve α-granule yield, ATP-depletion (using 2-deoxy-2-D-glucose and rotenone), and limited proteolysis of the intact platelet (using chymotrypsin) were included in the procedure as these two steps have been shown to contribute towards greater recovery of secretory granules during platelet sub-fractionation [32].

Platelets, prepared and disrupted as described in section 2, separated on discontinuous sucrose gradients in a reproducible pattern comprising 5 distinct bands (Fig. 1). The top two bands (i.e. B1, B2), occurring as a closely spaced doublet within the 1.2 M sucrose phase, were pooled (see below) and designated as fraction I. The third band (i.e. B3), designated as fraction II, formed in the region of the 1.2–1.4 M sucrose interphase. B4 and B5 formed at the 1.4–1.6 M and 1.6–1.8 M interphases respectively. B5 appeared as a fine aggregate and could be readily dispersed. B4 and B5 were pooled (see below) and designated as fraction III. A pellet, presumed to be enriched in dense granules [22,32], was observed in most preparations but was not recovered due to the small amount of material present.

A variety of markers were used to characterize the platelet subfractions. The tight binding of Concanavalin A to the outer aspect of the intact cell was used to follow plasma membrane during the sub-fractionation procedure [23]. In our preparation, maximal ^{125}I-labelled Concanavalin A binding (cpm/mg protein) was found in fraction I (comprising bands 1 and 2) (Fig. 2), consistent with previous observations that this low density fraction is enriched in plasma membrane [22,32]. Relative enrichment over lysate was 2.4-fold in a single experiment in which pelleted fractions I–III were simultaneously prepared for other marker studies. Identical results in terms of plasma membrane localization were

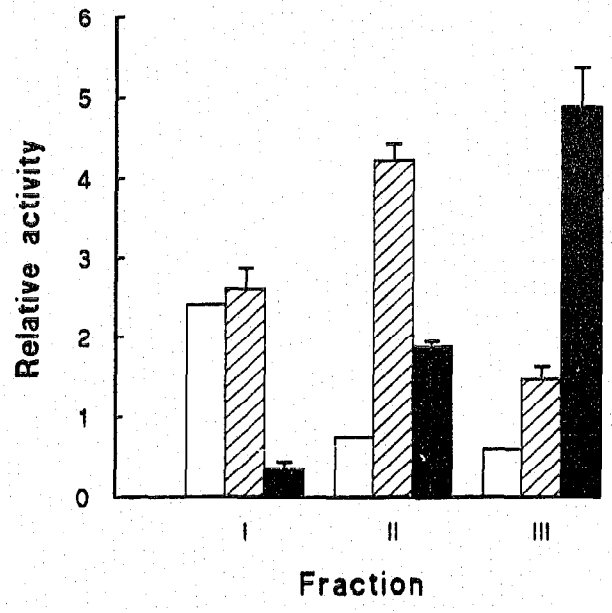
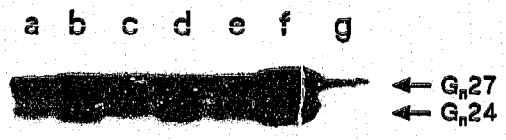


Fig. 2. Distribution of subcellular markers in platelet fractions. Plasma membrane was localized by following [^{125}I]Concanavalin A after incubation of intact platelets with the radiolabelled lectin (□). *N*-acetyl glucosaminidase (▨) and β-thromboglobulin (■) were used as markers for lysosomes and α-granules respectively. Specific activities (units per mg protein) were calculated for each fraction and are expressed relative to the specific activity measured in the whole platelet lysate. Results are from 3 independent experiments (mean ±SEM) except for the [^{125}I]Concanavalin A profile which is from a single experiment in which fractions were processed in parallel for the other markers.



Enrichment	1.7	1.4	1.6	1.8	4.8	0.3
Ratio: G_n24	(0.1)	(0.1)	(0.1)	(0.1)	(0.3)	(0.1)

Fig. 3. Detection of G_n-proteins in platelet subcellular fractions. Proteins (25 μg) in the various subcellular fractions (lane a, lysate; lane b, solution applied to gradient; lane c, fraction I; lane d, fraction II; lane e, fraction III; lane f, α-granule membrane; lane g, α-granule supernatant) were electrophoresed and blotted onto nitrocellulose. To detect G_n-proteins the blot was incubated with [α-^{32}P]GTP and an autoradiogram developed. The enrichment ratio for G_n24 was calculated by counting the ^{32}P associated with this band in various fractions. The enrichment value for the lysate was taken as 1. Values indicated represent the mean enrichment ± SEM (given in parentheses) for 4 independent experiments.

obtained when 0.6 ml fractions collected from the 30 ml density gradient were individually analyzed for [^{125}I]Concanavalin A and protein content. Using this approach the average enrichment calculated from the individual gradient fractions comprising fraction I was somewhat greater (3.7-fold), presumably reflecting the absence of the wash step which is included when pelleted fractions are prepared from the pooled sucrose gradient fractions. *N*-acetyl glucosaminidase, a marker for lysosomes [25], was found in all three fractions but was maximally enriched in fraction II (4.22 ± 0.2-fold, mean ±SEM, n=3) (Fig. 2). β-thromboglobulin, a secretory protein marker localized to the α-granules [26], was detectable in all three fractions but was maximally enriched in fraction III (4.9 ± 0.48-fold, mean ± SEM, n=3) (Fig. 2). B4 and B5 showed comparable enrichment in this marker (4.4- and 4.2-fold, respectively in a separate single experiment) and hence were routinely pooled as fraction III. The activity of the mitochondrial marker enzyme, cytochrome *c* oxidase, was detectable in the material applied to the gradient (10.1 ± 1.9), fraction I (20.48 ± 6.6) and fraction II (41.25. ± 10.7) (μmol product/mg/min, mean ±SEM, n=3–4) but not in fraction III or in whole platelet lysate (using 50–75 μg total protein per assay). This is consistent with the expected localization on sucrose density gradients of mitochondria from both pig [22] and human [32,33] platelets.

Electron microscopy was used to further characterize fraction III. This fraction consisted of morphologically pure α-granules, recognizable by their characteristic shapes and uniformly dense core, with very little evidence of contamination by other organelles. Treatment of intact granules with 0.1 M Na_2CO_3 produced membrane vesicles largely devoid of their electron dense contents (data not shown).

The presence of GTP-binding proteins in the various

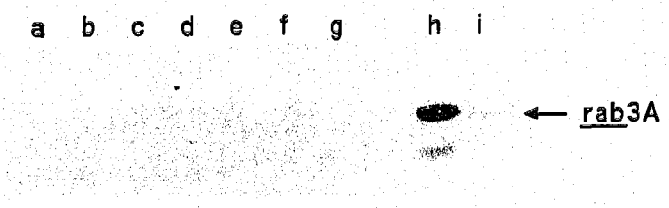


Fig. 4. Immunodetection of *rab*3A in platelet subcellular fractions and bovine brain membrane. Proteins (50 μg) in the various platelet subcellular fractions (lane a, lysate; lane b, solution applied to gradient; lane c, fraction I; lane d, fraction II; lane e, fraction III; lane f, α-granule membrane; lane g, α-granule supernatant), recombinant *rab*3A (lane h, 0.5 μg) and whole bovine brain membrane (lane i, 50 μg) were electrophoresed and blotted onto nitrocellulose. The blot was then incubated with antiserum against *rab*3A (1:250 dilution) and developed using [^{125}I]protein A as described in section 2.

platelet subcellular fractions was assessed using [α-^{32}P]GTP binding on nitrocellulose blots containing polypeptides separated by SDS-polyacrylamide gel electrophoresis. This procedure has been used previously for the detection of GTP-binding proteins (G_n-proteins) in platelets and other cells [1]. Two major GTP-binding proteins, G_n27 and G_n24, were detected in the various platelet subcellular fractions (Fig. 3). The binding of [α-^{32}P]GTP to G_n-proteins was not affected by the addition to the incubation medium of a 1000-fold excess of ATP or GMP but was completely abolished by the addition of GTP or GTPγS (results not shown). G_n27 was found to be present in all the fractions tested, with the plasma membrane-rich fraction I and the α-granule membrane fraction showing the greatest degree of enrichment (2.4- to 2.8-fold over lysate). However, the G_n24 protein was found to be maximally enriched (4.8-fold over lysate) in the α-granule membrane fraction and only minimally enriched (1.4- to 1.8-fold over lysate) in the other subcellular fractions (Fig. 3). The platelet cytosol has been previously shown to contain only trace amounts of this protein [1]. Furthermore, the G_n24 protein could not be recognized in the α-granule membranes by Western blotting using an antiserum prepared against the recombinant *rab*3A protein under conditions where the *rab*3A protein in whole bovine brain membrane and the recombinant *rab*3A protein were recognized by this antiserum (Fig. 4).

4. DISCUSSION

To the best of our knowledge, this represents the first report describing the presence of low molecular mass GTP-binding proteins in association with a platelet secretory granule. Furthermore, we have shown that, under our isolation conditions, one of the two GTP-binding proteins detected is highly enriched in the α-granule membrane fraction (Fig. 3). This GTP-binding protein, which we have designated as G_n24 on the basis of its apparent molecular weight on SDS-polyacrylamide gel electrophoresis, was capable of binding [α-^{32}P]GTP on incubation of nitrocellulose blots containing proteins separated by SDS-polyacrylamide gel electrophoresis. Lesser amounts of this protein could be detected in fractions I and II. The relative quantities of G_n24 detected in fractions I and II (Fig. 3) cannot be accounted for solely by contamination of these fractions with intact α-granules (Fig. 2) but could, nonetheless, be due to the presence of membranes derived from α-granules disrupted during the sub-fractionation procedure. Alternatively, it is possible that while G_n24 is clearly preferentially associated with the α-granule under our conditions, its distribution is not entirely restricted to this organelle.

*rab*3A, a GTP-binding protein which has an apparent molecular mass of 25 kDa, is associated with synaptic vesicles [9] and chromaffin cell secretory granules [10]. It has been speculated that the *rab*3A protein may be associated with secretory granules from other tissues [10]. The present results demonstrate that the platelet α-granule membrane contains a 24 kDa GTP-binding protein (G_n24) which is not recognized by a polyclonal antiserum to *rab*3A (Fig. 4). Further dissimilarity is underscored by the fact that, whereas *rab*3A is present in substantial amounts in cytoplasm [9,10], G_n24 is present only in trace amounts in platelet cytosol [1].

It cannot be concluded, however, that G_n24 is unrelated to *rab*3A. Fujioka et al. have previously isolated a 24-kDa GTP-binding protein (designated 24KG) from whole platelet membranes [21]. The 24KG protein is of special interest in the present context because: (i) it binds [α-^{32}P]GTP after transfer to nitrocellulose, (ii) it is capable of interacting with the GDP dissociation inhibitor (GDI) for brain *smq* p25A (= *rab*3A) in a manner which mimics *smq* p25A, (iii) platelet cytosol contains a GDI, immunologically indistinguishable from the bovine brain *smq* p25A GDI, which demonstrates activity towards both the platelet 24KG protein and *smq* p25A, and (iv) in spite of these similarities to *smq* p25A, 24KG is not recognized by antiserum against bovine brain *smq* p25A on Western blotting. These authors have speculated that 24KG and its associated GDI may be analogous to the *smq* p25A–*smq* p25A GDI system implicated in secretion by exocytosis in neurosecretory tissues. Our localization of a 24 kDa GTP-binding protein to the membrane of a platelet secretory granule is therefore significant, raising the possibility that G_n24 and 24KG may be identical, and complementing the data of Fujioka et al. which suggest that a 24-kDa GTP-binding protein may play a role in platelet secretion. Furthermore, the present data increase the probability that, in spite of not being recognized by specific antisera against *rab*3A, these 24-kDa proteins are related to the *rab* subfamily of GTP-binding proteins.

The significance of the association of low molecular mass GTP-binding protein(s) with exocytotic secretory granules is not well understood. However, it has been proposed that these proteins may relate to the function-

ally identified GTP-binding protein G_e, originally described in the context of Ca^{2+}-independent exocytosis from permeabilized cell systems [17,34]. While non-hydrolyzeable analogues of GTP (e.g. GTPγS) are generally inhibitory with respect to membrane fusion processes studied in cell-free systems [14,15], under certain conditions, GTPγS has been shown to stimulate membrane fusion [35]. The effector for the GTP-bound form of these low molecular mass GTP-binding protein(s) on the secretory granule is unknown at the present time. However, it may be of significance that phospholipase D, which has been indirectly linked with the exocytotic secretory process [36–39], has also been recently reported by us to be under regulation by a GTP-binding protein of inherently low GTPase activity [40]. This is a well-known property of GTP-binding proteins belonging to the *ras* superfamily [2]. The possibility that phospholipase D may be a target effector for a secretory granule GTP-binding protein would appear, therefore, worthy of further investigation.

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