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Association of *Ral* GTP-Binding Protein with Human Platelet Dense Granules

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GTP-binding proteins of molecular mass of 24–27 kDa were detected in the dense granule fraction of human platelets when nitrocellulose blots containing proteins separated by SDS-polyacrylamide gel electrophoresis were incubated with [α-^{32}P]GTP. Further analysis, using isoelectric focusing and two-dimensional polyacrylamide gel electrophoresis, resolved the dense granule 27 kDa and 24 kDa GTP-binding proteins into four distinct forms each. GTP-binding proteins in the total particulate fraction were resolved into seven 27 kDa and four 24 kDa forms. Immunoblotting with antiserum against known platelet low molecular mass GTP-binding proteins demonstrated that *rap*2 and *G25K/CDC*42Hs proteins, although present in platelets, were not detected in the dense granule fraction. However, *ral* was one of the proteins associated with dense granules. Association of specific low molecular mass GTP-binding proteins with dense granules suggests a potential role for these proteins in regulating the release of storage contents from this granule.

The existence in the eucaryotic cell of more than 50 GTP-binding proteins with molecular mass between 17–27 kDa has been established [1]. These proteins share varying degree of identity with the *ras* p21 protein [2,3] and are implicated in mediating the known GTP-dependency of several intracellular membrane trafficking processes [4], including secretion by exocytosis [5]. In particular, proteins coded for by the *rab* genes have been implicated in these pathways and ~30 genes belonging to this subfamily have been identified [6].

In platelets, the existence of ~30 low molecular mass GTP-binding proteins has been established. Included in this list are products of the *G25K/CDC42Hs* [7], *rac*1 and *rac*2 [8], *ral*A and *ral*B [7,9,10], *rho*A [11], *rap*1A and *rap*1B [12], *rap*2B [13], *ram* [14], *rab*1, *rab*3B, *rab*4, *rab*6 and *rab*8 [15] genes. In addition, the existence of other low molecular mass GTP-binding proteins has been established in the platelet but the identity of the gene(s) coding for these proteins is not known [9].

Platelets contain three recognized populations of secretory granules, namely, α granules, dense granules and lysosomes [16]. Knowledge about the association of low molecular mass GTP-binding proteins with various platelet secretory granules is limited. Previously, we have demonstrated the enrichment of a 24 kDa GTP-binding protein in human platelet α-granules [17]. Recently, products of *rab*4, *rab*6 and *rab*8 genes have been shown to be enriched with human platelet α-granules [15]. However, not much is known about the association of low molecular mass GTP-binding proteins in platelet dense granules. The current study was undertaken to establish the presence of low molecular mass GTP-binding proteins in human platelet dense granules. Results obtained have established the association of 24–27 kDa GTP-binding proteins with human platelet dense granules. Further analysis by two-dimensional polyacrylamide gel electrophoresis and GTP-binding on nitrocellulose blots resolved both the 27 kDa

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and 24 kDa proteins into four distinct forms each. Using antibodies raised against recombinant *ral*A protein, the presence of *ral* GTP-binding protein in dense granules was established.

MATERIALS AND METHODS

Materials. Quinacrine, leupeptin, benzamidine, phenylmethylsulfonyl fluoride (PMSF), bovine serum albumin (BSA), metrizamide, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES) and protein standard solution were obtained from Sigma Chemical Company (St. Louis, MO, USA). Prestained protein markers for SDS-PAGE, nitrocellulose membrane (0.20 μm poresize) and ampholines for isoelectric focusing (Bio-Lytes 3/10) were from Bio-Rad Laboratories (Mississauga, ON, Canada). [α-^{32}P]GTP ($\sim$3000 Ci/mmol) was obtained from ICN Radiochemicals (Irvine, CA, USA). Freund's complete and incomplete adjuvant were purchased from GIBCO-BRL (Burlington, ON, Canada). Enhanced Chemiluminescence (ECL) Western blotting developing solutions and horseradish peroxidase conjugated anti-mouse and anti-rabbit secondary antibodies were obtained from Amersham International (Mississauga, ON, Canada). *rap*2 primary antibody was obtained from Transduction Laboratories (Lexington, KY, USA). Antibody (D545) against the 40 kDa dense granule specific protein was kindly provided by Dr. S. J. Israels of the University of Manitoba. All other reagents were of laboratory grade.

Preparation of platelet particulate, cytosolic and dense granule fractions. To obtain particulate and cytosolic fractions, outdated platelets (obtained from the local Red Cross) were washed three times in a buffer containing 13 mM-trisodium citrate, pH 6.5, 5 mM-dextrose and 135 mM NaCl. The final platelet pellet was resuspended in 100 mM Tris-HCl, pH 7.5, 5 mM EDTA, 1 mM PMSF, 10 μg/ml leupeptin, 1 mM benzamidine and sonicated (5 $\times$ 10 sec) on ice at power setting 5 using a Microson sonifier (Heat Systems Inc., Farmingdale, NY, USA.) [9]. After centrifuging at 100,000 $\times$ g for 1 hr at 4°C, the particulate fraction was resuspended in the above buffer and various fractions were kept frozen at $-$20°C until required. The platelet dense granule fraction was prepared using the method described in [18] and electron microscopy performed on whole mounts using a Philips 400 Transmission Electron microscope as described in [19].

*Generation of antibodies against recombinant RalA. Ral*A was expressed as a fusion protein coupled to the C-terminal end of flag peptide of the expression vector, pFLAG-1, and purified as described elsewhere [20]. To raise antibodies in rabbits, 0.25 mg of purified recombinant *ral*A protein was added to an equal volume of Freund's complete adjuvant and, after thorough mixing, the solution was injected subcutaneously at multiple sites. Three weeks later, 0.25 mg of the antigen was mixed with an equal volume of Freund's incomplete adjuvant and the suspension injected subcutaneously at multiple sites. This step was repeated two more times at three week intervals and one week after the final injection a blood sample was taken and allowed to clot at room temperature. The serum was collected by centrifugation and stored at $-$20°C until further use.

Electrophoresis and transfer of resolved proteins onto nitrocellulose. Proteins in individual platelet fractions were separated by one-dimensional SDS-PAGE as described elsewhere [21]. However, to achieve antigen recognition by the D545 antibody, platelet proteins were electrophoresed using Laemmli's sample buffer that lacked β-mercaptoethanol [18]. For two dimensional separation of proteins by isoelectric focusing (IEF) in the first dimension and SDS-PAGE in the second dimension, the method of O'Farrell [22] was used with modifications [9]. Separating gels contained 13% acrylamide. Gels containing resolved polypetides were shaken for 30 min in a buffer containing 25 mM Tris, 192 mM glycine, 20% methanol (v/v), pH 8.3, and 0.1% SDS and electroblotted (16 hr at 30V) onto nitrocellulose using the same buffer [23]. To determine the pI of various GTP-binding proteins, one gel from the isoelectric focusing step was sliced (0.5 cm/slice) and each gel slice was mixed with 0.5 ml of distilled water. After 1 hr incubation, the pH was measured and a standard plot representing gel length versus pH was generated. The pI of various forms of the 27 kDa GTP-binding protein were determined using this plot.

[α-^{32}*P*]*GTP overlay assay and Western blot analysis.* The procedure for the detection of low molecular mass GTP-binding proteins after SDS-PAGE and transfer onto nitrocellulose has been described in detail elsewhere [20,24]. For immunodetection of dense granule associated low molecular mass GTP-binding proteins, nitrocellulose blots containing resolved polypeptides were left for 16 hr at 4°C in a buffer containing 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1% Tween 20 (w/v) and 5% milk powder (buffer A). The blots were further incubated with shaking for 1$-$2 hr in buffer A containing the primary antibody. Finally the blot was washed in buffer A (lacking milk powder) and incubated with anti-rabbit- or anti-mouse-IgG-horseradish peroxidase conjugate (1:2000 dilution) for 1 hr at room temperature. After washing in buffer A (lacking milk powder), the antigen-antibody complex was visualized using ECL.

Protein assay. The protein content in various platelet fractions was determined by the method of Lowry *et al.* [25], using a standard solution that contained 5% (w/v) human albumin and 3% (w/v) human globulin.

RESULTS

The final dense granule preparations were assayed for purity by 1) observing a representative sample via electron microscopy and 2) Western blot analysis. Visual inspection of the micrograph revealed that the procedure used resulted in a fraction which was enriched in dense

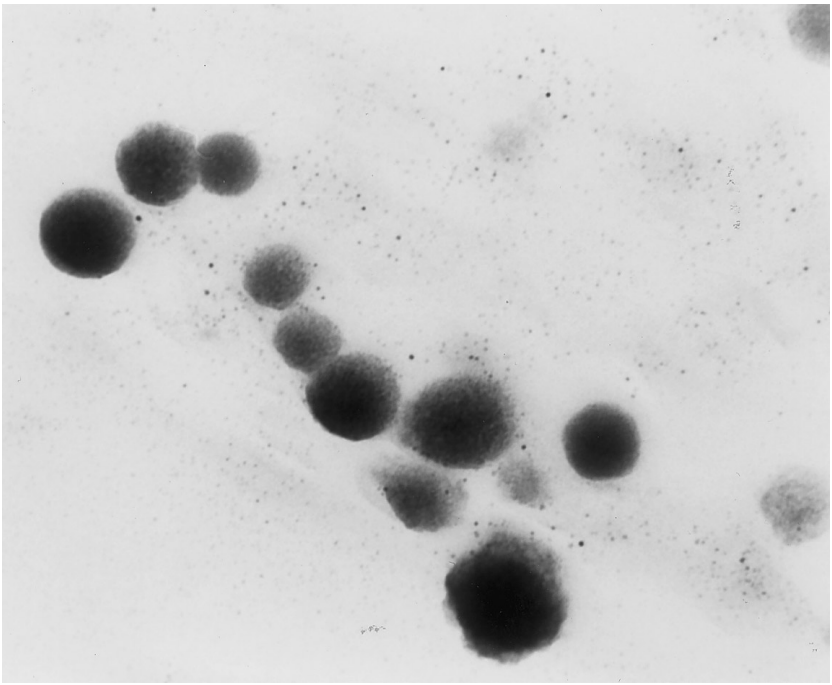

FIG. 1. Electron micrograph (whole mount) of the platelet dense granule preparation. The dense granule sample was pipetted directly onto a carbon- stabilized formvar grid and allowed to incubate for 1 min at room temperature. The grid was washed once with distilled water, allowed to dry and viewed under the electron microscope (original magnification, ×102,000).

granules and that there appeared to be little, if any, contamination by α-granules, lysosomes, whole platelets or membranous debris (Fig. 1). Further analysis using antibody (D545) against the 40 kDa dense granule specific protein [18] demonstrated enrichment of this protein in the dense granules (Fig. 2). The smearing effect observed in Fig. 2 was caused by electrophoresis of proteins under non-reducing conditions. This was necessitated by the fact that the D545 antibody does not recognize the reduced form of the antigen [18].

Assessment of platelet cytosol, particulate and dense granule fractions for the presence of low molecular mass GTP-binding proteins was carried out by incubating blots containing

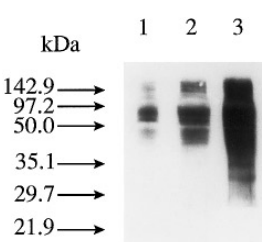

FIG. 2. Immunodetection of 40 kDa dense granule specific protein using D545 antibody. 15 μg of protein from platelet lysate (lane 1), dense granule depleted membrane (lane 2), and dense granule (lane 3) fractions was separated by SDS-PAGE under nonreducing conditions and then transferred to nitrocellulose. The blot was incubated with D545 antibody (10 μg/ml) and antigen–antibody complex detected using the ECL system. Mobility of prestained protein markers is indicated on the left.

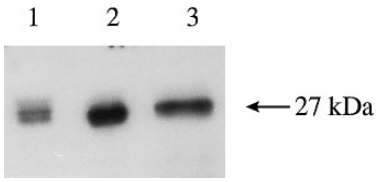

FIG. 3. Detection of low molecular mass GTP-binding proteins. 30 μg of human platelet cytosolic (lane 1), particulate (lane 2), and dense granule (lane 3) proteins were separated by SDS-PAGE and transferred to nitrocellulose. GTP-binding proteins were detected using [α-^{32}P]GTP overlay assay as described under Materials and Methods. Mobility of a 27 kDa prestained protein marker is indicated on the right.

polypeptides separated using SDS-PAGE with [α-^{32}P]GTP [20]. The results demonstrated the presence of a major 27 kDa GTP-binding protein and varying amounts of 24 kDa GTP-binding protein in all three fractions (Fig. 3). GTP-binding was least in the cytosolic fraction (Fig. 3, lane 1). Thus, further analysis was carried out on GTP-binding proteins associated with the particulate and dense granule fractions.

Previously, we have demonstrated that the human platelet 27 kDa and 24 KDa GTP-binding proteins can be resolved into seven and four distinct forms respectively [9]. Thus, GTP-binding proteins associated with total platelet particulate and dense granule fractions were resolved using isoelectric focusing and two-dimensional polyacrylamide gel electrophoresis. The platelet particulate fraction contained 7 distinct forms of the 27 kDa protein and the 24 kDa protein was resolved into four distinct forms (Fig. 4A). The dense granule 27 kDa and 24 kDa proteins were separated into four distinct forms each (Fig. 4B). The two most acidic 27 kDa proteins (Fig. 4B, 27f and 27g) associated with dense granules demonstrated the greatest GTP-binding

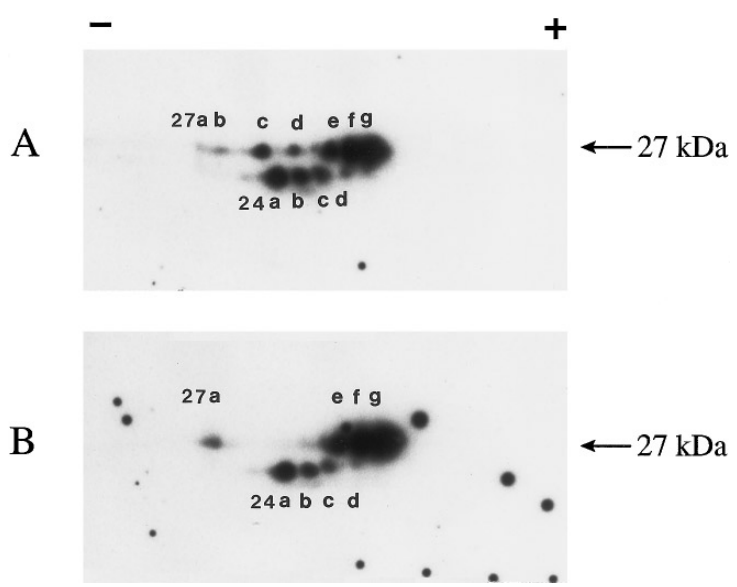

FIG. 4. Analysis of low molecular mass GTP-binding proteins by two-dimensional polyacrylamide gel electrophoresis. 50 μg of platelet (A) total particulate and (B) dense granule proteins were subjected to isoelectric focusing (pH 3–10) in the first dimension followed by SDS-PAGE in the second dimension. The proteins were transferred to nitrocellulose, and GTP-binding proteins were detected as described under Materials and Methods. The major species of the 27 and 24 kDa GTP-binding proteins are labeled. The orientation of the cathode (−) and anode (+) during IEF is indicated. The mobility of a 27 kDa prestained protein marker is given on the right.

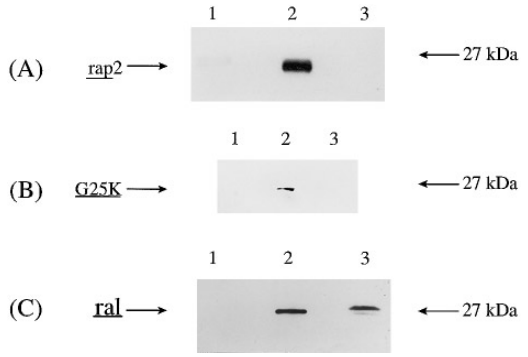

FIG. 5. Immunodetection of (A) *rap*2, (B) *G25K/CDC42*Hs, and (C) *ral* GTP-binding proteins in platelet fractions. 15 μg of protein from cytosol (lane 1), particulate (lane 2), and dense granule (lane 3) fractions was separated by SDS-PAGE, transferred to nitrocellulose, and incubated with the respective antibody as described under Materials and Methods. The antigen–antibody complex was visualized using the ECL protocol. Mobility of a 27 kDa prestained protein marker is indicated on the right.

capacity. By measuring pH along the length of the isoelectric focusing gel (results not shown), the pI of these two proteins were estimated to be 4.80 (27f) and 4.60 (27g). Some of the 27 kDa GTP-binding proteins detected in the platelet particulate fraction (Fig. 4A, 27b, 27c and 27d) were not associated with dense granules (Fig. 4B). The four forms of the 24 kDa GTP-binding protein present in the total particulate fraction were also associated with the dense granule fraction (compare Fig. 4A and 4B). Thus, of the 11 low molecular mass GTP-binding proteins detected in the total platelet particulate fraction (Fig. 4A), eight were also associated with the dense granules (Fig. 4B).

To further characterize the low molecular mass GTP-binding proteins associated with dense granules, antibodies specific for some of the known platelet GTP-binding proteins were used in Western blot analysis. Antibodies specific for *rap*2 and G25K/CDC42Hs demonstrated that these proteins were not present in the platelet cytosol (Fig. 5A, lane 1 and Fig. 5B, lane 1) but were associated with the particulate fraction (Fig. 5A, lane 2 and Fig. 5B, lane 2). These proteins were not detected in dense granules (Fig. 5A, lane 3 and Fig. 5B, lane 3). This further supports the observation that the dense granules were not contaminated with other particulate debris. Analysis using antibody raised against recombinant *ral*A protein demonstrated that this protein was not present in the platelet cytosol (Fig. 5C, lane 1) but was detected in the platelet particulate fraction (Fig. 5C, lane 2) and the dense granule fraction (Fig. 5C, lane 3). The 27a dense granule associated protein detected by two dimensional electrophoresis (Fig. 4B) was recognized by the antibody against *ral*A (results not shown) confirming our previous observations with the platelet particulate fraction [9]. This also suggests that other forms of low molecular mass GTP-binding proteins associated with dense granules are not coded for by *ral*-related gene(s).

DISCUSSION

As demonstrated in this study, and elsewhere [24], human platelets contain appreciable amounts of 24–27 kDa low molecular mass GTP-binding proteins. We have now also demonstrated the association of 24–27 kDa GTP-binding proteins with platelet dense granules (Fig. 3). With the aid of two-dimensional polyacrylamide gel electrophoresis, we have resolved the dense granule associated GTP-binding proteins into four forms of 27 kDa and four forms of 24 kDa (Fig. 4B). Thus, of the 11 low molecular mass GTP-binding proteins detected in the

total platelet particulate fraction (Fig. 4A), eight were also associated with the dense granules (Fig. 4B).

The identity of the majority of the dense granule associated GTP-binding proteins is not known at the present time. However, in the present study we have demonstrated that *ral* protein is one of the GTP-binding proteins associated with platelet dense granules (Fig. 5C, lane 3). Of all the different forms of GTP-binding proteins associated with the dense granules (Fig. 4B), only the 27a species is coded for by the *ral* gene since this protein was recognized by an antibody raised against recombinant *ral*A protein and the pI value of 7.2 determined for the 27a protein matched the predicted pI for *ral* protein(s) [26]. The identity of the gene(s) coding for the remaining forms of 27 kDa and 24 kDa proteins associated with the dense granules is not known at the present time. Previously, we had proposed that only *ral* and *rab* gene products will easily bind GTP on incubation of nitrocelluose blots containing proteins separated using SDS-PAGE [27]. Thus, the remaining forms of 27 and 24 kDa proteins associated with dense granules could represent products of one or more *rab* genes. In this regard, it is of interest to note that recently products of *rab*1, *rab*3B, *rab*4, *rab*6 and *rab*8 genes have been demonstrated to be present in human platelets [15]. However, some of these *rab* proteins can be ruled out as candidates for dense granule associated GTP-binding proteins. Thus, *rab*3B was found to be mainly cytosolic in the platelet [15] and *rab*6 and *rab*8 were found to be associated with platelet α-granules and not with the dense granules [15]. In addition, the predicted pI for *rab*1 (6.0), *rab*4 (5.2), *rab*6 (5.3) and *rab*8 (9.5) proteins [26] is not in the range of the pI for the 27f (4.80) and 27g (4.60) proteins that we found associated with the dense granules (Fig. 4B). From the predicted values, the only *rab* protein that has a pI in the range of the 27f and 27g proteins is, *rab*18 [26]. However, it is not known if *rab*18 is present in human platelets. We are currently investigating this possibility.

The precise role of *ral* and other dense granule associated GTP-binding proteins in platelet physiology is not known. However, considering that *ral*-related proteins are associated with synaptic [28] and cholinergic [29] vesicles and now platelet dense granules, the possibility exists that these proteins may have a function in the regulated exocytotic pathway and that different types of secretory granules will have associated with them their own unique set of GTP-binding protein(s). The precise identification of these GTP-binding proteins should help in defining their function in platelet physiology.

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