Identification of small GTP-binding rab proteins in human platelets: Thrombin-induced phosphorylation of rab3B, rab6, and rab8 proteins

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ABSTRACT The activation of platelets by specific agonists is a tightly regulated mechanism that leads to the secretion of the dense- and α -granule contents. Platelets have been shown to possess small GTP-binding proteins thought to be involved in central biological processes; however, no rab proteins, which may regulate the exocytic process at different stages, have been reported. This study has shown that rab1, rab3B, rab4, rab6, and rab8 proteins, but not rab3A protein, were present in platelets and in endothelial cells. To probe their functional significance in platelets, rab3B, rab6, and rab8 proteins were further characterized with regard to their intracellular localization and their phosphorylation properties. Whereas rab3B protein was found to be mainly cytosolic, rab6 and rab8 proteins were preferentially targeted to the plasma membrane and to the α granules. The activation of platelets by thrombin, a potent inducer of secretion, resulted in the phosphorylation of rab3B, rab6, and rab8 proteins, whereas no phosphorylation was observed in the presence of prostaglandin E₁, which stimulates cAMP-dependent protein kinase and inhibits the secretion process. These findings provide evidence that members of the subfamily of rab proteins, rab6 and rab8, are localized in platelets to one type of specific secretory vesicle, the α granule, and would suggest their possible implication in the secretion process through phosphorylation mechanisms.

Primary hemostasis and thrombosis result from the adhesion of circulating platelets to subendothelium exposed after a lesion of the vessel wall. The subsequent stimulation of specific receptors (1, 2) in response to vascular injury alters platelet metabolism and leads to secretion of the α - and dense-granule contents. Platelet activation is a tightly regulated mechanism involving many membrane associated and cytoplasmic enzymes. In this regulation, a key role seems to be played by members of the heterotrimeric guanine nucleotide-binding protein family, which mediate the interaction between agonist receptors and intracellular effectors (3, 4), and possibly by the family of small GTP-binding proteins whose identity and function within platelets remain unsettled and are just beginning to emerge in other cells. The ras-related superfamily, which includes the R-ras, rap, ral/rac, and rho/rab subfamilies, exhibits 30–60% homology with p21^{ras}. They are thought to regulate central biological processes. Some of them are involved in signal transduction and in the control of regulatory pathways critical for normal proliferation or differentiation (5–7). Interesting data have suggested an important role for rab proteins either in endocytosis or in biosynthetic protein transport and stimulus-secretion coupling (8–11). The transport of newly synthesized proteins from the endoplasmic reticulum to the various stacks of the Golgi complex and to the secretory vesicles involves, at each stage, the movement of carrier vesicles.

There is growing evidence that rab proteins, localized to different subcompartments of this process, exert roles in such targeting machineries. The idea that rab proteins may also direct the exocytosis from secretory vesicles to the plasma membrane has received support from the observation that in yeast the SEC4 protein, which is 40% homologous to rab proteins, is associated with secretory vesicles (12, 13). Direct support for the involvement of the GTP-binding protein rab3A, specifically localized to synaptic vesicles, in the regulated secretion process in brain has also been reported (14, 15).

By their ability to specifically store some proteins and secrete them upon specific stimulation by agonists such as collagen or thrombin, platelets present a great interest for studying the stimulus-secretion coupling process. They are populated by two specific organelles, differing by their typical ultrastructure and biochemical components (16): (i) the dense granules, which contain essentially nucleotides, calcium ions, and serotonin, and (ii) the α granules, which contain various specific macromolecules such as von Willebrand factor, fibrinogen, and thrombospondin. The mechanism of exocytosis is only partially understood in platelets. The existence of different activation pathways directing dense- and α -granule secretion prompted us to investigate the presence and subcellular localization of the rab proteins in platelets as well as in endothelial cells. Endothelial cells, known to contain secretory organelles as the Weibel Palade bodies, a specific storage site of the von Willebrand factor, also behave as regulatory secretory cells.

Many studies have demonstrated the importance of phosphorylation events in various aspects of cellular function. In platelets, a number of kinases are stimulated upon platelet activation and are thought to play a key role in regulating the secretion process (17, 18). Phosphorylation studies of the rab proteins were therefore performed after activation or inhibition of platelet functions; this study might provide insights into platelet biology and the mechanism of platelet secretion.

MATERIALS AND METHODS

Antisera. Rabbit antisera to the rab proteins used in this work were developed against the human rab1, rab3A, rab4, and rab6 proteins expressed in *Escherichia coli* as described elsewhere (19) and against the following C-terminal peptides: Pro-Ser-Met-Leu-Gly-Ser-Ser-Lys-Ser-Asp-Thr, corresponding to residues 195–209 of the rab3B protein, and Pro-Gln-Gly-Ser-Asn-Gln-Gly-Val-Lys-Ile-Thr-Asp-Asp-Gln-Gln-Lys, corresponding to residues 182–197 of the Rab 8 protein. Both peptides were synthesized and coupled to ovalbumin via their C-terminal amino acid (Neosystemes, Strasbourg, France). Purified proteins or peptides coupled to ovalbumin (200 μ g) were emulsified in Freund's complete adjuvant and injected subcutaneously into New Zealand

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Abbreviation: PGE₁, prostaglandin E₁.

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White rabbits. They were reinjected with the same antigen in Freund's incomplete adjuvant at 4-week intervals and bled from the ear 10 days after each booster. Mouse monoclonal antibodies directed to glycoprotein Ib and rabbit antibodies to Ca^{2+} -ATPase were generous gifts of M. C. Berndt (Westmead, Australia) and of A. M. Lompré (Paris), respectively.

Subfractionation of Platelets. Fresh human platelets were isolated as described elsewhere (20).

Preparation of soluble and particulate fractions. Isolated platelets were disrupted in homogenization medium [25 mM Hepes/0.25 M sucrose/12 mM trisodium citrate/1 mM EDTA/1 mM phenylmethylsulfonyl fluoride/leupeptin (10 $\mu\text{g}/\text{ml}$)/aprotinin (0.15 unit/ml)] at pH 6.5 and 4°C and sonicated twice, in fractions of 2 ml (2×10^9 platelets per ml), for 5 sec on ice (setting of 40 W, frequency of 3.25 kHz; Branson B12 sonifier). They were then centrifuged at $250 \times g$ to eliminate the unlysed platelets. Platelet lysates (1 ml) were fractionated at $160,000 \times g$ for 1 hr in an air-driven microcentrifuge (Beckman) into cytosolic and particulate fractions. The particulate pellet was resuspended in the same volume as that of the cytosol. Equal volumes (50 μl) of total lysates and particulate and cytosol fractions were loaded on each lane of the gel.

Preparation of α - and dense-granule-enriched fractions. To isolate the plasma membranes and the enriched fractions of α and dense granules, two different methods described elsewhere were used and modified as follows (21–23): platelet lysates, obtained as described above, were submitted to centrifugation at $19,000 \times g$ to recover the bulk of platelet granules in the pellet, while plasma and internal membranes remained in the supernatant. The $19,000 \times g$ pellet was resuspended in a 20% (wt/vol) metrizamide solution and applied onto a two-step discontinuous gradient consisting of 1 ml of 38% metrizamide overlaid with 1 ml of 35% metrizamide. Centrifugation was performed at $100,000 \times g$ for 1 hr. The dense granule-enriched fraction was recovered as the pellet, and the α granules were recovered as a heavy band at the interface between the 35% and 38% metrizamide solution. The $19,000 \times g$ supernatant was centrifuged at $100,000 \times g$ for 60 min. This $100,000 \times g$ membrane pellet was characterized by different parameters as described elsewhere and referred to plasma and intracellular (dense tubular system) membranes (23).

Preparation of plasma membrane fractions. Plasma membranes were also isolated by a modified method of Barber and Jamieson as described elsewhere (20).

Endothelial Cell Cultures. Endothelial cells were isolated from umbilical veins by collagenase digestion as described (26). Cells were cultured in plastic flasks precoated with collagen in the presence of M199 medium containing 20% (vol/vol) fetal calf serum. Only primary cultures were used.

Electrophoresis and Western Immunoblots. Platelet or endothelial cell samples were run on an SDS/12% PAGE gel under reduced conditions and electrotransferred onto a nitrocellulose membrane sheet (BA85; Schleicher & Schuell). The blotted filters were probed with rabbit antisera directed against the different studied rab proteins. After incubation with ^{125}I -labeled protein A (0.25 $\mu\text{Ci}/\text{ml}$; 1 Ci = 37 GBq), the filters were dried and autoradiographed by exposing the gels to Hyperfilm- ^3H (Amersham).

Immunofluorescence Studies. Platelets in suspension or endothelial cells grown on collagen-covered glass coverslips were fixed with 2% or 4% paraformaldehyde, respectively, in phosphate-buffered saline for 30 min at room temperature. After washing, the free aldehyde groups were quenched for 15 min in 50 mM NH_4Cl . Platelets or endothelial cells were then permeabilized with 0.5% saponin for 30 min and incubated with specific anti-rab protein rabbit antibodies for 1 hr at room temperature. After a 5-min wash, glycoprotein Ib and rab proteins were visualized, respectively, with goat anti-

mouse and goat anti-rabbit tetramethylrhodamine isothiocyanate- or fluorescein isothiocyanate-conjugated IgGs (Biosys, Compiègne, France). Cells were examined with a Zeiss Axioptot microscope.

Immunoprecipitation Studies. Platelets ($10^9/\text{ml}$) were metabolically labeled with [^{32}P]orthophosphate (1 mCi/ml) for 90 min at 37°C. Platelets were then lysed in 50 mM Tris, 150 mM NaCl, and 1% Nonidet P-40 (pH 8) in the presence of 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, aprotinin (0.15 unit/ml), and 1 mM orthovanadate for 30 min at 4°C. Insoluble material was removed by centrifugation at 4°C for 10 min in a Microfuge (Beckman). Platelet aliquots (0.5 ml) were then precleared with 10 μl of nonimmune rabbit serum prior to adding 5 μl of the anti-rab protein antisera at 4°C for overnight. The bound antigen-IgG complexes were then incubated with 10% (vol/vol) protein A-Sepharose beads (CL4B; Sigma) and collected by centrifugation at $10,000 \times g$ for 15 sec at 4°C. After washings, the extracts were analyzed by SDS/PAGE, and the phosphorylated proteins were visualized by autoradiography.

RESULTS

Identification of rab-Encoded Proteins in Human Platelets and Endothelial Cells. The presence of the following family of rab-encoded proteins, rab1, rab3A, rab3B, rab4, rab6, and rab8, was investigated in platelets and endothelial cells. For this purpose, rabbit polyclonal antisera raised against purified rab1, rab3A, rab4, and rab6 proteins expressed in *E. coli* or against rab3B- and rab8-specific peptides were used. These antibodies were specific for their respective proteins as they did not recognize the other rab-encoded purified proteins (0.5 μg) or rab proteins expressed in *E. coli* (data not shown). Using these specific antibodies, immunoblot analysis of cell lysates showed that rab1, rab4, rab6, and rab8 proteins were present in both platelets (Fig. 1A) and endothelial cells (Fig. 1B). The same rab3A antibody, which had been previously shown to recognize specifically the synaptic vesicles in brain cells (25), failed to detect any rab3A protein in platelets and endothelial cells. In contrast, rab3B protein, which shares 80% amino acid identity with rab3A protein, was present in both types of cells. Among this set of rab-encoded proteins identified in platelets as well as in endothelial cells, rab3B, rab6, and rab8 proteins were further characterized.

Cellular Localization of rab3B, rab6, and rab8 Proteins in Platelets. To determine whether these rab proteins are mem-

FIG. 1. Identification of rab1, rab3A, rab3B, rab4, rab6, and rab8 proteins in platelets and endothelial cells by Western immunoblot analysis. Total proteins from platelets (A) and endothelial cells (B) were immunoblotted with specific rabbit sera raised against the different rab proteins as mentioned or with a nonimmune serum (control). Bands were visualized by autoradiography using ^{125}I -labeled protein A.

brane associated or cytosolic, soluble and particulate platelet fractions were prepared as described in *Materials and Methods*.

rab6 and rab8 proteins were essentially membrane associated (Fig. 2). In contrast, the majority of rab3B protein was found in the cytosol; only a faint signal could be detected in the membrane fraction.

Localization of rab6 and rab8 Proteins in Platelets and Endothelial Cells by Indirect Immunofluorescence. Since rab3B protein was essentially cytosolic, only rab6 and rab8 protein localization was investigated in saponin-permeabilized platelets by indirect immunofluorescence. A strong reactivity of anti-rab6 and anti-rab8 antibodies on platelets was observed; no fluorescence was detected with nonimmune serum. The distribution of a specific antigen of the plasma membrane, glycoprotein Ib, was, as shown in Fig. 3, exclusively restricted to the cell rim. In contrast, the staining pattern of rab6 and rab8 showed an intracellular localization of these proteins: it was possible to detect labeling of small dots distributed throughout the cytoplasm, closely resembling the appearance of cytoplasmic secretion vesicles. The same punctate pattern was observed by staining the α granules with anti-von Willebrand factor antibodies.

The localization of rab6 protein was also investigated in endothelial cells. As visualized by immunofluorescence, the anti-rab6 antibodies specifically stained a region beside the nucleus, typical of the Golgi complex (Fig. 4D). The association of this protein with organelles in the Golgi region was further studied by double immunofluorescence using a monoclonal antibody, CTR 433, specifically recognizing the Golgi complex (26); the staining pattern exhibited the same localization (Fig. 4C). The specificity of the anti-rab6 labeling was tested by competition with the specific protein against which the antibody was raised, clearly showing an extinction of the fluorescent signal (Fig. 4B).

Subcellular Distribution of rab Proteins in Human Platelets. The subcellular distribution of rab3B, rab6, and rab8 proteins was investigated. For this purpose, a subfractionation procedure, designed to yield greatly enriched α - and dense-granule fractions was developed as described in *Materials and Methods*. Another fractionation protocol based on a discontinuous sucrose density gradient was performed to obtain a highly enriched fraction of plasma membranes as described elsewhere (20). We therefore analyzed the distribution of rab3B, rab6, and rab8 proteins on the plasma membrane fraction and on both secretory vesicles (i.e., the dense and α granules) (Fig. 5). rab3B protein was poorly revealed on these fractions, confirming that most of rab3B protein is predominantly cytosolic and not associated with plasma or granule membranes in platelets. Interestingly, the subcellular fractionation analysis showed that rab6 and rab8 proteins were predominantly associated with the enriched α -granule fraction (α G) while they were not, or very poorly, associated with the dense-granule fraction (DG) (shown to contain 0.5% α granules) (21). rab6 and rab8 proteins were also detected on the plasma membranes (PM); their presence

FIG. 2. Distribution of rab3B, rab6, and rab8 proteins in particulate and cytosolic fractions in human platelets. Proteins from total platelet lysates (TOT.), particulate (PART.), and cytosolic (CYT.) fractions were immunoblotted with specific anti-rab3B, -rab6, and -rab8 antibodies. Bands were visualized by autoradiography using 125 I-labeled protein A.

FIG. 3. Localization of rab6 and rab8 proteins in human platelets by indirect immunofluorescence. Permeabilized intact platelets were stained with anti-glycoprotein Ib mouse monoclonal antibodies, as a marker of plasma membranes (A), anti-von Willebrand factor rabbit antibodies, as a marker of α granules (C), anti-rab6 rabbit antibodies (D), anti-rab8 rabbit antibodies (E), and nonimmune sera (B and F). Immunofluorescence labeling of platelets was performed using rhodamine-conjugated goat anti-rabbit or anti-mouse IgGs.

in the enriched α -granule fraction was therefore investigated by using, as markers, antibodies to a Ca^{2+} -ATPase, shown to be associated to plasma membranes (26), and to glycoprotein Ib. As shown in Fig. 5B, plasma membranes were almost totally absent in the enriched α -granule fraction.

The enriched α -granule fraction was not contaminated by dense granules. By using a fluorochrome, mepacrine, that is a specific marker of the dense vesicles, only a very faint background fluorescence was observed on this α -granule fraction (Fig. 6B), whereas dense vesicles were strongly fluorescent, exhibiting a characteristic punctate vesicular pattern (Fig. 6A). However, the α -granule fraction has been

FIG. 4. Localization of rab6 protein in endothelial cells by indirect immunofluorescence. Permeabilized endothelial cells were incubated with nonimmune serum (A), anti-rab6 antibodies adsorbed with the immunizing rab6 peptide (10 μ g) (B), anti-rab6 antibodies plus mouse monoclonal CTR 433 anti-Golgi antibodies (C), or affinity-purified anti-rab6 antibodies (D). Double-labeling immunofluorescence was performed with a mixture of rhodamine-conjugated goat anti-rabbit antibodies and fluorescein-conjugated sheep anti-mouse antibodies.

FIG. 5. Localization of rab proteins in the subcellular fractions of human platelets by immunoblot analysis. Proteins from total platelet extracts (T), plasma membrane (PM), and enriched- α -granule (α G) and dense-granule (DG) fractions were separated by SDS/PAGE and analyzed by immunoblotting using anti-rab3B, -rab6, and -rab8 specific antibodies (A) or anti-glycoprotein Ib or anti- Ca^{++} -ATPase monoclonal antibodies (B). Bands were visualized with ^{125}I -labeled protein A and autoradiographed.

shown to contain some mitochondria and lysosomes (21); the colocalization of rab6 and rab8 proteins with von Willebrand factor, which is specifically stored in these granules, was therefore investigated by double immunofluorescence studies. In Fig. 6, rab6 and rab8 proteins appeared, by double immunofluorescence, to be present on the same dispersed grouped vesicles as the von Willebrand factor, thus exhibiting exactly the same staining pattern and strongly suggesting, in the absence of consistent plasma membrane contamination (Fig. 5B), their specific colocalization on α granules.

Phosphorylation of the rab Proteins upon Platelet Activation. To investigate whether the identified rab proteins could be phosphorylated under certain conditions, platelets were metabolically labeled with ^{32}P orthophosphate before their stimulation either by thrombin, an activator of protein kinase C and inducer of secretion, or by prostaglandin E_1 (PGE_1), which, by elevating intracellular cAMP levels, inhibits platelet functions.

Phosphorylation of the different rab gene products was not, or very faintly, observed in unstimulated platelets. Fig. 7 shows that rab3B, rab6, and rab8 proteins became strongly phosphorylated upon activation of platelets by thrombin. However, they were not phosphorylated by PGE_1 , which stimulates cAMP-dependent protein kinase and inhibits the secretion process. In contrast, rap1 protein was phosphorylated after cAMP elevation by PGE_1 , whereas no phosphorylation was observed in response to thrombin.

DISCUSSION

In the past 2 years, platelets have been shown to possess distinct small GTP-binding proteins, which have been identified from protein or cDNA sequence homologies to the ras proteins. Among these, *ral*-, *rac1*-, *rap1A*-, *rap1B*-, *rap2*-, and possibly *rho*-encoded proteins were described (27–30). Their possible physiological significance in platelets has not yet been elucidated. However, rap1B protein has been shown to be selectively phosphorylated by cAMP-dependent protein kinase A and consecutively translocated from plasma membrane to cytosol (31–33). rap1B protein has been proposed to be involved in the regulation of the transduction mechanism; in particular, a possible role for this protein was recently assigned in epinephrine-stimulated signal transduction (34). No rab protein has been reported as yet in platelets even though they constitute a distinct subgroup of the ras family and may regulate the various steps of the secretion process. The present study provides evidence that a set of rab proteins, rab1, rab4, rab3B, rab6, and rab8, are present in human platelets, as well as in endothelial cells. rab3A pro-

FIG. 6. Colocalization of rab6 and rab8 proteins with von Willebrand factor on the enriched α -granule fraction by indirect immunofluorescence. The enriched dense- (A) and α - (B) granule fractions were first subjected to mepacrine, a fluorochrome that specifically stains the dense granules. Double-labeling of the enriched α -granule fraction was performed either with rabbit anti-rab6 peptide IgGs (C) and sheep anti-von Willebrand factor IgGs (D) or with rabbit anti-rab8 protein IgGs (E) and sheep anti-von Willebrand factor IgGs (F). Controls were nonimmune rabbit serum (G) and sheep antiserum (H). Fluorescein-conjugated anti-rabbit antibodies and rhodamine-conjugated anti-sheep antibodies were used.

tein, which is specific for synaptic vesicles in neural tissue, is absent.

In some cells, rab1 or rab2 protein has been suggested to be involved in the vesicular transport from endoplasmic reticulum to Golgi or through the Golgi saccules (8, 11, 35), whereas rab4 protein would be required for endocytosis (36). However, little was reported on rab6 or on rab3B and rab8 proteins. To probe their functional significance in platelets, rab3B, rab6, and rab8 proteins were first characterized with regard to their intracellular localization and their phosphorylation properties.

With regard to the immunofluorescence experiments in intact platelets and to the cellular subfractionation analysis, this study strongly suggests that rab6 and rab8 proteins are associated not only with the platelet plasma membrane but also with secretory vesicles and are preferentially targeted to one type of secretory vesicle—the α granule. This preferential localization of rab6 and rab8 proteins differs from that of rab3B protein, which was mainly cytosolic. In endothelial

FIG. 7. Phosphorylation of rap1 and rab proteins induced by thrombin or PGE₁ in human platelets. ³²P-labeled platelets that were unstimulated (CONT.) or exposed to thrombin (THR.) at 1 unit/ml or to 10 μM PGE₁ for 1 min at 37°C were lysed, and ³²P-labeled proteins were immunoprecipitated with anti-rap1, -rab3B, -rab6, and -rab8 rabbit antibodies. Immunoprecipitates were subjected to SDS/PAGE and autoradiographed.

cells, rab6 protein was associated with the Golgi apparatus. This is consistent with the previously described localization of rab6 protein to medial- and trans-Golgi cisternae of several cell types (37). The association of this protein with the platelet α granules is however not unexpected since, in megakaryocytes, these granules were directly derived from the posttrans-Golgi vesicles, whereas dense granules were not (38). A possible implication of rab6 protein in the transport of soluble or membrane proteins from the trans-Golgi vesicles to the α granules cannot be discarded and could explain its presence on these granules; however, this is not the case for rab8 protein, which did not exhibit any localization on the Golgi complex (data not shown). It would be interesting to determine whether rab8 protein or rab6 protein may function as a heterotrimeric guanine nucleotide-binding protein on the secretory vesicle to transduce an intracellular signal needed for transport of the vesicle and/or its fusion with the plasma membrane. Only the yeast rab-related SEC4 protein, also localized on the cytoplasmic surface of secretory vesicles and on the plasma membrane, has been shown to be involved in the final stage of the secretory pathway—i.e., the fusion of secretory vesicles with the plasma membrane. The preferential localization of rab6 and rab8 proteins in the α granules and not in the dense granules also suggests that the delivery of both secretory granules may occur by distinct mechanisms.

A number of kinases are stimulated upon the activation of platelets and are thought to play an important role in regulating the secretion process. This is the case, in particular, of protein-tyrosine kinases, which are widely regarded as key regulators and of protein kinase C, a calcium and phospholipid-dependent kinase whose activation is associated with cell-signaling events leading to secretion in thrombin-induced platelets. In this regard, the activation of platelets results in the phosphorylation of the rab3B protein. By taking into consideration their localization on secretory vesicles, the phosphorylation of rab6 and rab8 proteins by thrombin but not by PGE₁, which inhibits secretion, is of particular interest. Elucidation of the functions of these rab proteins, as well as the identification of the kinase(s) responsible for their phosphorylation, may bring new insights in the understanding of the secretion process.

In conclusion, our study clearly identified in platelets rab3B protein, which was mainly cytosolic, and rab6 and rab8 proteins, which were found preferentially associated to a particular type of specific secretory vesicle, the α granule; this would support a possible role for rab6 and rab8 proteins in the secretion process through phosphorylation mechanisms.

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