Rab6 is phosphorylated in thrombin-activated platelets by a protein kinase C-dependent mechanism: effects on GTP/GDP binding and cellular distribution

Michael L. FITZGERALD and Guy L. REED¹

Cardiovascular Biology Laboratory, Harvard School of Public Health, 677 Huntington Avenue, Boston, MA 02115, U.S.A.

In platelets and other secretory cells, protein kinase C (PKC) plays a role in exocytosis stimulated by physiological extracellular signals, although its linkage to the secretory machinery is poorly understood. We investigated whether Rab6, a GTP-binding protein that fractionates with platelet α -granules, may be involved in linking these processes. We found that Rab6 contains two PKC consensus phosphorylation sites that are evolutionarily conserved. In platelets metabolically labelled with [³²P]P_i, Rab6 phosphorylation was induced by phorbol esters or by thrombin. This phosphorylation was blocked by a specific PKC inhibitor (Ro-31-8220), but not by a p38 mitogen-activated protein kinase inhibitor (PD-169316). Physiological stimulation of platelets caused a PKC-dependent translocation of Rab6 from platelet particulate fractions, nearly doubling the fraction of Rab6 in the cytosol. A human Rab6 isoform (Rab6C) that is preferentially expressed in human platelet RNA was cloned and its

phosphorylation by PKC was characterized. Rab6C incorporated up to 2 mol of [³²P]P_i per mol of active protein. Rab6C bound GDP and GTP with K_d values of 113 ± 12 and 119 ± 27 nM respectively, and hydrolysed GTP at a rate of 100 ± 15 μ mol of GTP/mol of Rab6C per min. PKC phosphorylation of Rab6C increased the affinity for GTP by 3-fold, although it had lesser effects on GDP (1.6-fold). Phosphorylation did not alter the GTPase activity. In summary, thrombin activation of platelets leads to PKC-dependent phosphorylation of Rab6 and a translocation of Rab6 to the cytosol. We suggest that PKC phosphorylation may be an important mechanism through which Rab functional interactions in vesicle trafficking and secretion can be altered in response to an external stimulus.

Key words: exocytosis, intracellular signalling, thrombin.

INTRODUCTION

Platelets are anucleate cells that play a pivotal role in thrombus formation in both normal and pathological settings. Activation of platelets by extracellular molecules generated during vascular injury leads to platelet cytoskeletal reorganization, aggregation and secretion of granules [1]. Thrombin, a proteolytic enzyme of the coagulation cascade, is a potent physiological activator of platelets through its interaction with seven-transmembrane receptors [2,3]. Induction of protein kinase C (PKC) activity is an important component downstream of thrombin signalling. When PKC is activated indirectly by thrombin or other agonists, or directly stimulated by phorbol esters, it induces platelet vesicle release [4–6]. Conversely, inhibitors of PKC activity attenuate secretion [7–9].

How thrombin signalling and PKC activation directly couple to platelet degranulation is not clear. We and others have recently identified members of the SNARE [soluble *N*-ethylmaleimide-fusion-protein attachment-protein (SNAP) receptor] family of proteins in human platelets [10–12]. The SNARE machinery [e.g. syntaxin, 25-kDa synaptosome-associated protein (SNAP-25), vesicle-associated membrane protein (VAMP), SNAP and *N*-ethylmaleimide-sensitive fusion protein (NSF)] has been identified in neurons and other secretory cells as mediating vesicle trafficking events such as the exocytosis of neurotransmitters [13,14]. Moreover, functional evidence indicates that both α - and dense-granule secretion depends on NSF activity [15], and the SNARE complex [12]. The actions of the SNARE

machinery in vesicle trafficking and secretion are further regulated by direct and indirect interactions with the Rab proteins [16–18]. The Rab proteins are modulated in part through a cycle of GTP binding and hydrolysis that controls the membrane/cytosolic partitioning of these proteins and their interaction with effector molecules [19]. Human platelets express Rab6, which fractionates with α -granules and the plasma membranes, suggesting a potential role for the protein in the process of granule secretion [20]. The phosphorylation of Rab6 during activation of human platelets by thrombin further links Rab6 to signalling processes and platelet activation.

Because of their well-characterized pattern of cell activation, intracellular signalling and secretion, platelets represent a unique system to investigate how cell signalling may modulate Rab function. Here we have investigated the linkage between thrombin stimulation of platelets and the phosphorylation of Rab6 by analysing its effects on nucleotide binding and cellular translocation. Human Rab6 contains two PKC consensus phosphorylation sites that have been conserved strongly during the evolution of this gene. We find that activation of platelets with a physiological agonist like thrombin induces phosphorylation of platelet Rab6 through a PKC-dependent mechanism. Analyses of Rab6 nucleotide binding and cellular distribution during platelet activation indicate how phosphorylation may alter the cellular activity of the protein. These results have implications for how extracellular signals that activate cells may be transmitted through PKC to affect the proteins implicated in the secretory process.

Abbreviations used: PKC, protein kinase C; GDI, GDP-dissociation inhibitor; MAP, mitogen-activated protein; SNARE, soluble *N*-ethylmaleimide-fusion-protein attachment-protein receptor; NSF, *N*-ethylmaleimide-sensitive fusion protein; RT, reverse transcriptase; NTA, nitrilotriacetic acid.

¹ To whom correspondence should be addressed (e-mail reed@cvtlab.harvard.edu).

EXPERIMENTAL

Materials

Bovine thrombin was purchased from Parke-Davis (Morris Plains, NJ, U.S.A.); hirudin, prostaglandin E_1 and PMA were from Sigma (St. Louis, MO, U.S.A.); the PKC inhibitor Ro-31-8220 and the p38 mitogen-activated protein (MAP) kinase inhibitor PD-169316 were from Calbiochem (San Diego, CA, U.S.A.); PKC was from Upstate Biotechnology (Lake Placid, NY, U.S.A.); [32 P]orthophosphoric acid was from ICN (Irvine, CA, U.S.A.); GTP radionucleotides were from Dupont NEN (Boston, MA, U.S.A.); *Taq* polymerase and restriction enzymes were from Boehringer Mannheim (Indianapolis, IN, U.S.A.); Rab6 affinity-purified antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.); guanosine-dissociation inhibitor (GDI) affinity-purified antibodies were from Zymed Laboratories (San Francisco, CA, U.S.A.); and Nonidet P40 was from United States Biochemical (Cleveland, OH, U.S.A.).

Platelet isolation, metabolic labelling, cellular fractionation and RNA isolation

Human platelets were isolated from outdated plasma (Massachusetts General Hospital, Boston, MA, U.S.A.) or from the platelet-rich plasma of freshly drawn citrated blood by gel filtration over a Sepharose-2B column in a modified Tyrode's buffer (3.5 mM Hepes, 137 mM NaCl, 2.7 mM KCl, 3 mM NaH_2PO_4 , 8.5 mM glucose and 0.35% BSA, pH 7.35). For metabolic labelling, gel-filtered platelets (1×10^9) in Tyrode's buffer (no phosphate) were incubated with 1 mCi of [32 P]orthophosphoric acid at 37 °C for 1 h. Cytosolic and membrane/particulate fractions were produced essentially as described by Karniguian et al. [20]. In brief, gel-filtered platelets were lysed in 2 ml of lysis buffer (25 mM Hepes, pH 6.5, 250 mM sucrose, 12 mM sodium citrate, 1 mM EDTA, 1 mM PMSF, 1 $\mu\text{g}/\text{ml}$ leupeptin, 15 units/ml aprotinin, 1 mM sodium orthovanadate and 1 mM sodium fluoride) by mild sonication (twice for 5 s each, 40 W, 3.25 kHz; Branson Sonifier 450). Unlysed platelets were pelleted by spinning at 250 *g*, and the supernatant of this spin was centrifuged at 160 000 *g* for 1 h at 4 °C. The cytosolic fraction (supernatant) was precipitated with 10% trichloroacetic acid, and the resulting precipitate and the particulate pellet were dissolved in equal volumes of 2 $\times$ sample buffer (125 mM Tris base, 20% glycerol and 2% SDS). Equal volumes of cytosolic and particulate lysate were boiled and run on 12% non-reducing SDS/polyacrylamide gels. The mass of platelet Rab6 in the fractions was quantified by immunoblotting, by comparison with standard curves of known amounts of purified Rab6C, using anti-Rab6 antibody, [125 I]Protein A (7.81 $\mu\text{Ci}/\mu\text{g}$; Dupont NEN) and PhosphorImaging (Molecular Dynamics, Sunnyvale, CA, U.S.A.). Analysis of variance was performed using Statview 4.0 software (Abacus Concepts, Berkeley, CA, U.S.A.) to determine the statistical significance of the Rab6 recruitment to the cytosol.

To isolate human platelet RNA, 30 units of outdated platelet-rich plasma were subjected to three low-speed spins (150 *g*) to remove aggregated platelets and contaminating red and white blood cells. The resulting platelets were pelleted at 1500 *g* and washed twice in Tyrode's buffer/EDTA, selecting only the top 66% of the pellet with each cycle. Microscopic examination of the resuspended platelets revealed no contaminating nucleated cells. RNAzol extraction, as described by the manufacturer (Tel-Test, Friendswood, TX, U.S.A.), and extensive phenol/chloroform extractions yielded 37 μg of RNA with an $A_{260/280}$ absorbance ratio of 2.0.

Cloning and recombinant expression of Rab6C

The sense primer, 5'-TTTCCATGGGATCCACGGGCGGAG-ACTTCG-3', and the antisense primer, 5'-CCATGG-ATCCAAGCTTAGCAGGAACAGCCTCC-3', were designed to amplify full-length Rab6 [21]. The underlined sequences contained *Nco*I, *Bam*HI and *Hind*III restriction sites for the purpose of cloning. By PCR, the Rab6 primers amplified a product of appropriate size (about 650 bp) from a cDNA of the DAMI megakaryocyte-like cell line (American Type Culture Collection, Rockville, MD, U.S.A.). The PCR product was ligated directly into the pCR-II plasmid (Invitrogen, San Diego, CA, U.S.A.) and sequenced on both strands. The sequence data confirmed that the insert coded for a Rab6 isoform (Rab6C, see the Results section). To quantify the relative expression of Rab6C and Rab6A, sense (5'-AGCAAAGCGTTGGAAAG-ACATC-3') and antisense (5'-CAGCTCTTTGGCTTTCCTC-TCTC-3') primers, complementary to sequences in both Rab6C and Rab6A, which flanked the Rab6C region of divergence, were used in reverse transcriptase (RT)-PCR. The primers amplified a 382-bp fragment for Rab6C, which contains a unique *Pst*I site at nucleotide 261 (relative to the ATG initiation codon). To quantify Rab6C expression, the PCR product was 32 P-end-labelled, followed by digestion of the product with *Pst*I and separation of the digests on agarose gels. To test for GDI isoform expression, the following degenerate primers were used: 5'-TCCRAYG-AGGARTACGAYGYGATCGTGC-3' and 5'-GGRATYAR-GTCAACRTTCCACTC-3'. The purity of the platelet-derived cDNA was verified by using primers specific for the CD-68/macrosialin open reading frame expressed by cells in the monocyte/macrophage lineage [22].

To express recombinant protein, the Rab6C insert from pCR-II was cloned into the His-tagged pQE-30 plasmid between the *Bam*HI and *Hind*III sites (Qiagen, Santa Clarita, CA, U.S.A.). Positive clones (JM109 bacterial host) were selected by restriction digestion of plasmid DNA. For induction of protein, overnight cultures were diluted 1/40 in fresh Luria-Bertani medium and grown to a D_{600} of 0.7, induced with 2 mM isopropyl β -D-thiogalactoside, and grown for an additional 3 h. Soluble His-tagged Rab6C was purified on a Ni-nitrilotriacetic acid (NTA)-agarose matrix according to the manufacturer's protocol (Qiagen), and a pH gradient was used to elute the protein. Peak fractions were adjusted to pH 7.4 with Tris base and analysed by SDS/PAGE. After pooling of purified fractions, recombinant Rab6C was dialysed (two 6-litre changes) overnight against 50 mM Hepes, pH 7.4, 150 mM NaCl, 1 mM EDTA and 1 mM dithiothreitol.

Immunoprecipitation of platelet Rab6

Gel-filtered platelets (4.3×10^8) were lysed in 0.5 ml of immunoprecipitation buffer (150 mM NaCl, 10 mM EDTA, 10 mM NaN_3 , 10 mM Tris, pH 8.0, 1.0% Nonidet P40, 1 mM PMSF and 1 $\mu\text{g}/\text{ml}$ aprotinin). Lysates were cleared of insoluble material by centrifugation (16 000 *g*) for 5 min and then incubated for 1 h at 4 °C with Protein A-Sepharose beads. The cleared lysate was then incubated with the Rab6 antibody (2 μg) bound to Protein A-Sepharose beads for 2 h at 4 °C. Precipitates were washed twice in a high-salt solution (10 mM Tris, pH 8, 500 mM NaCl, 0.5% Triton X-100, 0.5% deoxycholate and 0.05% SDS) and once in a lower-salt solution (as above, but with 150 mM NaCl). An enhanced chemiluminescence system (Amersham, Little Chalfont, Bucks., U.K.) was used to detect protein by Western blotting. Relative protein levels in the immunoprecipitations were measured with the NIH Image program (developed by the United States' National Institutes of Health and available on the

Worldwide Web at <http://rsb.info.nih.gov/nih-image/>) on a Macintosh 7100/80 computer. Western-blot films (linear range of exposure) were scanned on a Howtek Scanmaster 3+ equipped with an adapter for transparent media.

PKC phosphorylation of Rab6

Purified PKC (25 ng; mix of α , β and γ rat brain isoforms) was incubated with immunoprecipitated platelet Rab6 or recombinantly expressed Rab6C (molar ratio of 1:57) for 1 h at 30 °C in a buffer recommended by the supplier (Upstate Biotechnology; 20 mM Mops, pH 7.2, 25 mM β -glycerophosphate, 13 mM MgCl_2 , 1 mM NaF, 1 mM dithiothreitol, 1 mM CaCl_2 , 4 mM ATP, 0.1 mg/ml phosphatidylserine and 0.1 mg/ml diacylglycerol). Phosphorylation efficiency was estimated by quantification of phosphorimages of PKC-phosphorylated Rab6C after SDS/PAGE analysis by comparison with phosphorimages of known molar quantities of [^{32}P]ATP. This method yielded an estimate of 0.51 ± 0.07 mol of phosphate incorporated/mol of total protein (mean $\pm$ S.D., $n = 4$), as determined by the Bradford assay (Bio-Rad, Richmond, CA, U.S.A.), and 1.7–2.0 mol of phosphate/mol of active protein (as determined by GDP or GTP saturation-binding studies, see below).

Nucleotide-binding and GTPase assays

To assay solution binding of [8,5- ^3H]GTP and [8,5- ^3H]GDP (25 Ci/mmol; Dupont NEN) by phosphorylated Rab6C, 25 μl of phosphorylated protein was diluted to 40 μl in nucleotide-binding buffer (50 mM Hepes, pH 7.4, 150 mM NaCl, 8 mM MgCl_2 , 1 mM EDTA, 1 mM dithiothreitol and 0.1% BSA). The final concentration of Rab6C in the reaction mixture was 400 nM. Specific binding was assessed as described in [23] by spotting aliquots of the reaction mixture on Millipore filters (Millipore, Bedford, MA, U.S.A.) and washing the filters in cold binding buffer, followed by scintillation counting. Rab6C complex stability in the filter assay was determined by Sephadex G-25 filtration (PD-10 Pharmacia column) as described previously [23]. In our studies, the stability of GDP complexes was 71%, which was identical with that reported for Rab9, whereas the stability of GTP complexes in our experiments was 84%, compared with a reported value of 75% [23]. The conditions for maximal stable binding of GTP and GDP to Rab6C were established by investigating the influence of incubation time (2, 4, 6, 8 and 24 h) and temperature (4, 25 and 37 °C) on binding. Estimates for the dissociation constants and number of binding sites were derived using the EBDA and Ligand, version 2.0, programs from Biosoft (Milltown, NJ, U.S.A.). By these estimates the preparation bound 0.25 mol of GTP/mol of Rab6C and 0.3 mol of GDP/mol of Rab6C, determined by a total protein assay. For GTP-overlay assays, Rab6C that had been transferred to nylon membranes after SDS/PAGE was incubated in 50 mM sodium phosphate, pH 7.0, 5 mM MgCl_2 , 1 mM ethylene glycol-bis (β -aminoethyl ether)- N,N,N',N' -tetraacetic acid, 2 μM ATP and 0.3% Tween-20 for 6 h at room temperature. 5'-(γ -thio)-GTP (1000 Ci/mmol) was then added to a concentration of 2.5 μM and the mixture was incubated overnight at room temperature. The GTPase activity of Rab6C was assayed by the charcoal-separation assay [24].

RESULTS

In intact activated platelets, Rab6 is phosphorylated by a PKC-dependent mechanism

We investigated whether phosphorylation of Rab6 in platelets treated with thrombin depended on activation of PKC. Gel-

Figure 1 Thrombin-induced phosphorylation of Rab6 proceeds through activated PKC

Platelets, metabolically labelled with [^{32}P]orthophosphate, were activated with thrombin (1 unit/ml), thrombin (1 unit/ml) in the presence of Ro-31-8220 (10 μM), thrombin (1 unit/ml) in the presence of PD-169316 (10 μM), or PMA (100 nM) in the presence of hirudin (0.1 unit/ml). Rab6 was immunoprecipitated, separated by SDS/PAGE and transferred to PVDF membranes. The three panels show immunoprecipitated Rab6 protein (top panel), the phosphorylation signal of the precipitated protein (middle panel) and the level of pleckstrin phosphorylation in the crude lysates (bottom panel). The left three lanes and the right three lanes are from two independent experiments. Detection of immunoprecipitated protein was by enhanced chemiluminescence. Phosphorylated Rab6 and pleckstrin were detected by phosphorimaging. Migration of molecular-mass markers is indicated on the left.

filtered platelets were labelled metabolically with [^{32}P]orthophosphate and treated with thrombin, thrombin in the presence of Ro-31-8220 (a specific inhibitor of PKC), or thrombin in the presence of PD-169316 (a specific inhibitor of p38 MAP kinase). In thrombin-treated platelets, Rab6 was phosphorylated (Figure 1, lane 1, middle left panel). The specificity of the PKC inhibition by Ro-31-8220 was confirmed by analysing the phosphorylation of pleckstrin (a known substrate of activated PKC). Ro-31-8220 inhibited 70% of the pleckstrin phosphorylation signal, whereas PD-169316 was without effect (Figure 1, bottom left panel). Blocking PKC activity by Ro-31-8220 inhibited thrombin-induced phosphorylation of Rab6 by 50% (Figure 1, lane 2, middle left panel, normalized for the amount of immunoprecipitated Rab6), whereas the p38 MAP kinase inhibitor PD-169316 did not affect the level of Rab6 phosphorylation (Figure 1, lane 3, middle left panel).

The phorbol ester PMA, a direct activator of PKC and platelet secretion, was used to demonstrate further the relation between platelet activation, PKC activity and Rab6 phosphorylation. Thrombin induced the phosphorylation of Rab6 and Ro-31-8220 inhibited it (by 70%; Figure 1, middle right panel, lanes 1 and 2). PMA also induced the phosphorylation of Rab6 (Figure 1, lane 3, middle right panel, 194% of the thrombin signal) despite the presence of hirudin, which blocks any thrombin that may be generated as a result of platelet activation and granule secretion. As before, the phosphorylation of pleckstrin (Figure 1, bottom right panel) paralleled Rab6 phosphorylation, confirming the activity of PKC and its inhibition by Ro-31-8220.

Platelet Rab6 is a direct substrate for PKC

Because PKC activation leads to Rab6 phosphorylation, we next tested whether Rab6 could serve as a direct substrate of PKC. Nonidet P40 lysates from resting platelets were first immunoprecipitated with non-immune rabbit IgG or affinity-purified rabbit anti-Rab6 antibody. The washed immunoprecipitates were

Figure 2 Platelet Rab6 is a substrate of PKC

Nonidet P40 lysates from resting platelets were immunoprecipitated with non-immune rabbit IgG or affinity-purified anti-Rab6 antibody. Immunoprecipitated proteins were incubated with purified PKC and [γ - 32 P]ATP, separated by SDS/PAGE and transferred to PVDF membranes. Top panel, Rab6 protein in the immunoprecipitates as detected by immunoblotting with anti-Rab6 antibody. Bottom panel, phosphorylation of immunoprecipitated proteins as detected by phosphorimaging.

then incubated with purified PKC in the presence of [γ - 32 P]ATP and lipid activators. After separation by SDS/PAGE and transfer to nylon membranes, the immunoprecipitated proteins were analysed by immunoblotting with Rab6 antibody (Figure 2, top panel, Rab6 protein) and phosphorimaging (Figure 2, bottom panel, phosphorylated Rab6). The immunoblot detected Rab6 only in the anti-Rab6 precipitation, and the phosphorimage indicated that the Rab6 could be phosphorylated by PKC. In the control IgG precipitation, no phosphorylated protein was detected at the molecular mass of Rab6 (24 kDa), indicating that the phosphorylated protein was indeed Rab6 and not a non-specifically precipitated protein.

Human platelets express a Rab6 variant (Rab6C) that maintains conserved PKC consensus phosphorylation sites

In order to assess functional effects of phosphorylation we cloned and expressed Rab6 as a His-tagged bacterial protein. Initially, mRNA harvested from the human DAMI megakaryocyte-like cell line was used as the template for RT-PCR with Rab6-specific primers [25]. Sequence analysis of the cloned product confirmed that it coded for the complete open reading frame of Rab6. However, beginning at nucleotide 184 (relative to the initiation ATG codon), the DAMI sequence diverged from the reported sequence of human Rab6 for 96 bp. In this region of divergence, the megakaryocyte Rab6 was only 68% identical with the reported nucleotide sequence of Rab6 cloned from a human pheochromocytoma library [21]. The remaining 447 bp were 100% identical. The divergent sequence of Rab6C was used in a search of the GenBank database. The sequence was found to be 100% identical with human expressed sequence tags (THC125791 contig, TIGR database), independently verifying that Rab6C is expressed in the human genome.

RT-PCR was used to quantify the relative expression of Rab6C in human platelets. A product of the expected size was obtained and cloned. Restriction-enzyme analysis (Rab6C contains a unique *Pst*I site) and the sequences of nine clones showed eight to be Rab6C, and one to be Rab6A. The expression level of Rab6C in human platelet RNA was compared with RNA from DAMI cells and total human brain tissue by *Pst*I digestion of the respective 32 P-end-labelled PCR products (Figure 3A). Quantification of the results showed that 93% of the human platelet PCR products contained the *Pst*I sequence, whereas 33 and 41% of the DAMI and brain sequences contained the *Pst*I site, respectively (Figure 3A). To verify the purity of our platelet RNA preparation we used primers specific for the monocyte/

Figure 3 Human platelets predominately express Rab6C

(A) Analysis of Rab6 isoform expression levels by *Pst*I digestion. 32 P-labelled Rab6-specific PCR products from human platelet RNA, DAMI RNA and human brain-tissue RNA (control and *Pst*I-digested) were separated by agarose-gel electrophoresis and quantified by phosphorimaging. The top panels show the phosphorimages, and the graph shows the percentages of products containing the *Pst*I site carried by the Rab6C variant, as determined from two independent digests (means $\pm$ S.D.). (B) Demonstration that the platelet RNA preparation did not support amplification of the monocyte/macrophage-specific gene product CD-68. Equivalent amounts of RNA from human platelets and the human monocyte/macrophage U937 cell line were subjected to RT-PCR using CD-68-specific primers. Lane 1, DNA markers; lane 2, negative control (complete reaction with no added RNA); lane 3, platelet RNA reaction; lane 4, U937 monocyte/macrophage reaction.

macrophage CD-68 gene product in RT-PCR reactions [22]. Figure 3(B) shows that the human platelet mRNA did not support amplification of a CD-68-specific product, whereas RNA from the human U937 monocyte/macrophage line did support amplification of a specific product. These results indicate that human platelets predominately express the Rab6C isoform, but that the expression of Rab6C is not restricted to the megakaryocyte/platelet lineage.

The divergent nucleotide sequence introduces three changes in amino acid residues flanking the second GTP-binding domain of Rab6C. Figure 4 shows the alignment of the amino acid sequences of human Rab6A and Rab6C (huRab6A, huRab6C), *Drosophila* Rab6 (drosRab6), yeast Rab6 homologues (Rhy1, YPT6) and the partial reported sequences of mouse and *Caenorhabditis elegans* Rab6 (mRab6 and C.eleRab6). The differences in amino acid sequences between human Rab6A and Rab6C are indicated by '+' and the potential PKC phosphorylation sites are underlined. The first PKC site (PKC-I) just upstream of the second GTP-binding region is conserved in yeast, *C. elegans*, *Drosophila*, mice and humans. It is noteworthy that Rab6C maintains this consensus PKC site in spite of an isoleucine $\rightarrow$ valine substitution.

				GTPBind-I		

	1					50
huRab6A	MSTGGDFGNP	LRKFKLVFLG	EQSVGKTSLI	TRFMYDSFDN	TYQATIGIDF	
huRab6C	MSTGGDFGNP	LRKFKLVFLG	EQSVGKTSLI	TRFMYDSFDN	TYQATIGIDF	
drosRab6	.MSSGDFGNP	LRKFKLVFLG	EQSVGKTSLI	TRFMYDSFDN	TYQATIGIDF	
Rhy1	..MSENYSFS	LRKFKLVFLG	EQSVGKTSLI	TRFMYDQFDN	TYQATIGIDF	
Ypt6	...MSRSGKS	LTKYKIVFLG	EQGVGKTSLI	TRFMYDTFDD	HYQATIGIDF	
mRab6	MSAGGDFGNP	LRKFKLVFLG	EQSVGKTSLI	TRFMYDSFDN	TYQATIGIDF	
C.eleRab6	...MSDFGNP	LKKFELVFLG	EQSVGKTSLI	TRFMYDSFDN	TYQATIGIDF	
		PKC-I	GTPBind-II			

	51					100
huRab6A	LSKTMYLEDR	<u>TVRLQLWDTA</u>	QGERFRSLIP	SYIRDSTVAV	VVYDITNVNS	
huRab6C	LSKTMYLEDR	<u>TIRLQLWDTA</u>	QGERFRSLIP	SYIRDSAAV	VVYDITNVNS	
drosRab6	LSKTMYLEDR	<u>TVRLQLWDTA</u>	QGERFRSLIP	SYIRDSTVAV	VVYDITNVNS	
Rhy1	LSKTMYLEDR	<u>TVRLQLWDTA</u>	QGERFRSLIP	SYIRDSVAI	IVYDITNVNS	
Ypt6	LSKTMYLEDR	<u>TIRLQLWDTA</u>	QGERFRSLIP	SYIRDSVAI	IVYDITNRKS	
mRab6	LSKTMYLEDR	<u>TIRLQLWDTA</u>	QGE			
C.eleRab6	LSKTMYLEDR	<u>TVRLQLWDTA</u>				
			GTPBind-III			
		PKC-II	*****			
	101					150
huRab6A	FQOTTKWIDD	VTERGSDVI	IM.LVGNKTD	LADKRQVSIE	EGERKAKELN	
huRab6C	FQOTTKWIDD	VTERGSDVI	IM.LVGNKTD	LADKRQVSIE	EGERKAKELN	
drosRab6	FHQTSKWIDD	VTERGSDVI	IM.LVGNKTD	LSDKRQVSTE	EGERKAKELN	
Rhy1	FVNTEKWIED	VRAERGDDVI	IV.LVGNKTD	LADKRQVTQE	EGEKKAKELK	
Ypt6	FEYIDKWIED	VKNERGDENV	ILCIVGNKSD	LSDERQISTE	EGEKKAKLLG	
mRab6						
C.eleRab6						
	151					200
huRab6A	V.MFIETSAK	AGYNVQLFR	RVAAALPGME	STQ.....	DRSRE	
huRab6C	V.MFIETSAK	AGYNVQLFR	RVAAALPGME	STQ.....	DRSRE	
drosRab6	V.MFIETSAK	AGYNVQLFR	RVAAALPGMD	STE.....	NKPSE	
Rhy1	I.MHMETSAT	AGHNKLLFR	KIAQMLPGME	NVE.....	TQS.T	
Ypt6	AKIFMETSTK	AGYNVQLFK	KIAKSLPEFQ	NSESTPLDSE	NANSANQNKP	
mRab6						
C.eleRab6						
	201		223			
huRab6A	DMIDIKLEKP	QEQQVSEGGC	SC.			
huRab6C	DMIDIKLEKP	QEQQVSEGGC	SC.			
drosRab6	DMQEVVLKDS	PNETKDPEGG	CAC			
Rhy1	QMIDVSIQPN	ENESSCNC..	...			
Ypt6	GVIDISTAE	QEQSACQC..	...			
mRab6						
C.eleRab6						

Figure 4 Rab6 contains highly conserved consensus sites for PKC phosphorylation

Alignment of amino acid sequences of human Rab6A and Rab6C (huRab6A and huRab6C), *Drosophila melanogaster* Rab6 (drosRab6), Rhy1 (yeast Rab6 homologue), Ypt6 (yeast Rab6 homologue), mouse Rab6 (mRab6, partial sequence) and *Caenorhabditis elegans* Rab6 (C.eleRab6, partial sequence). + indicates amino acid differences between Rab6A and Rab6C. Underlined residues are consensus sites for PKC phosphorylation. The conserved residues of the GTP-binding domains are indicated by underlined asterisks.

Human Rab6 contains a second PKC site (PKC-II) near the third GTP-binding domain that is maintained in the *Drosophila* homologue, but not in the yeast homologues. Whether the second site is maintained in other mammalian homologues or *C. elegans* is not known because the available sequence is incomplete. The yeast Ypt6 homologue has an additional site at the N-terminus that is unique. Of the other Rab members analysed for potential PKC phosphorylation sites (Rabs 1–32, Nentrez search as of 17 February 1999), Rab1 and Rab11 were found to contain conserved sites homologous to PKC site I in Rab6. Human Rab13 contains a PKC site in a position near to, but not identical with, that of PKC site II in Rab6.

Rab6C expressed in bacteria was detected by the anti-Rab6 antibody and bound GTP, as assessed by the overlay assay (Figure 5A). The Rab6C protein purified with the Ni-NTA affinity resin by specific elution at pH 4.7 (Figure 5B, lane 6) and 4.6 (Figure 5B, lane 8) was estimated to be 90% pure by Coomassie Brilliant Blue staining. It was dialysed against an EDTA-containing buffer to reduce bound nucleotide, and used in the experiments described below [24,26].

PKC phosphorylation of Rab6C: effects on GTP/GDP binding

We first confirmed that PKC phosphorylated the Rab6C isoform by incubating purified PKC and [γ - 32 P]ATP with purified Rab6C

(Figure 5C). Estimation of the reaction efficiency indicated that approx. 2 mol of phosphate were incorporated per mol of active protein (see the Experimental section). Since the predicted PKC-phosphorylation sites in Rab6C are immediately upstream of GTP-binding domains, we tested whether PKC phosphorylation of Rab6C altered its nucleotide-binding activities. Purified Rab6C bound GTP and GDP in a saturable manner, with maximal equilibrium binding occurring after an 8-h incubation at 37 °C (results not shown). In saturation-binding studies the GTP and GDP dissociation constants for non-phosphorylated Rab6C were 119 ± 27 and 113 ± 12 nM, respectively (Figures 6A and 6B). For phosphorylated Rab6C the GTP and GDP dissociation constants were 38 ± 5 and 70 ± 9 nM, respectively. There was no significant difference between non-phosphorylated samples and phosphorylated samples in the number of binding sites for GTP (87 ± 17 versus 99 ± 9 nM) and GDP (110 ± 6 versus 111 ± 9 nM). In both experiments the extent of phosphorylation was 2 mol of phosphate/mol of active Rab6C, and the 8-h incubation at 37 °C did not diminish the level of phosphorylation (see the Experimental section, and results not shown). These studies show that phosphorylation increased the affinity of Rab6C for GTP by greater than 3-fold and increased the affinity for GDP to a lesser extent (1.6-fold).

We also tested whether phosphorylation altered the GTPase activity of Rab6C (Figure 6C). The GTP-hydrolysis rate for both

Figure 5 Recombinant expression, purification and PKC phosphorylation of Rab6C

(A) SDS-extracted bacterial proteins from JM109 bacteria carrying a Rab6C plasmid (uninduced or induced with isopropyl β -D-thiogalactoside, IPTG) were separated on SDS/polyacrylamide (12%) gels. The gels were stained with Coomassie Brilliant Blue dye (left panel) or transferred on to PVDF membranes for immunoblotting for Rab6 (middle panel) and ligand blotting with [γ - 35 S]GTP (right panel). (B) Coomassie Brilliant Blue-stained SDS gel of the purification of recombinant Rab6C using Ni-NTA resin; lanes 1 and 7, molecular-mass markers; lane 2, total soluble protein; lane 3, flow-through; lane 4, wash (pH 8.0); lane 5, elution (pH 4.9); lane 6, elution (pH 4.7); lane 8, elution (pH 4.6). (C) PKC phosphorylation of Rab6C with [γ - 32 P]ATP. Rab6C was separated by SDS/PAGE. Shown is the Coomassie Brilliant Blue-stained gel (left panel) and a phosphorimage of the gel (right panel). The phosphorylation efficiency was estimated to be 2 mol of phosphate/mol of active Rab6C.

Rab6C and phosphorylated Rab6C was estimated to be $100 \pm 15 \mu\text{mol}$ of P_i /mol of Rab6C per min. In these experiments, Rab6 phosphorylation stoichiometry was 2 mol of phosphate/mol of active Rab6C.

Human platelets express GDI- α , and during activation the fraction of cytosolic Rab6 is increased by a PKC-dependent process

Altered nucleotide-binding affinities of phospho-Rab6C suggest that phosphorylation of Rab6 may modulate its cycling between

Figure 6 GTP/GDP binding and GTPase activity of Rab6C and PKC-phosphorylated Rab6C

(A) Rab6C ($\square$) and PKC-phosphorylated Rab6C ($\blacksquare$) were incubated with increasing concentrations of [$8,5$ - ^3H]GTP for 8 h at 37°C . Bound counts were assessed using a filter assay. Background binding of filters and PKC alone is indicated (Control), which was not different from filters alone. (B) As for (A) except that [$8,5$ - ^3H]GDP was assessed. Values are the means $\pm$ S.E.M. of triplicate measurements. Results are representative of three independent experiments. (C) GTPase activity of PKC-phosphorylated ($\blacktriangle$) and non-phosphorylated ($\square$) Rab6C. Rab6C (400 nM) was incubated in the presence of [γ - 32 P]GTP ($3 \mu\text{M}$) at 37°C . At the indicated times, 40- μl aliquots were removed and the amount of hydrolysed phosphate was determined by charcoal separation. Incubations with PKC alone served as control for non-specific hydrolysis. Results are representative of two independent experiments. For all three experiments, the means $\pm$ S.E.M. of Rab6C-specific nucleotide binding or hydrolysis are shown.

membrane-bound and cytosolic states during activation. First we determined whether platelets express GDI, since in neurons this Rab accessory protein has been shown to mediate the cycling of Rab3A/C on and off the membranes of neurotransmitter vesicles during calcium-stimulated exocytosis [27]. Immunoblotting of human platelet lysates with an anti-GDI antibody showed two bands of approx. 50 kDa, which is consistent with expression of both GDI- α (50.5 kDa) and GDI- β (49.8 kDa). To confirm GDI expression in human platelets, RT-PCR of platelet RNA (with degenerate primers that would amplify the first 240 bp of the open reading frames of GDI- α or GDI- β) yielded nine cloned PCR products, all of which coded for GDI- α when sequenced.

Next we developed an assay to quantify the amount of cytosolic Rab6 in resting and activated platelets. Unstimulated gel-filtered

Figure 7 Rab6 is recruited to the cytosol during platelet activation

(A) A quantitative immunoblot of Rab6C. Purified Rab6C was separated by SDS/PAGE, transferred to PVDF membranes and detected using a primary anti-Rab6 antibody and [125 I]Protein A. (B) A graph of the immunoblot in (A) demonstrating the linear relation between mass of Rab6C (ng) and immunoreactivity of anti-Rab6 antibody (detected by [125 I]Protein A and phosphorimaging). Error bars represent the S.E.M. of the duplicate samples. (C) Cellular translocation of Rab6. Samples of gel-filtered platelets (4.3×10^6) were pretreated with Ro-31-8220 (10 μ M) for 10 min at 37 $^{\circ}$ C as indicated and then treated with the following agents for 1 min at 37 $^{\circ}$ C: EDTA (1 mM, Control), thrombin (1 unit/ml) or PMA (100 nM). The platelets were separated into cytosolic and particulate fractions, and volumes of the fractions [adjusted to fall within the range of the Rab6C standard curves run in parallel, see (B) above] were separated by SDS/PAGE (12% gel), transferred on to nylon membranes and probed for Rab6 as in (A). The percentages of Rab6 molecules in the platelet cytosol are shown ($\pm$ S.E.M. of four samples from two independent experiments).

platelets, or platelets activated with thrombin (in the presence or absence of the PKC inhibitor Ro-31-8220) or with PMA were lysed by mild sonication in a Hepes/sucrose buffer and separated into cytosolic and crude membrane/particulate fractions by ultracentrifugation (as described in the Experimental section). To measure the amount of Rab6 in the isolated fractions we

performed quantitative immunoblotting using known amounts of purified Rab6C to generate linear standard curves that related immunoreactivity to the mass (ng) of Rab6C (Figures 7A and 7B). Samples of the platelet fractions were immunoblotted in parallel, adjusting the volumes of the cytosolic and particulate fractions such that the amount of platelet Rab6 fell within the range of the standard curve. Treatment with thrombin nearly doubled the fraction cytosolic Rab6 relative to resting platelets (Figure 7C, control versus thrombin). Pretreatment of platelets with the PKC inhibitor Ro-31-8220, before thrombin stimulation, attenuated the thrombin-induced recruitment of Rab6 to the cytosol. Stimulation of platelets with the PKC activator PMA again nearly doubled the amount of cytosolic Rab6. Analysis of variance showed that the thrombin and PMA treatments significantly increased the percentage of cytosolic Rab6 as compared with the matched control samples ($P < 0.005$ in both cases), whereas the samples pretreated with Ro-31-8220 were not significantly different from the matched control samples ($P = 0.99$), but did differ from the samples treated with thrombin alone ($P < 0.005$).

DISCUSSION

Platelets and other secretory cells including neurons, polarized epithelial cells and pancreatic β -cells are able to regulate secretion in response to extracellular signals [28–30]. In platelets, as with other secretory cells, the intracellular signalling pathways that lead to exocytosis involve PKC activation [4,6,7], but the direct linkage between PKC activity and molecules involved in secretion remains poorly understood. In this study, we found that, in intact thrombin-stimulated platelets, Rab6 phosphorylation depended largely on activation of PKC. This conclusion is based on the observations that PMA induces Rab6 phosphorylation and that the thrombin-induced phosphorylation of Rab6 was inhibited up to 70% in the presence of the PKC inhibitor Ro-31-8220 but not by the p38 MAP kinase inhibitor PD-169316. That PD-169316 did not affect Rab6 phosphorylation argues for the specificity of the inhibition by Ro-31-8220, since p38 MAP kinase is expressed in platelets and is maximally activated after 1 min of thrombin stimulation, the time frame used in our experiments [31]. Moreover, the phosphorylation of immunoprecipitated platelet Rab6 with purified PKC indicates that Rab6 may be directly phosphorylated by PKC during platelet activation and degranulation.

To explore further the PKC phosphorylation of Rab6 we cloned and characterized a novel Rab6 variant, which is the major form expressed in human platelets. We termed this isoform Rab6C because a partial cDNA for another Rab6 isoform (Rab6B) has recently been cloned from human melanoma cells [32]. Rab6C and Rab6A are distinct from Rab6B and are natural variants, since we confirmed their expression in RNA from both human platelets and brain tissue. Rab6C sequence diverges from Rab6A within the PKC phosphorylation site I, but the site is maintained as a consensus sequence despite an isoleucine $\rightarrow$ valine substitution. Of the two PKC sites in human Rab6, PKC site I has been strongly conserved as a consensus site in a variety of eukaryotic organisms, including yeast, nematodes, *Drosophila*, mice and humans. Although PKC site II is not maintained in the yeast Rab6 homologues, it is conserved in *Drosophila* and thus may also be functionally important.

Our experiments demonstrate that Rab6C is a functional isoform with GTP/GDP-binding affinities and GTPase activity that are very similar to that reported for unmodified Rab6A [26]. Phosphorylation of Rab6C by PKC increased the affinity of the protein for GTP and GDP, but did not affect the intrinsic

GTPase activity of the molecule. The recent crystal structure of Rab3A with its effector molecule rabphilin-3A suggests how phosphorylation of Rab6C by PKC may alter nucleotide-binding affinities through longer-range allosteric effects [33]. Threonine-60 of PKC site I is predicted to reside at the beginning of β -strand 3 and is not in the immediate topological vicinity of the GDP/GTP-binding domain. However, PKC site I and β -strand 3 are in close topological contact with α -helix 2, which tethers the switch region II loop, a domain involved in nucleotide binding and metal-ion chelation. We speculate that phosphorylation of threonine-60 may alter nucleotide binding by influencing switch region II through effects on β -strand 3 and α -helix 2.

The altered nucleotide binding of Rab6C, induced by PKC, may also affect Rab6's interactions with accessory and effector molecules. In particular, the interactions of Rab6 with GDI may be altered since the highly conserved PKC site I resides in sequences critical to the functional interaction of Rab6 with GDI [34,35]. That PKC phosphorylation of platelet Rab6 modulates such an interaction seems plausible since platelets express GDI- α and phosphorylation of Rab6 is associated with a partial translocation of Rab6 to the cytosol, an effect expected to be mediated by GDI. The translocation of Rab6 during degranulation parallels the cycling of Rab3A/C on and off the membranes of neurotransmitter vesicles during calcium-stimulated exocytosis [27]. GDI-mediated extraction of Rab proteins in the GDP-bound state has been proposed as a mechanism that allows Rab proteins to participate in multiple rounds of membrane recruitment and GTP-dependent effector-molecule interaction [36]. Unfortunately, nothing is known about Rab6 effector molecules in platelets. However, the recently identified effector molecule, rabkinesin-6, which binds microtubules and Rab6 in a GTP-dependent fashion, suggests the hypothesis that Rab6 in platelets may be involved in granule centralization and microtubule reorganization, which are prominent features of platelet exocytosis [37,38].

We are grateful for the editorial assistance of Thomas McVarish and Janos Polgar, and thank Jose Alberto Betances for his help in developing the nucleotide-binding assays. This work was supported in part by National Institutes of Health grants R01 HL57314 and HL57301 to G. L. R. and by a grant from Bristol-Myers Squibb to Harvard School of Public Health.

REFERENCES

- Gordon, J. L. (1976) *Platelets in Biology and Pathology*, North Holland Publishing, New York
- Ishihara, H., Connolly, A. J., Zeng, D., Kahn, M. L., Zheng, Y. W., Timmons, C., Tram, T. and Coughlin, S. R. (1997) *Nature (London)* **386**, 502–506
- Vu, T. K., Hung, D. T., Wheaton, V. I. and Coughlin, S. R. (1991) *Cell* **64**, 1057–1068
- Gerrard, J. M., McNicol, A. and Saxena, S. P. (1993) *Biochem. Soc. Trans.* **21**, 289–293
- White, J. G., Rao, G. H. and Estensen, R. D. (1974) *Am. J. Pathol.* **75**, 301–314
- Zucker, M. B., Troll, W. and Belman, S. (1974) *J. Cell Biol.* **60**, 325–336
- Gerrard, J. M., Beattie, L. L., Park, J., Israels, S. J., McNicol, A., Lint, D. and Cragoe, Jr., E. J. (1989) *Blood* **74**, 2405–2413
- Shattil, S. J. and Brass, L. F. (1987) *J. Biol. Chem.* **262**, 992–1000
- Niyya, K., Hodson, E., Bader, R., Byers-Ward, V., Koziol, J. A., Plow, E. F. and Ruggeri, Z. M. (1987) *Blood* **70**, 475–483
- Reed, G., Hough, A. and Fitzgerald, M. (1999) *Blood* **93**, 2617–2626
- Lemons, P. P., Chen, D., Bernstein, A. M., Bennett, M. K. and Whiteheart, S. W. (1997) *Blood* **90**, 1490–1500
- Flaumenhaft, R., Croce, K., Chen, E., Furie, B. and Furie, B. (1999) *J. Biol. Chem.* **274**, 2492–2501
- Sudhof, T. C. (1995) *Nature (London)* **375**, 645–653
- Rothman, J. E. (1994) *Nature (London)* **372**, 55–63
- Polgar, J. and Reed, G. (1999) *Blood*, in the press
- Schimmoller, F., Simon, I. and Pfeffer, S. R. (1998) *J. Biol. Chem.* **273**, 22161–22164
- Novick, P. and Zerial, M. (1997) *Curr. Opin. Cell Biol.* **9**, 496–504
- Fischer von Mollard, G., Stahl, B., Li, C., Sudhof, T. C. and Jahn, R. (1994) *Trends Biochem. Sci.* **19**, 164–168
- Shapiro, A. D. and Pfeffer, S. R. (1995) *J. Biol. Chem.* **270**, 11085–11090
- Karniguan, A., Zahraoui, A. and Tavittian, A. (1993) *Proc. Natl. Acad. Sci. U.S.A.* **90**, 7647–7651
- Zahraoui, A., Touchot, N., Chardin, P. and Tavittian, A. (1989) *J. Biol. Chem.* **264**, 12394–12401
- Holness, C. L. and Simmons, D. L. (1993) *Blood* **81**, 1607–1613
- Shapiro, A. D., Riederer, M. A. and Pfeffer, S. R. (1993) *J. Biol. Chem.* **268**, 6925–6931
- Kabcenell, A. K., Goud, B., Northup, J. K. and Novick, P. J. (1990) *J. Biol. Chem.* **265**, 9366–9372
- Greenberg, S. M., Rosenthal, D. S., Greeley, T. A., Tantravahi, R. and Handin, R. I. (1988) *Blood* **72**, 1968–1977
- Yang, C., Mollat, P., Chaffotte, A., McCaffrey, M., Cabanie, L. and Goud, B. (1993) *Eur. J. Biochem.* **217**, 1027–1037
- Fischer von Mollard, G., Stahl, B., Khokhlatchev, A., Sudhof, T. C. and Jahn, R. (1994) *J. Biol. Chem.* **269**, 10971–10974
- Eliasson, L., Renstrom, E., Ammala, C., Berggren, P. O., Bertorello, A. M., Bokvist, K., Chibalin, A., Deeney, J. T., Flatt, P. R., Gabel, J. et al. (1996) *Science* **271**, 813–815
- Cardone, M. H., Smith, B. L., Song, W., Mochly-Rosen, D. and Mostov, K. E. (1994) *J. Cell Biol.* **124**, 717–727
- Coffey, E. T., Sihra, T. S. and Nicholls, D. G. (1993) *J. Biol. Chem.* **268**, 21060–21065
- Kramer, R. M., Roberts, E. F., Striffler, B. A. and Johnstone, E. M. (1995) *J. Biol. Chem.* **270**, 27395–27398
- Chen, D., Guo, J. and Gahl, W. A. (1997) *Biochim. Biophys. Acta* **1355**, 1–6
- Ostermeier, C. and Brunger, A. (1999) *Cell* **96**, 363–374
- Beranger, F., Cadwallader, K., Porfiri, E., Powers, S., Evans, T., de Gunzburg, J. and Hancock, J. F. (1994) *J. Biol. Chem.* **269**, 13637–13643
- Schalk, I., Zeng, K., Wu, S. K., Stura, E. A., Matteson, J., Huang, M., Tandon, A., Wilson, I. A. and Balch, W. E. (1996) *Nature (London)* **381**, 42–48
- Rybin, V., Ullrich, O., Rubino, M., Alexandrov, K., Simon, I., Seabra, C., Goody, R. and Zerial, M. (1996) *Nature (London)* **383**, 266–269
- Echard, A., Jollivet, F., Martinez, O., Lacapere, J. J., Rousselet, A., Janoueix-Lerosey, I. and Goud, B. (1998) *Science* **279**, 580–585
- Morgenstern, E. (1997) in *Platelets and their Factors*, vol. 126 (von Bruchhausen, F. and Walter, U., eds.), pp. 27–52, Springer, Berlin

Received 4 March 1999/25 May 1999; accepted 15 June 1999