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A *ras*-related protein is phosphorylated and translocated by agonists that increase cAMP levels in human platelets

(*ras*-encoded protein antibodies/G proteins/cAMP-dependent protein kinase/platelet activation/signal transduction)

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ABSTRACT **The antigenicity of platelet proteins was assayed against various monoclonal antibodies (mAbs) that recognize specific epitopes of the *ras*-encoded p21 protein. mAb M90, which detects the region of p21 protein within amino acids 107–130 and inhibits its GTP-binding activity, strongly reacted with a 22-kDa protein present in the particulate fraction of human platelets. Other mAbs against *ras*-encoded proteins, including Y13-259, which efficiently detects *ras* proteins from a variety of organisms, did not recognize the platelet 22-kDa protein. Transfer of the platelet 22-kDa protein to nitrocellulose paper showed that the protein binds [α-^{32}P]GTP. Moreover, preincubation of the transferred protein with mAb M90 drastically reduced its GTP-binding activity. Treatment of platelets with iloprost, a prostacyclin analog, caused (*i*) a time-dependent increase of a 24-kDa protein that is recognized by mAb M90 in particulate and cytosolic fractions and (*ii*) the gradual decrease of the 22-kDa protein from the particulate fraction. When platelets were labeled with ^{32}P and then treated with iloprost, the 24-kDa protein was found to be phosphorylated. The ^{32}P-labeled 24-kDa protein was specifically immunoprecipitated by mAb M90. These results suggest that appearance of the 24-kDa protein results from phosphorylation of the 22-kDa protein, which shifts its mobility to a higher molecular mass area.**

In the past two years platelets have been shown to possess distinct GTP-binding proteins with molecular masses between 21 and 31 kDa (1–3). These low molecular weight guanine nucleotide-binding regulatory (G) proteins can be electrophoretically transferred from SDS/polyacrylamide gels to nitrocellulose blots, where they are detected by the binding of [α-^{32}P]GTP (2, 3). One of them, a 21-kDa protein, was recently isolated from platelet membranes; however, this protein is not recognized with monoclonal antibody (mAb) Y13-259 (4), which recognizes all known *ras*-encoded p21 proteins (1). Also, this antibody did not cross-react with the other low molecular mass G proteins present in platelets (3). The possible physiological significance of these proteins has not yet been elucidated; they could be correlated with regulation of phospholipase C (2) and, recently, we reported that a platelet *ras*-related protein is phosphorylated by a cAMP-dependent protein kinase (5).

Lacal and Aaronson (6) have developed a series of mAbs that recognize epitopes located at different regions in the Harvey (Ha) *ras*-encoded p21 protein. We have screened platelet proteins against those mAbs and found that a 22-kDa platelet protein is recognized by two of those mAbs. These mAbs, designated M3 and M90, recognize a region of *ras* p21 protein containing residues 116–119, involved in the interaction with the guanine base (7). By contrast the 22-kDa protein was not recognized by the *ras*-specific mAb Y13-259. These results suggest that the 22-kDa platelet protein is a member of the *ras* family. Finally, the 22-kDa protein is a substrate for cAMP-dependent protein kinase. Phosphorylation of the protein affects its mobility on SDS gels and, also, the phosphorylated protein seems to be translocated from the membrane fraction to the cytosol.

EXPERIMENTAL PROCEDURES

Isolation of Human Platelets. Human blood (100–200 ml) was drawn from healthy volunteers, using trisodium citrate (0.38%, wt/vol) as an anticoagulant, and centrifuged at 180 × *g* for 20 min to yield platelet-rich plasma. Platelets were obtained by centrifuging the platelet-rich plasma at 1200 × *g* for 15 min in the presence of prostacyclin (0.1 μg/ml) and were washed twice by centrifugation at 800 × *g* for 10 min in 40 ml of a Hepes-buffered Tyrode's solution (138 mM NaCl/12 mM $NaHCO_3$/0.36 mM NaH_2PO_4/2.9 mM KCl/1 mM $MgCl_2$/1 mM EGTA/10 mM glucose/5 mM Hepes, pH 7.4) containing prostacyclin (0.3 μg/ml). At this stage the platelets were resuspended in cold hypotonic buffer for fractionation. In initial experiments (see Figs. 1 and 2) prostacyclin was used during isolation of platelets. However, in later experiments (see Figs. 3 and 4) we used 5 mM EDTA during centrifugation, instead of prostacyclin, to avoid the stimulation of cAMP-dependent protein kinases. In these experiments, platelet-rich plasma was treated with 1 mM aspirin for 15 min to further prevent platelet activation. If platelets were isolated in the presence of prostacyclin (Fig. 1) a greater percentage of the 24-kDa protein was seen than in platelets that were isolated in the absence of prostacyclin (see Figs. 3 and 4). All experiments presented here represent at least three others that produced similar results.

Fractionation of Platelets. Modification of a published method (8) was used. The platelet pellet was quickly resuspended at 4°C in 1 ml of hypotonic buffer (5 mM Tris·HCl/5 mM EDTA, pH 7.5) and frozen in liquid nitrogen. Five cycles of rapid freezing and thawing lysed the cells, and after centrifugation at 800 × *g* for 10 min to remove any unbroken cells the homogenate was centrifuged at 160,000 × *g* for 15 min in an air-driven microcentrifuge. The resulting supernatant (300 μl), representing the platelet cytosol fraction, was removed. Pellets were washed once and resuspended in 50 μl of Tris/EDTA buffer. Equal amounts (50 μl) of cytosolic and particulate fractions were loaded into the gels (see Figs. 3 and 4).

Protein Separation and Transfer to Nitrocellulose Blots. The proteins (homogenate, cytosol, or particulate) were separated by SDS/PAGE (9) in 11% gels (either 12 or 16 cm long) and then electrophoretically transferred to nitrocellulose paper using the LKB NovaBlot semidry blotting method. The 16-cm gels (see Figs. 3 and 4) gave better separation of the 22- and 24-kDa proteins than did the 12-cm gels (see Fig. 1).

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Abbreviation: mAb, monoclonal antibody.

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GTP Binding to Platelet Proteins. This procedure was done as described (2). The transfer blots were rinsed briefly in binding buffer (50 mM Tris·HCl/0.3% Tween 20/5 mM $MgCl_2$/1 mM EGTA, pH 7.5), and the gels were stained with Coomassie blue to reveal residual proteins. Blots were incubated 60–90 min at 25°C with [α-^{32}P]GTP (1 μCi/ml; specific activity, 2903 Ci/mmol; 1 Ci = 37 GBq) in binding buffer. The blots were rinsed with several changes of binding buffer over 1–2 hr and air-dried. [α-^{32}P]GTP binding was visualized by autoradiography using Kodak XAR-5, BB-5, or DEF-5 x-ray film. The proteins bound to the nitrocellulose paper were stained by using a Janssen Ferri dye kit (Janssen Pharmaceutica, Beerse, Belgium).

Antibody Localization and Immunoprecipitation of a *ras*-Related Protein. Nitrocellulose blots were washed for 90 min with buffer (50 mM Tris/0.2 M NaCl/ bovine serum albumin at 1 mg/ml/polyethylene glycol at 1 mg/ml, pH 7.4) containing an additional 30 mg of bovine serum albumin per ml. The papers were then incubated either for 1 hr at room temperature or overnight at 4°C with different mAbs that recognize *ras* p21 protein. Papers were then washed several times with buffer and incubated with goat anti-mouse IgG, goat anti-rat IgG, or Fc fragment linked to alkaline phosphatase, and the bands were visualized by a phosphatase reaction. In experiments in which platelets were prelabeled with $^{32}P_i$ (10), nitrocellulose blots were subsequently dried and exposed to x-ray film to examine correlation of the immunobands with the phosphorylated proteins. Immunoprecipitation of the platelet protein that is recognized by mAb M90 was done as reported (6, 11). For these experiments cytosolic and particulate fractions were prepared as above, but both fractions were diluted to the same final volume. Therefore, in Fig. 5 the cytosolic fraction contained six times more protein than the particulate fraction when compared with Figs. 3 and 4.

Materials. Prostacyclin was from Wellcome (Beckenham). Iloprost was from Schering (Berlin). Alkaline phosphatase goat anti-mouse IgG, anti-rat IgG, and Fc were from Jackson ImmunoResearch. [α-^{32}P]GTP [tetra(triethylammonium) salt] was from ICN. Other materials were as described (2, 10).

RESULTS AND DISCUSSION

A series of mAbs that recognize different epitopes of the *ras* p21 protein (4, 6) were used to detect *ras*-like proteins in platelet homogenates. Fig. 1 shows strong reaction of mAbs M3, M90, and Y13-259 with a standard of bacterially expressed and purified H-*ras* p21 protein (6). Strong reactivity was observed between mAb M90 and two specific platelet proteins with apparent molecular masses of ≈22–24 kDa. This mAb recognized an epitope within one of the major GTP-binding regions of the *ras* p21 protein between amino acids 107–130 (6). By contrast mAb M3, which recognized an epitope between amino acids 131–152 including residue 144—thought to interact with guanine base (7)—had a slight reaction with the same platelet proteins recognized by mAb M90; mAb Y13-259, which recognizes a region from residue 63–73 (11, 12), did not react with the platelet proteins (Fig. 1). In addition, other mAbs raised against *ras* p21, protein such as mAbs M8 and M9 (6), which recognized the region of amino acids 23–69 of *ras* p21 protein, and mAb M70, which recognized the region between amino acids 89–106, did not react with platelet proteins (data not shown). Taken together, this information indicates that the 22- to 24-kDa proteins from platelets share epitopes that are present within the region of *ras* p21 protein between amino acids 107–152 but do not share epitopes with the *ras* p21 protein region including amino acids 23–106.

The *ras* p21 protein contains several noncontiguous regions related to the binding of GTP (7). These regions are localized around residue 12 (which also constitutes the catalytic site), residue 30 (interacting with the ribose sugar), residue 59 (interacting with the γ phosphate), and residues 116, 117, 119, 120, and 145–147 (which interact with the guanine base). At least two of the epitopes in the Ha-*ras* p21 proteins recognized by mAbs M90 and M3 are also present in the 22- to 24-kDa platelet proteins. We have previously shown that mAbs M3 and M90 efficiently block GTP-binding to *ras* p21 protein (6). Therefore, we investigated whether the platelet proteins recognized by M3 and M90 bind [α-^{32}P]GTP. Several platelet proteins with apparent molecular masses ranging from 21–29.5 kDa have been reported as GTP-binding proteins (1–3). Fig. 2 shows that under conditions in which the *ras* p21 protein binds [α-^{32}P]GTP, some binding of [α-^{32}P]GTP seems associated with the 22-kDa protein recog-

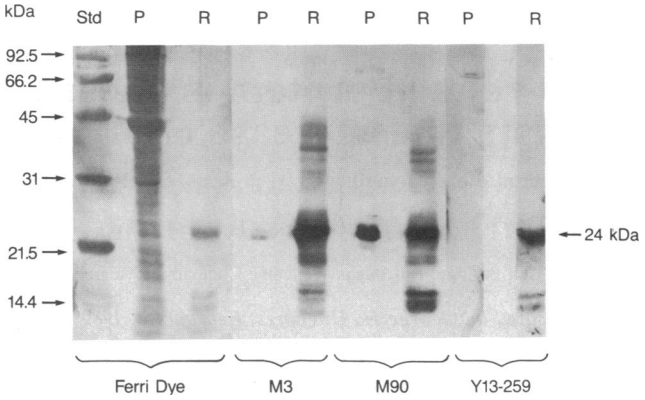

FIG. 1. Antibody localization of *ras*-related proteins of human platelet homogenates. Platelet proteins (P) were separated using SDS/PAGE and electrophoretically blotted to nitrocellulose paper. The same procedure was used for proteins of known molecular mass (std) and *ras* p21 protein (R). Blots were then either stained with Janssen Ferri dye kit or incubated with different monoclonal antisera (M3, M90, or Y13-259) that localize specific regions of the *ras* p21 protein.

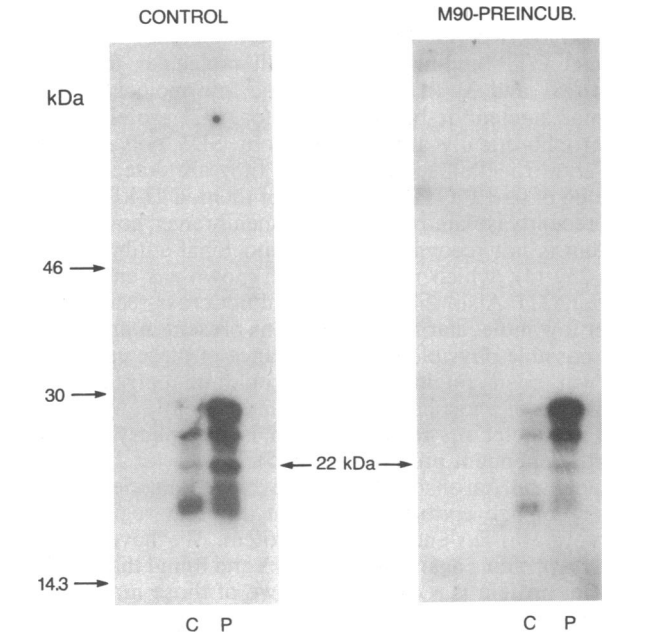

FIG. 2. Binding of [α-^{32}P]GTP to a *ras*-related protein of human platelets. Proteins were separated by SDS/PAGE and electrophoretically transferred to nitrocellulose paper as in Fig. 1. Antiserum M90 was used to localize a platelet *ras*-related protein in cytosol (C) and particulate (P) fractions. Autoradiography shows various proteins that bind [α-^{32}P]GTP in control platelet fractions and fractions preincubated with antiserum M90.

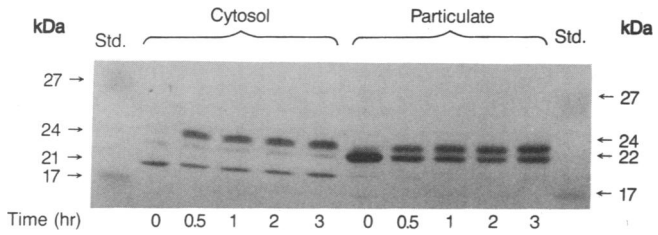

FIG. 3. Time course of the effect of iloprost on a platelet *ras*-related protein that reacts with mAb M90. Intact platelets were treated with 10 μM iloprost for up to 3 hr. Then platelets were lysed, and the cytosolic and particulate fractions were separated as explained in text. Proteins from cytosol and particulate were separated by SDS/PAGE and electrophoretically transferred to nitrocellulose paper. The *ras*-related proteins were localized by antiserum M90.

nized by mAb M90, but most of the binding correlates with proteins having molecular masses of 28–30 kDa, as has been reported (2). However, binding of [α-^{32}P]GTP to the 22-kDa protein is substantially reduced when the nitrocellulose blots are preincubated with mAb M90 (Fig. 2).

The increase of cAMP levels in platelets produced the phosphorylation of several proteins with molecular masses from 20 to 250 kDa (13–20). Two of those proteins have estimated molecular masses of 22 and 24 kDa, and they are rapidly phosphorylated by cAMP-dependent protein kinase (15, 16). A 22-kDa protein is phosphorylated in platelet microsomes treated with cAMP (21–23), and this correlates with increased uptake of Ca^{2+} by the microsomal fraction (21–26). This 22-kDa protein is peripherally associated with the membrane (20). We thought there might be some correlation between the 22- and 24-kDa proteins that are phosphorylated by cAMP-dependent protein kinase and the platelet proteins that are detected by mAb M90. Therefore, we looked for the effect of iloprost, a prostacyclin analog that increases platelet cAMP levels, on the proteins that are recognized by mAb M90 in human platelets. (In these experiments we isolated platelets in the absence of prostacyclin to avoid possible effects of prostacyclin during platelet preparation.) Fig. 3 shows that in the absence of iloprost (t_o) the predominant protein detected by mAb M90 in platelet membranes is the 22-kDa protein. Very small amounts of the 22-kDa protein were found in the cytosolic fraction. Treatment of platelets with 10 μM iloprost induced the appearance in platelet membranes of a protein with an apparent molecular mass of 24 kDa. This parallels a decrease of the 22-kDa protein from the membranes (Fig. 3). Both proteins are recognized by mAb M90. In the cytosol of platelets not treated with iloprost (t_o), a major band was observed with an apparent molecular mass of 21 kDa, which was also detected by mAb M90. Treatment with iloprost produced an increase in the cytosolic fraction of the 24-kDa protein recognized by mAb M90 (Fig. 3). This effect was already evident at 0.01 μM iloprost and reached a plateau at 0.1 μM iloprost (data not shown). A similar effect was produced by 1 mM dibutyryl cAMP (data not shown).

The change of mobility of the 22-kDa protein to a region of higher molecular mass might indicate a covalent modification such as phosphorylation. Therefore, we prelabeled platelets with $^{32}P_i$ and treated them with iloprost with subsequent mAb M90 probing. One of these experiments is shown in Fig. 4. The appearance of the 24-kDa protein in particulate and cytosol, as determined by mAb M90, correlated exactly with the gradual increase of a ^{32}P-labeled protein with an apparent molecular mass of 24 kDa (Fig. 4). These effects may provide a biochemical basis for the understanding of the general inactivation of platelet responses that are known to be caused by cAMP (27, 28).

The phosphorylated 24-kDa protein was immunoprecipitated by mAb M90 (Fig. 5). The radioactivity of this protein is dramatically increased in the cytosolic and particulate fractions of platelets treated with iloprost (Fig. 5). The immunoprecipitation of the ^{32}P-labeled 24-kDa protein by mAb M90 is also shown in Fig. 6, where it can be observed that this protein is not immunoprecipitated by mAb Y13–259 or two other polyclonal antisera that recognize proteins derived from the *rho* genes (29, 30).

It is well established that *ras*-encoded p21 proteins are synthesized as soluble cytosolic polypeptides that are translocated to the plasma membrane by palmitoylation at cys-186 (29, 30). This translocation is associated with an increase in the mobility of the *ras* proteins as analyzed by SDS/PAGE. Palmitoylation of the *ras* region induces its translocation to the plasma membrane. Both palmitoylation and association with the membrane are reversible (31). Our evidence favors the possibility that the *ras*-related platelet protein is phosphorylated.

Phosphorylation of *ras* proteins has been shown in cell-free systems by the action of protein kinase C and cAMP-dependent protein kinase (32–34). Addition of phorbol esters can induce protein kinase C-mediated phosphorylation of the Ki-*ras* p21 in whole cells (32). Previous reports indicated that epidermal growth factor or insulin could increase the autophosphorylation of viral Ha-*ras* p21 protein (35, 36). However, no indication of an agonist-induced phosphorylation of *ras* proteins in intact cells has been reported yet. Our information provides the basis for a coupling mechanism involving the phosphorylation and translocation to the cytoplasm of a GTP-binding protein that is related to an oncogene product. Phosphorylation and translocation of the platelet

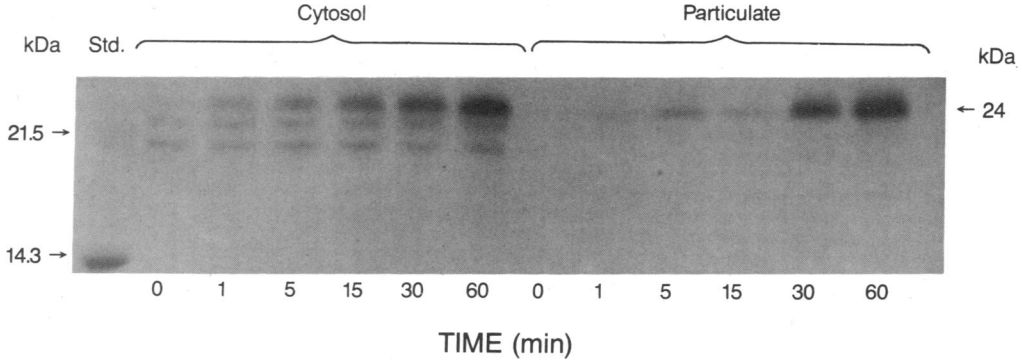

FIG. 4. Phosphorylation of a *ras*-related protein of human platelet homogenate. Platelets were prelabeled with $^{32}P_i$ and then treated with 10 μM iloprost for the indicated time periods. Proteins from cytosol and particulate were separated by SDS/PAGE and electrophoretically transferred to nitrocellulose paper. The blot was probed with antiserum M90 and, subsequently, an autoradiograph was obtained. There was exact correlation between the ^{32}P-labeled 24-kDa phosphoprotein and the 24-kDa protein detected by antiserum M90.

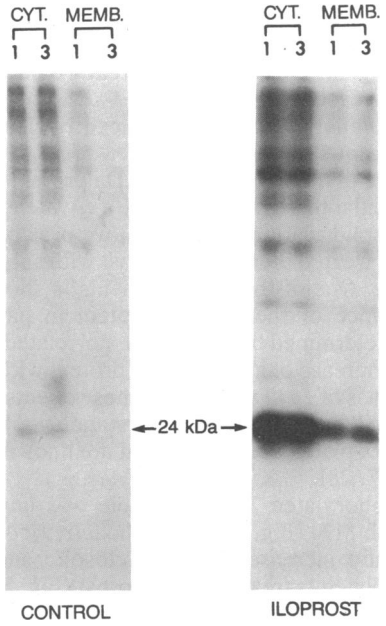

FIG. 5. Immunoprecipitation of ^{32}P-labeled 24-kDA protein by mAb M90. Human platelets were labeled with ^{32}P, treated without (control) or with 10 μM iloprost (for 30 min, and then cytosolic (CYT.) and membrane (MEMB.) fractions were isolated, immunoprecipitated by antiserum M90, and separated on SDS/PAGE as described. In each case 1 or 3 μl of antiserum was used as indicated; these are saturating amounts of antiserum to produce complete immunoprecipitation. Note that the relative proportion of cytosolic to membrane protein is six times greater than the proportion shown in Figs. 3 and 4.

ras-related protein by iloprost could be involved in the regulation of transmembrane signaling. The fact that agonists that increase cAMP levels in platelets inhibit the activation of phospholipase C might indicate that phosphorylation and translocation of the 22-kDa *ras*-related phosphoprotein could be involved in the regulation of phospholipase C.

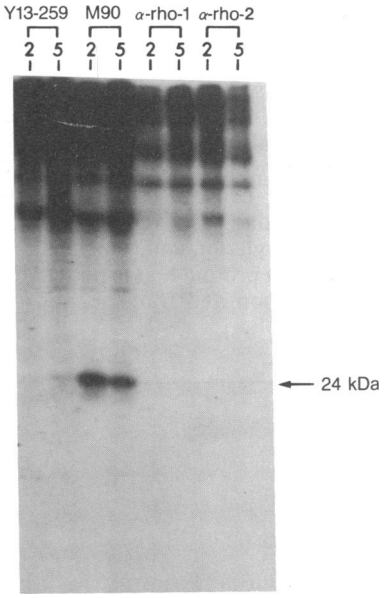

FIG. 6. Specificity of the immunoprecipitation of the cytosolic ^{32}P-labeled 24-kDa protein. The experiment was carried out as for Fig. 5. Antisera Y13–259, M90, and two polyclonal antisera that recognize proteins derived from *rho* genes (α-rho-1 obtained from rabbits and α-rho-2 obtained from goats) were used. In each case, 2 or 5 μl of antiserum were used as indicated. Only antiserum M90 immunoprecipitated the ^{32}P-labeled 24-kDa protein from the cytosolic fraction of platelets treated with 10 μM iloprost for 30 min.

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