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LOW Mr GTP-BINDING PROTEINS IN HUMAN PLATELETS: CYCLIC AMP-DEPENDENT PROTEIN KINASE PHOSPHORYLATES m22KG(I) IN MEMBRANE BUT NOT c21KG IN CYTOSOL

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Summary: We have purified and characterized two kinds of GTP-binding proteins with Mr of 22,000 in human platelet membrane (main; m22KG(I), minor; m22KG(II))(Nagata, K. and Nozawa, Y. (1988) FEBS Lett. 238, 90-94). In this study, the main GTP-binding protein (m22KG(I)) was found to be phosphorylated by cyclic AMP-dependent protein kinase (A-kinase), but not by protein kinase C. About 0.5 mol of phosphate was maximally incorporated into one mol of the protein and this phosphorylation was inhibited in the presence of A-kinase inhibitor. Phosphorylation of m22KG(I) did not alter either its GTP-binding or GTPase activity. When m22KG(I) was incubated alone or in the presence of 100 μM guanosine 5'-(3-O-thio)triphosphate (GTPγS) and then exposed to A-kinase, no significant changes in the level of phosphorylation were observed. On the other hand, the most abundant GTP-binding protein with Mr of 21,000 (c21KG) in human platelet cytosol, which was identified as a transformation suppressor gene product (rap 1 protein, smg p21 and Krev-1 protein), was not phosphorylated by A-kinase under the same condition. However, c21KG was phosphorylated by A-kinase after pretreatment with alkaline phosphatase.

It is widely accepted that there is a family of GTP-binding proteins (G-proteins) with a subunit structure of $\alpha\beta\gamma$ which serves as transducers for membrane receptors in mammalian tissues (1-3). These G-proteins communicate between a variety of membrane receptors and effectors including adenylate cyclase and cyclic GMP-dependent phosphodiesterase. Recently, evidence is accumulating that G-proteins of this family are involved in phospholipase C and A_2 activa-

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tion, and K^+-, Ca^{2+}-channels (2). In addition to this family of G-proteins, there is another family of monomeric GTP-binding proteins with Mr values of between 20,000 and 30,000. This family includes ras, rho, ral, R-ras, ypt1, rab2 and smg gene products as well as ARF (4-7). Among these GTP-binding proteins with low Mr values, c-Ki-ras p21 has been known to be phosphorylated by both cyclic AMP-dependent protein kinase (A-kinase) and protein kinase C (8), and v-Ha-ras p21 by protein kinase C (9). In yeast, RAS2 gene product was phosphorylated by A-kinase in vivo but not by protein kinase C. When the RAS2 protein was phosphorylated by A-kinase, its ability to activate the adenylate cyclase was diminished (10). However, it has not been reported whether other GTP-binding proteins with low Mr values are phosphorylated by these protein kinases.

In platelets, several GTP-binding proteins with low Mr values have been reported (11) and in addition to Gi2 (12) we purified two of them (main; m22KG(I) and minor; m22KG(II)) from the membrane fraction (13). Takai and coworkers purified a GTP-binding protein with Mr of 22,000 from human platelet membranes and identified it as smg p21 (14), which was recently reported to be phosphorylated by A-kinase (15). It is well known that protein phosphorylation by A-kinase or protein kinase C plays an important role in the regulation of platelet functions (16, 17). Platelets respond to several extracellular stimulants, e.g., thrombin, collagen and platelet-activating factor (PAF), resulting in the shape change, aggregation and release of dense body constituents, e.g., ADP and serotonin (16). Activation of protein kinase C by diacylglycerol, which is derived from the receptor-linked breakdown of phosphoinositides, is thought to be essential for these reactions, while cyclic AMP-elevating agents, including prostaglandin E_1, I_2 and D_2, inhibit these reactions via activation of A-kinase (16).

In this communication, we report that A-kinase phosphorylates m22KG(I) purified from membrane, but not c21KG from cytosol of human platelets which was identified as a transformation suppressor gene product[1] (rap1 (18), smg 21 (14) and Krev-1 (19) proteins). Moreover, it was found that c21KG was phosphorylated only after treatment with alkaline phosphatase.

MATERIALS AND METHODS

Materials: A-kinase, protein kinase inhibitor Type-II, histone (Type IIIS), and insoluble alkaline phosphatase were obtained from Sigma (St. Louis, MO). [γ-^{32}P]ATP, [γ-^{32}P]GTP, [^{3}H]GTP, [^{35}S]GTPγS were all purchased from New England Nuclear (Boston, Mass). AppNHp and GTPγS were obtained from Boehringer Mannheim GmbH (West Germany). N-[2-(methylamino)ethyl]-5-isoquinoline sulfonamide dihydrochloride (H-8) was from Seikagaku Kogyo Co., Ltd. (Tokyo). All other chemicals were of reagent grade from various sources. Protein kinase C was purified from rat brain cytosol as described (20).

Phosphorylation Assay: Phosphorylation by A-kinase was carried out in a reaction mixture (final volume 125 µl) containing 20 mM Tris/HCl (pH 7.5), 10 mM Mg acetate, 20 µM [γ-^{32}P]ATP (1000-2000 cpm/pmol), 1 µM cyclic AMP and 5 µg partially purified bovine heart A-kinase. Where indicated, 10 µg protein kinase inhibitor Type-II or 10 µM H-8 was added. The reaction was conducted for various time periods indicated in each experiment at 30 °C. Treatment with protein kinase C was carried out in a reaction mixture (final volume 100 µl) containing 25 mM Tris/HCl (pH 7.5), 5 mM Mg acetate, 0.5 mM $CaCl_2$, [γ-^{32}P]ATP (1000-2000 cpm/pmol), 20 µg phosphatidylserine, 0.4 µg diolein and sample protein. Reactions were carried out for 5 min at 30 °C. These reactions were terminated by addition of 25 % trichloroacetic acid. The precipitates were collected with a nitrocellulose filter (pore size, 0.45 µm) and the radioactivity was counted. Otherwise, the reaction was stopped by addition of 0.5 vol. SDS-stop solution containing 150 mM Tris/HCl (pH 6.8), 7.5 % sodium dodecyl sulfate (SDS), 12 % glycerol and 4.5 % 2-mercaptoethanol (w/v). Then, the phosphorylated proteins were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (21) and stained with Coomassie blue. The gels were then dried and autoradiographed at -80 °C for 4-8 h using Kodak X-Omat films.

Assays for [^{35}S]GTPγS- or [^{3}H]GTP- binding and GTPase Activities: Binding of [^{35}S]GTPγS or [^{3}H]GTP, and GTPase activity were assayed according to the methods described earlier (22).

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[1] Nagata, K., Itoh, H., Katada, T., Takenaka, K., Kaziro, Y. and Nozawa, Y., manuscript in preparation.

Phosphatase Treatment: c21KG (2 μg) was well mixed with insoluble alkaline phosphatase in reaction mixture (0.2 ml) containing 20 mM Tris/HCl (pH 10.5), 0.1 mM EDTA and 0.1 mM dithiothreitol for 2.5 min at 37 °C, diluted with 10 vol. of 20 mM Tris/HCl (pH 7.4) and then centrifuged. The supernatant was concentrated by Centricon-10 (Amicon). Phosphorylation assay was carried out as described above.

Protein Determination: Protein was measured by the method of Bradford (23) using bovine serum albumin as standard.

RESULTS

We have purified two GTP-binding proteins with Mr of 22,000 (main; m22KG(I) and minor; m22KG(II)) to homogeneity from platelet membranes (12). Addition of A-kinase to m22KG(I) resulted in a pronounced incorporation of ^{32}P into the protein (Fig. 1A and B). The phosphorylated band at 22,000 Da corresponds to the band of m22KG(I) (Fig. 1A). The phosphorylation was observed in time-dependent manner and maximally about 0.5 mol of phosphate was incorporated into 1 mol of protein at 10 min, with no increase in phosphorylation at 30 min (Fig.

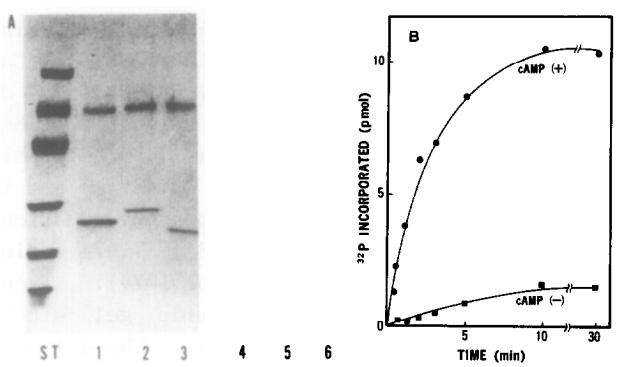

Fig. 1. Phosphorylation by A-kinase of GTP-binding proteins with low Mr from human platelets. Phosphorylation of the sample (500 ng) by A-kinase was performed as described in "Materials and Methods". (A) Coomassie blue-stained gel of the phosphorylated GTP-binding proteins with low Mr (lanes 1-3), and autoradiogram of the gel showing ^{32}P incorporation (lane 4-6). Lane ST indicates protein standards (Pharmacia); phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (20,100) and α-lactalbumin (14,400). Lanes 1 and 4, m22KG(I); lanes 2 and 5, c25KG; lanes 3 and 6, c21KG. (B) Time course of m22KG(I) phosphorylation by A-kinase. m22KG(I) (500 ng) was incubated with A-kinase for various incubation periods under the standard condition (●) or in the absence of cyclic AMP (■).

Table 1

Effect of protein kinase inhibitors on the phosphorylation of m22KG by A-kinase

Inhibitor	^{32}P-incorporated cpm	% of control
None	3,096	100
Protein kinase inhibitor (10 μg)	1,006	32.5
H-8 (10 μM)	649	21.0

After inhibitors were incubated with A-kinase (5 μg) for 15 min at 4 °C, m22KG(I) (250 ng) was added to the reaction mixture and incubation was further performed for 1 min at 30 °C. The results shown are representatives of three independent experiments.

1B). Such phosphorylation did not occur in the absence of cyclic AMP. In order to ensure that this phosphorylation is selectively elicited by A-kinase, protein kinase inhibitors (Type II and H-8) were added in some incubations. The phosphoprylation was inhibited by these inhibitors (Table 1). Interestingly, no

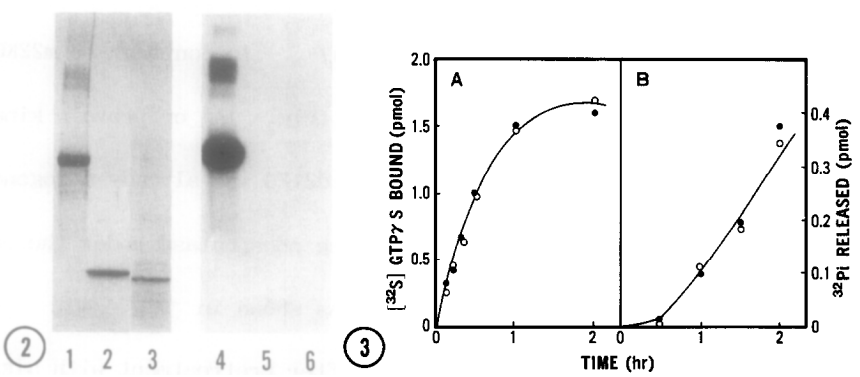

Fig. 2. Incubation of m22KG(I) with protein kinase C. Phosphorylation was performed as described in "Materials and Methods". Coomassie blue-stained gel of histone type IIIS (lane 1), m22KG(I) (lane 2) and c21KG (lane 3) after phosphorylation, and autoradiogram of the gel showing ^{32}P incorporation (lane 4-6). Lane 4, histone type IIIS; lane 5, m22KG(I); lane 6, c21KG.

Fig. 3. Effects of phosphorylation of m22KG(I) by A-kinase on its GTP-binding and GTPase activities. (A) Time course of the GTPγS-binding activity of phosphorylated (●) or nonphosphorylated (○) m22KG(I). (B) Time course of the GTPase activity of phosphorylated (●) or nonphosphorylated (○) m22KG(I).

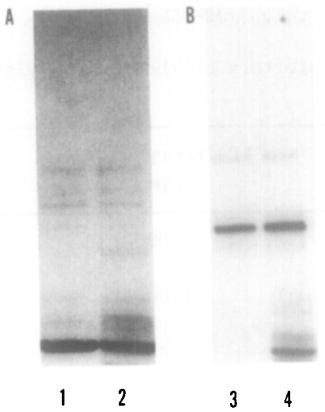

Fig. 4. Phosphorylation of c21KG after pretreatment with alkaline phosphatase. c21KG was pretreated with alkaline phosphatase as described in "Materials and Methods". (A) Coomassie blue staining of c21KG. Lane 1, control; lane 2, treatment with phosphatase. (B) Autoradiogram. Lane 3, control; lane 4, treatment with phosphatase.

detectable phosphorylation of m22KG(I) was seen with protein kinase C (Fig. 2). The phosphorylation of m22KG(I) affected neither its GTPγS-binding (Fig. 3A) nor GTPase activities (Fig. 3B).

On the other hand, we purified two GTP-binding proteins with Mr values of 21,000 (c21KG) and 25,000 (c25KG)[1] from the cytosolic fraction of human platelets. c21KG was identified as a transformation suppressor gene product (rap 1 (18), smg 21 (14) and Krev-1 (19) proteins)[1]. In contrast to m22KG(I), c21KG was not phosphorylated by either A-kinase (Fig. 1A) or protein kinase C (Fig. 2). However, since it was possible that c21KG was already endogenously phosphorylated, we pretreated c21KG with alkaline phosphatase under the condition that removes endogenous phosphate groups. As shown in Fig. 4, phosphorylation of c21KG by A-kinase was observed after pretreatment with alkaline phosphatase.

DISCUSSION

In the present study, we reported the specific phosphorylation by A-kinase of a major membranous GTP-binding protein with Mr of 22,000, termed m22KG(I).

It has already been shown that c-Ki-ras p21 is phosphorylated by A-kinase (8). The platelet m22KG(I) did not cross-react with a monoclonal antibody against ras p21 (12). Recently, an A-kinase substrate, termed thrombolamban, with Mr of 22,000 was partially purified from human platelet membranes (24). This protein was functionally similar to phospholamban (25), which was the A-kinase substrate in muscle and may play a role in Ca^{2+} transport by Ca^{2+}-ATPase. Thus, it is tempting to speculate that m22KG(I) may be associated with Ca^{2+}-ATPase, though it is not clarifed whether m22KG(I) is identical to thrombolamban itself.

The phosphorylation of m22KG(I) by A-kinase did not affect its [^{35}S]GTPγS- or [^{3}H]GTP-binding activity, and GTPase activity (Fig. 3). However, it is possible that this phosphorylation would affect the function of the target protein of m22KG(I). Recently, it was reported that smg p21 purified from platelet membrane was phosphorylated by A-kinase (15). We should await identification of these two proteins.

In addition to membranous GTP-binding proteins, we have purified two GTP-binding proteins with Mr of 21,000 (c21KG) and 25,000 (c25KG)[1] from human platelet cytosol. And c21KG was identified as rap1 (18), smg 21 (14) or Krev-1 protein (19), which is reported as a transformation suppressor gene product. It is to be noted that c21KG was not phosphorylated by A-kinase under the condition that m22KG(I) was able to be phosphorylated, since smg p21 was known to be phosphorylated by A-kinase (15). Moreover, it was observed that c21KG was phosphorylated by A-kinase only after pretreatment with alkaline phosphatase. This finding leads us to speculate that c21KG is translocated from membrane to cytosol after A-kinase-mediated phosphorylation.

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