We have purified, characterized, and identified two GTP-binding proteins with $M_0$ of 25,000 (c25KG) and 21,000 (c21KG) from the cytosol fraction of human platelets. These two proteins were copurified with the $\beta$ subunits of heterotrimeric GTP-binding proteins. Amino acid sequences of tryptic fragments of c21KG completely matched with those of rap1 protein (Pizon, V., Chardin, P., Lerosey, I., Oflofsson, B., and Tavitian, A. (1989) Oncogene 3, 201-204). c25KG bound about 1 mol of [35S]guanosine 5'-dithiotriphosphate (GTPyS)/mol of protein, with a $K_d$ value of about 45 nM. The activities of the already purified low $M_0$ GTP-binding proteins having $M_0$ of about 21,000 (ras) have been found in mammalian tissues (9-11). Recently, a ras-related gene, YPT1, encoding a protein with $M_0$ of 23,500, has been found in yeast (12). Other ras-related genes, rho (13), rat (14), and SEC4 (15), have also been reported. A GTP-binding protein with $M_0$ of about 21,000 required for the cholera toxin-dependent ADP-ribosylation of G, has been purified to near homogeneity from rabbit liver (16) and bovine brain (17). This protein has been designated as ADP-ribosylation factor. New species of GTP-binding protein with $M_0$ of about 21,000, termed as $G_p$, has been found and partially purified from human placenta (18). More recently, a GTP-binding protein with $M_0$ of 24,000 has been purified from bovine brain membrane (19). Moreover, another species of GTP-binding protein was purified from bovine brain membrane (20) and cytosol of bovine adrenal gland (21) as a substrate for botulinum ADP-ribosyltransferase, which was identified as rho gene product (20, 22).

As for the platelet, several kinds of GTP-binding proteins with $M_0$ values ranging 20,000-30,000 were detected in both cytosol and membrane fractions (23, 24). Recently, we have purified two GTP-binding proteins with $M_0$ of 22,000 from membrane fraction, which were biochemically and immunochemically distinguishable from ras p21 (25). Thereafter, Ohmori et al. (26) also purified a major low $M_0$ GTP-binding protein from human platelet membranes and identified as smg p21 (27). The physiological functions of these low $M_0$ GTP-binding proteins are unknown, but by analogy with the G proteins with heterotrimeric structure such as $G_{o}$, $G_{i}$, $G_{o}$,
and Go, it can be speculated that these GTP-binding proteins with low M, values would also be involved in transmembrane signaling.

In the present investigation, we have purified two GTP-binding proteins with M, of 25,000 (c25KG) and 21,000 (c21KG) to near homogeneity from the cytosol fraction of human platelet, identified, and characterized them. Partial amino acid sequence analysis of c25KG compared with other GTP-binding proteins with low M, values indicates that c25KG is a novel GTP-binding protein distinct from other GTP-binding proteins described previously. On the other hand, the determined amino acid sequences of c21KG were completely agreed with those of rap1 protein (28), smg p21 (29), and Kreu-1 protein (30).

**EXPERIMENTAL PROCEDURES AND RESULTS**

**DISCUSSION**

Several recent discoveries indicate that the ras genes belong to a large family consisting of about 20 genes. The amino acid sequences of these proteins predicted from the cDNAs have the consensual sequence responsible for GTP-binding activity, which has been found in heterotrimeric G proteins. All low M, GTP-binding proteins (M, 20,000–30,000) so far purified reveal GTP-binding activity. The C terminus of the encoded proteins includes a cysteine that seems to become fatty-acylated, suggesting the distribution of these proteins both in membranes and cytosol: the acylated proteins may locate in membranes whereas the proteins located in cytosol may not be acylated. In fact, several kinds of the proteins including ras, rho, and smg p25A proteins were purified from the membrane fraction and some of them such as rho (21) and smg p25A (31) proteins have been found in the cytosol fraction. In this paper, we have detected in the cytosol fraction of human platelets at least three low M, GTP-binding proteins which were separated on the MonoQ HR 5/5 column chromatography. Among these three major proteins, two proteins with M, values of 25,000 and 31,000, tentatively termed as c25KG and c21KG, respectively, were purified to homogeneity.

To identify the purified c25KG and c21KG, we determined the partial amino acid sequences of the nine peptides containing 76 amino acid residues of c25KG and of the six peptides containing 58 amino acid residues of c21KG. The results indicated that c25KG is a novel low M, GTP-binding protein, although the protein is closely related to ras and smg 25 families: the sequence revealed 60% homology to ras proteins both in membranes and cytosol: the acylated proteins may locate in membranes whereas the proteins located in cytosol may not be acylated. In fact, several kinds of the proteins including ras, rho, and smg p25A proteins were purified from the membrane fraction and some of them such as rho (21) and smg p25A (31) proteins have been found in the cytosol fraction. In this paper, we have detected in the cytosol fraction of human platelets at least three low M, GTP-binding proteins which were separated on the MonoQ HR 5/5 column chromatography. Among these three major proteins, two proteins with M, values of 25,000 and 31,000, tentatively termed as c25KG and c21KG, respectively, were purified to homogeneity.

It was reported that GDP is tightly associated with many GTP-binding proteins such as PT-substrates (1) and ras gene proteins (35), even in their highly purified preparations. And it is also known that the addition of 250 mM (NH₄)₂SO₄ to the reaction mixture caused the release of GDP probably due to the conformational change in the vicinity of guanine nucleotide-binding site (36). It was the case with c25KG and consequently the binding rate of GDP-S exogenously added apparently increased (Fig. 10B). The apparent first order rate constants were calculated to be 0.28/min and 0.016/min in the presence and absence of 250 mM (NH₄)₂SO₄, respectively, by the analysis of first order plots (data not shown). On the other hand, c25KG was found to be distinct from other low M, GTP-binding proteins in the GAP activity. The GAP activity of c25KG was calculated to be 1.8 mmol of P/mol of protein/min in the absence of (NH₄)₂SO₄. These results suggest that the slow GAP rate is due to the slow hydrolysis rate.

The structure of the GTP-binding domain or its vicinity in c25KG may be similar, if not identical, to that in smg p25A but evidently distinct from those in c21KG and smg p21. N-Ethylmaleimide inhibited the GTP-binding activities of c21KG and smg p21 (29) but did not affect those of c25KG and smg p25A (19). Thus, c25KG and smg p25A appear to contain cysteine residue(s) in the GTP-binding domain or its vicinity, but c21KG and smg p21 do not.

Moreover, it is noteworthy that smg p21, which is abundant in human platelet membranes (26, 27), was able to be phosphorylated by cAMP-dependent protein kinase (37), whereas the same gene product c21KG in cytosol was not phosphorylated (38). Considering the fact that smg p21 and c21KG are the same gene product of Kreu-1, it seems feasible to anticipate that smg p21 in membranes may be fatty-acylated and c21KG in cytosol may not be. Accordingly, it is conceivable that the former is associated with membranes and is the preferred substrate for phosphorylation by cyclic AMP-dependent protein kinase whereas the latter is not phosphorylated.

The biological observations of Kreu-1 indicate that its product may be involved in the negative growth regulation of Kirsten sarcoma virus-transformed NIH/3T3 (30). In fact, Kreu-1 is observed to suppress the transformed phenotype of NIH/3T3 when highly expressed (30). It could be considered that c21KG and smg p21 play an important role in proliferation of some cell types. However, since the platelet represents terminal differentiation stage in hematoepoietic development, the physiological significance of c21KG abundant in cytosol remains unknown in this nonproliferating cell. In mammalian cells, the effects of GTP and its analogues on phosphoinositide metabolism and exocytosis have suggested the existence of two putative G proteins, G, and G, that are involved in phospholipase C activation and secretion processes, respectively (39). This hypothesis is supported by the findings that 1) the soluble phospholipase C from human platelets is stimulated by a low M, GTP-binding protein (29,000) (40) and 2) low M, GTP-binding proteins (18,000–24,000) may be implicated in exocytosis in the adrenal chromaffin cell (41). Thus, it is tempting to speculate that c25KG and c21KG purified from the human platelet cytosol may function in the regulation of the activity of phospholipase C and/or the process of exocytosis in the human platelet.

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Fig. 1. DEAE-Sephadex column chromatography of the purified fraction. The purified fraction of human platelets was applied to a DEAE-Sephadex column and eluted as described under "Materials." "[3H]GTP-γS-binding activity (B) of 0.5 µl of each fraction was assayed as described in "Experimental Procedure."" Active fractions, indicated by a bar, were pooled. "Nonspecific" for further explanation.

Fig. 2. Ultragel AcA-45 column chromatography. "[3H]GTP-γS-binding activity (B) of 0.5 µl of each fraction was assayed as described in "Experimental Procedure."" Active fractions, indicated by a bar, were pooled. "Nonspecific" for further explanation.

Fig. 3. DEAE-Sephadex column chromatography. The active fraction of the DEAE-Sephadex chromatography was dialyzed for 6 h against 1.0 M of NaCl, then dialyzed against a column (1.5 x 10 cm) of DEAE-Sephadex A-50 (previously conditioned with 0.1 M of NaCl, containing 0.1 M of NaCl) and finally against a column (1.5 x 10 cm) of DEAE-Sephadex A-50 (previously conditioned with 0.1 M of NaCl, containing 0.1 M of NaCl). After the purification procedure, the protein was isolated in an amount of 1 mg/10 mg of NaCl. The active fractions (Nos. 34-45, 30.6 µg of protein) were pooled and concentrated to a final volume of 500 µl.

Fig. 4. Ultragel AcA-45 column chromatography. The active fraction of the DEAE-Sephadex chromatography was dialyzed for 6 h against 1.0 M of NaCl, then dialyzed against a column (1.5 x 10 cm) of DEAE-Sephadex A-50 (previously conditioned with 0.1 M of NaCl, containing 0.1 M of NaCl) and finally against a column (1.5 x 10 cm) of DEAE-Sephadex A-50 (previously conditioned with 0.1 M of NaCl, containing 0.1 M of NaCl). After the purification procedure, the protein was isolated in an amount of 1 mg/10 mg of NaCl. The active fractions (Nos. 34-45, 30.6 µg of protein) were pooled and concentrated to a final volume of 500 µl.
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Fig. 5. Mono Q HPLC column chromatography of cGMP. "^{[35]P]GTP binding activity (•) of 5 μl of each fraction was assayed as described in "Experimental Procedures". Absorbance at 280 nm (□) was also monitored. Fractions eluted from the columns were analyzed by SDS-PAGE (15% polyacrylamide), and the Coomassie blue-stained protein was visualized in the top. The number of each lane corresponds to the fraction number. Active fractions, indicated by a bar, were pooled. The lane "0" indicates protein standards (bovine testes) the numbers used were phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (45,000), carbonic anhydrase (30,000), trypsin inhibitor (20,100) and a-lactalbumin (14,000).

Fig. 6. Mono Q HPLC column chromatography of cGMP. "^{[35]P]GTP binding activity (•) of 5 μl of each fraction was assayed as described in "Experimental Procedures". Absorbance at 280 nm (□) was also monitored.

Fig. 7. ATPase of cGMP and cGMP isozymes of human platelet-cytosol. About 500 μg of cGMP (lane 1), cGMP (lane 2) was incubated in a buffer containing 20 mM of human platelet-washed (lane 2), cGMP (lane 3) and ATP (3 μg each) for 30 min. The proteins were visualized with Coomassie blue. The protein markers used were the same as in Fig. 5. The protein bands were excised and analyzed by 

Table 1. Purification of cGMP and cGMP from human platelet-cytosol

| Purification step | Total volume | Total protein | Total GTP-binding activity | Specific activity | Yield |
|------------------|--------------|---------------|---------------------------|------------------|-------|
| Cytosol          | 100          | 3,380.0 μg    | 90.0 μg                   | 0.26%            | 100   |
| DEAE-Sepharose    | 200          | 780.0 μg      | 93.1 μg                   | 0.36%            | 48    |
| Ultracentrifuged  | 15            | 87.5 μg       | 331.4 μg                  | 3.79%            | 37    |
| DEAE-Sepharose G50 | 15            | 26.8 μg       | 174.2 μg                  | 6.5%             | 19    |
| HPLC-HPLC (1)    | 11.5         | 7.8 μg        | 113.9 μg                  | 18.8%            | 12.0  |
| HPLC-HPLC (2)    | 12           | 2.5 μg        | 66.3 μg                   | 28.5%            | 7.3   |

Partial Amino Acid Sequences of cGMP and cGMP

Primary sequence information of cGMP and cGMP was needed to identify those GTP-binding proteins. Partially digested proteins were digested with Trypsin-trypsin or Staphylococcus aureus V8 proteinase digested peptides were separated by reverse-phase HPLC. Identical peaks were not observed for Trypsin digestion of cGMP and cGMP (Fig. 7A and 8B). Several peaks were subjected to amino acid sequence analysis. From the analysis of the V8-digested cGMP fragments, two sequences (LLEUGGTPP and TATATGQNK) were obtained (data not shown). After the Trypsin-trypsin digestion, six peaks of the peptide fragments were obtained as shown in Fig. 7A. Peaks 1, 2, 3, 4, 5 and 6 contained a single peptide, while peak 5 was a mixture of two peptides. The sequences determined were as followed: 1. ASHPIRP, 2. TVPADD, 3. PLLAGSNQG, 4. QGGFAGAVT, 5. GAVWKTMAAG, 6. NTTVNMEYQ, 7. FITVQNM. The sequences of the fragments in peak 5 were deduced from the results of V8-digested fragment sequence analysis. Three sequences from cGMP were similar to, but distinct from, any published sequence of the cGMP-binding proteins (Fig. 7A). The amino acid sequences of peak 1 to 4 of cGMP were determined and compared with those of the known cGMP binding proteins. The sequences of peak 1 to 4, NAHR, HARN, TPTVGGK, LTLDSQVKG, VDTPQHM, and GAVWKTMAAG, were identical with those of cGMP.
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Fig. 9. Alignment of the amino acid sequences of the two molecular weight GTP-binding proteins. The sequences of the GTP-binding proteins have been taken from the literature (8, 9, 10). The protein (GGP) is in the sequence of GTP-binding proteins from (8, 9). Amino acid sequences of GTP and ATP-depleted proteins obtained from (10) are indicated. (Figure not shown for alignment.) The sequences of GTP-binding proteins are based on the restriction enzyme banding pattern of the enzymes by gelatin gel electrophoresis.

Characterization of GTP

(1) [14C]GTP-binding Activity. [14C]GTP binds to GTP in a dose- and time-dependent manner as shown in Fig. 10. A, B, C, D, and E, Enzyme plot on GTP in the reaction mixture. The GTP-binding activity was calculated to be 0.3 pmol of GTP binding per mg of protein in the presence of 50 mM ATP, 200 mM cGMP, and 0.1 mM GTP. The results are the average of three independent experiments.

(2) Effect of ATP on GTP-binding Activity. The results in Fig. 11 show that, in the presence of 50 mM ATP, the specific activity of GTP-binding activity is reduced to 0.3 pmol of GTP binding per mg of protein in the absence of ATP. The results are the average of three independent experiments.

(3) [14C]GTP-binding Activity. The results in Fig. 12 show that, in the presence of 50 mM ATP, the specific activity of GTP-binding activity is reduced to 0.3 pmol of GTP binding per mg of protein in the absence of ATP. The results are the average of three independent experiments.

(4) Effect of cGMP on GTP-binding Activity. The results in Fig. 13 show that, in the presence of 50 mM cGMP, the specific activity of GTP-binding activity is reduced to 0.3 pmol of GTP binding per mg of protein in the absence of cGMP. The results are the average of three independent experiments.