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Purification and characterization of two GTP-binding proteins of 22 kDa from human platelet membranes

Koh-ichi Nagata and Yoshinori Nozawa

Department of Biochemistry, Gifu University School of Medicine, Tsukasamachi-40, Gifu 500, Japan

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Two GTP-binding proteins (G-proteins) of 22 kDa were purified to near homogeneity from a sodium cholate extract of human platelet membranes by successive chromatographies on DEAE-Sephacel, Ultrogel AcA-44, phenyl-Sepharose CL-4B, Mono Q HR5/5 and hydroxyapatite columns. They bound maximally 0.89 mol of [^{35}S]guanosine 5′-(3-*O*-thio)triphosphate per mol of both purified proteins, and this binding was inhibited by GTP and GDP but not by ATP and AppNHp. Their molecular masses were somewhat lower than that of *ras* p21 and they were not recognized by an anti-v-Ki-*ras* p21 antibody. These results indicate that human platelet membranes contain at least two low-molecular-mass G-proteins distinct from *ras* p21, in addition to the heterotrimeric G-proteins, the α-subunits of which possess molecular mass values of about 40 kDa.

GTP-binding protein; Low-molecular mass; *ras* p21; (Human platelet)

1. INTRODUCTION

It is well known that structurally and functionally homologous membrane-bound GTP-binding proteins (G-proteins) occur in a variety of mammalian cells. Among them, the stimulatory (Gs) and inhibitory (Gi_1) G-proteins for hormone-sensitive adenylate cyclase [1] and transducin (Gt) involved in retinal light reception [2] are best characterized. In addition, Go and Gi_2 ($\alpha_{40}\beta\gamma$) have been purified from brain, though their functions remain unknown [3–7]. All these G-proteins have been shown to possess a heterotrimeric structure ($\alpha\beta\gamma$) [1–7]. Recent molecular cloning of genes and cDNAs encoding their α-subunits has revealed that in addition to the heterogeneity of the α-subunit according to which these G-proteins are classified into major classes (Gs, Gi and Gt), there are multiple forms of the subunit within each class [8–15].

Accumulating evidence indicates the occurrence of another group of G-proteins having molecular masses between 20 and 30 kDa, besides the above-mentioned heterotrimeric G-proteins. Proteins encoded by three *ras* genes, Ki-*ras*, Ha-*ras* and N-*ras*, appear to belong to this group, because these proteins exhibit GTP-binding and GTPase activities [16–18]. G-proteins belonging to this group have so far been purified and characterized from such sources as rabbit liver [19], bovine brain [20,21] and human placenta [22]. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of human platelet membranes and subsequent blotting of the separated polypeptide bands to nitrocellulose sheets have shown the presence of several low-molecular-mass G-proteins capable of binding [α-^{32}P]GTP (23–27 kDa [23]; 21, 27, 29 and 29.5 kDa [24]). However, none of the G-proteins from platelets have been purified. We report here the purification and characterization of two G-proteins both of which have a molecular mass of about 22 kDa from human platelet membranes.

Correspondence address: Y. Nozawa, Department of Biochemistry, Gifu University School of Medicine, Tsukasamachi-40, Gifu 500, Japan

Abbreviation: AppNHp, adenosine 5′-(β,γ-imido)triphosphate

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2. MATERIALS AND METHODS

2.1. *Materials*

Guanosine 5'-(3-*O*-thio)triphosphate (GTPγS) was purchased from Boehringer, Mannheim. [^{35}S]GTPγS and [*adenylate*-^{32}P]nicotinamide adenine dinucleotide ([^{32}P]NAD) were from Du Pont-New England Nuclear. Islet-activating protein (IAP) was from Kaken Pharmaceutical Co. v-*ras* p21 synthesized in *Escherichia coli* was a generous gift from Dr H. Nakano of Kyowa Hakko Kogyo Co., Tokyo. A monoclonal antibody against v-Ki-*ras* p21 was kindly supplied by Dr H. Shiku of Nagasaki University.

2.2. *Assays of activities and immunoblot procedures*

Binding of [^{35}S]GTPγS to GTP-binding proteins and IAP-mediated ADP-ribosylation were assayed according to the method described earlier [6]. Electrophoresis of polypeptides through SDS-polyacrylamide gels and the treatment of the sample with *N*-ethylmaleimide are described in [3]. Immunoblot analysis was performed essentially as described in [7].

2.3. *Preparation of human platelet membrane extracts*

All procedures were carried out at 4°C. Outdated human platelet concentrate was centrifuged at 200 × *g* for 5 min to remove contaminating erythrocytes. The supernatant was separated and centrifuged at 2000 × *g* for 20 min. The pelleted platelets were then washed twice with 20 mM Tris-malate buffer (pH 7.0)/5 mM EGTA/1 mM phenylmethylsulfonyl fluoride (PMSF) (buffer A) and were disrupted by sonication for a total of 10 min with 15-s burst of probe sonicator (Branson Sonifier B-12). The membrane fraction was obtained by centrifugation at 105000 × *g* for 1 h, washed once with buffer A, and extracted with 200 ml of 20 mM Tris-HCl (pH 8.0)/1 mM EDTA/1 mM dithiothreitol (DTT)/0.5 mM PMSF (TEDP) containing 2% sodium cholate. After incubation for 2 h with stirring, the cholate extract was obtained by centrifugation at 105000 × *g* for 1 h for purification of G-proteins.

3. RESULTS AND DISCUSSION

The sodium cholate extract of human platelet membranes (1165 mg protein) was applied to a DEAE-Sephacel (Pharmacia) column (4.4 × 23 cm) pre-equilibrated with TEDP/20 mM NaCl/1% sodium cholate. The column was washed with 500 ml of the same buffer and elution was conducted with 1000 ml of a linear NaCl gradient (20–250 mM) in the same buffer and then 500 ml of TEDP/500 mM NaCl/1% sodium cholate (fig.1). Fractions 62–74, containing high GTPγS-binding activities, were pooled and concentrated to 20 ml. The concentrate was fractionated on an Ultrogel AcA-44 (LKB) (2.8 × 100 cm) column in TEDP/100 mM NaCl/1% sodium cholate. Fractions containing GTPγS-binding activity eluted from Ultrogel AcA-44 were diluted with 4 vols of

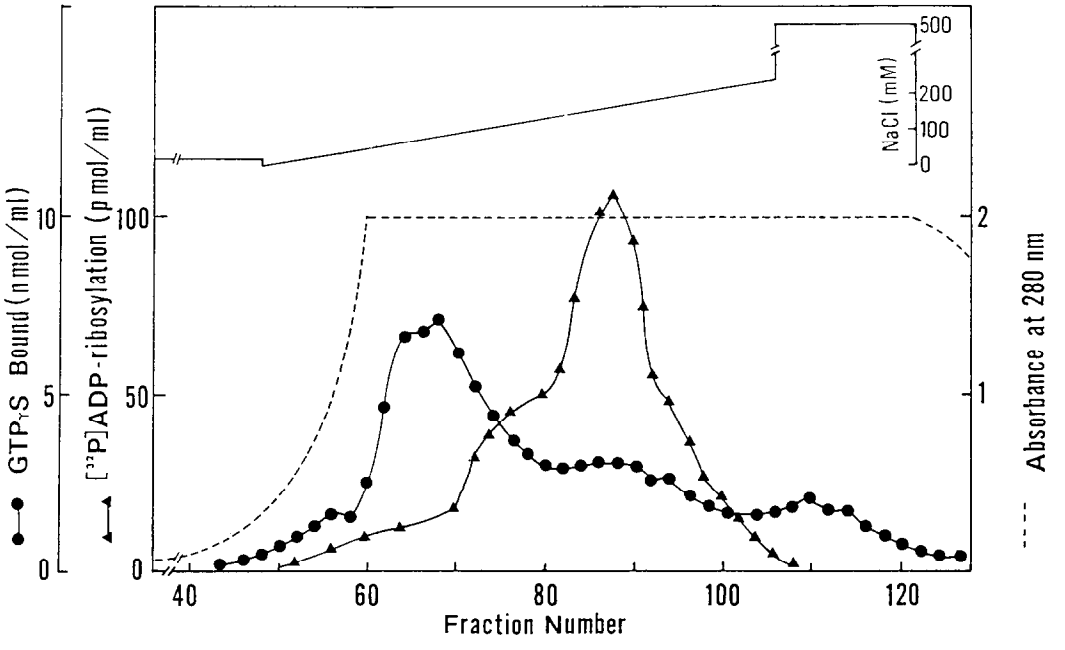

Fig.1. DEAE-Sephacel column chromatography. The activities of [^{35}S]GTPγS-binding and [^{32}P]ADP-ribosylation of each fraction of the cholate extract from human platelet membrane separated by DEAE-Sephacel column chromatography were assayed as described in [25].

TEDP/300 mM NaCl and then applied to a phenyl-Sepharose (Pharmacia) (1.6 × 25 cm) column pre-equilibrated with TEDP/250 mM NaCl/0.25% sodium cholate. After washing with 1 bed vol. of the same buffer, elution was conducted with 300 ml of a linear gradient of 500 mM NaCl/0.25% sodium cholate to 25 mM NaCl/1.5% sodium cholate in TEDP. The peak fraction of GTPγS-binding activity was collected and concentrated. The concentrate was diluted with 4 vols of TEDP and applied to a Mono Q HR5/5 (Pharmacia) column pre-equilibrated with TEDP/20 mM NaCl/0.7% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (Chaps) and elution was conducted (flow rate, 0.5 ml/min) by using a Pharmacia FPLC system with the following gradients of NaCl: 20 mM for 10 min; 20–300 mM for 40 min; 300–1000 mM for 5 min and 1000 mM for 5 min. After the Mono Q HR5/5 column chromatography step, pooled fractions containing high GTPγS-binding activities were diluted with 4 vols of 20 mM Tris-HCl (pH 8.0)/0.1 mM EDTA/1 mM DTT/100 mM NaCl/0.7% Chaps and applied to a hydroxyapatite HCA-100S column (1 × 10 cm) pre-equilibrated with the same buffer. The column was washed and elution was conducted (flow rate, 0.5 ml/min) by using a Pharmacia FPLC system with the following gradients of potassium phosphate (pH 7.5): 0 mM for 10 min; 0–50 mM for 50 min; 50–200 mM for 3 min; 200 mM for 10 min. This chromatography resulted in the separation of two protein peaks (peak I, fractions 26 and 27; and peak II, fractions 28–30) both exhibiting GTPγS-binding activity (fig.2).

As shown in fig.3, both peaks I and II were virtually homogeneous on SDS-PAGE. The molecular mass of peak I was slightly higher than that of peak II, but both were approximately 22 kDa. This value was somewhat lower than the estimated molecular mass for v-Ki-*ras* p21 synthesized in *E. coli* (23 kDa) by SDS-PAGE [25]. These two G-proteins were not ADP-ribosylated by islet-activating protein (IAP) (not shown). Their specific GTPγS-binding activities were 41.6 nmol/mg protein for both peak I and peak II. These proteins bound [^{35}S]GTPγS in a dose-dependent manner and Scatchard plot analysis showed that 1 mol each of both peaks I and II bound maximally 0.89 ± 0.3 mol (mean ± SE) of

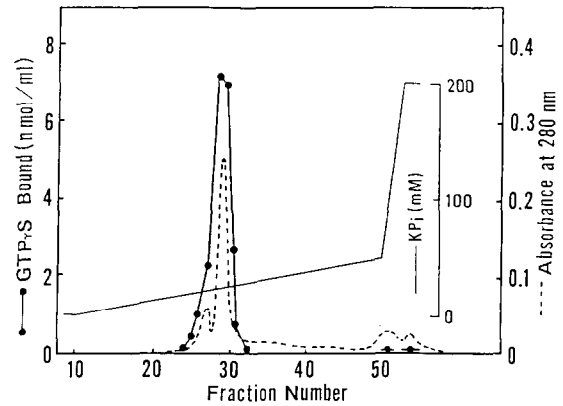

Fig.2. Hydroxyapatite (HCA-100S) column chromatography. The peak fraction with GTP-binding activity obtained from Mono Q HR5/5 column was applied to HCA-100S column and the elution was conducted with gradients of potassium phosphate (KP_i, pH 7.5). 0.5-ml fractions were collected. Further details are in the text. Aliquots (5 μl) of each fraction were assayed for GTP-binding activity.

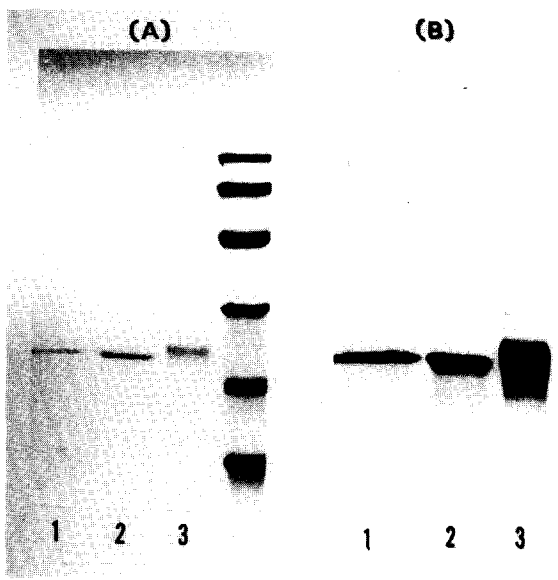

Fig.3. SDS-polyacrylamide gel electrophoresis of the G-proteins (peak I and peak II) with molecular mass values of 22 kDa from human platelet membranes and *ras* p21. Samples (750 ng protein) were subjected to gradient (8–16%) SDS-polyacrylamide gel electrophoresis. The gel was then stained with Coomassie blue (A) or silver (B). Lanes: 1, peak I; 2, peak II; 3, *ras* p21. The markers (Pharmacia) were phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa).

GTPγS with a K_d of 46 ± 5 nM (mean ± SE) (fig.4). The K_d value for peaks I and II is almost the same as that determined for a low-molecular-mass G-protein, termed 24K G, from bovine brain [21] and is 2–4-fold higher than those for Gs, Gi, Gt, Go and *ras* p21 [2,3,6,16,17,26]. As shown in fig.5, [^{35}S]GTPγS-binding activity of peak II was inhibited by GTP and GDP, but not significantly by ATP and AppNHp. The concentrations of GTP and GDP required for 50% inhibition were 0.8 and 1.6 μM, respectively. Although data are not shown, the binding activity of peak I was similarly inhibited by GTP and GDP, but not by ATP and AppNHp; 50% inhibition was attained with 0.56 μM GTP and 2.5 μM GDP. Finally, it was found that a monoclonal antibody raised against v-Ki-*ras* p21, which recognized not only the antigen but also v-Ha-*ras* p21 and v-N-*ras* p21, did not cross-react with both peaks I and II (not shown), indicating that the latter proteins were distinct from *ras* p21 proteins.

From these results it can be concluded that human platelet membranes contain at least two G-proteins of about 22 kDa in addition to the heterotrimeric G-proteins whose α-subunits have values of about 40 kDa. Since the GTPγS-binding capacity is practically the same between these two low-molecular-mass G-proteins, the possibility cannot be ruled out that peak II has been produced from peak I by limited proteolysis. This possibility as well as their functions remain to be explored.

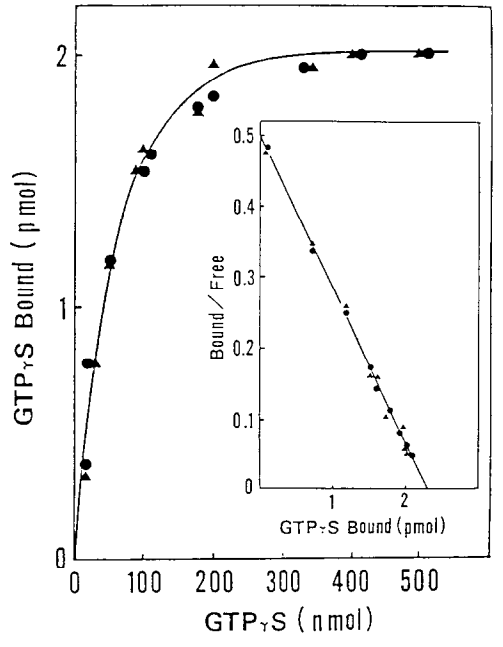

Fig.4. Dose-dependence of [^{35}S]GTPγS-binding of the G-proteins (peak I and peak II). Peak I or peak II (approx. 62 ng protein) was incubated with various concentrations of [^{35}S]GTPγS for 2 h at 30°C for measurement of its binding activity. The inset shows the Scatchard plots. The results shown are representatives of three independent experiments.

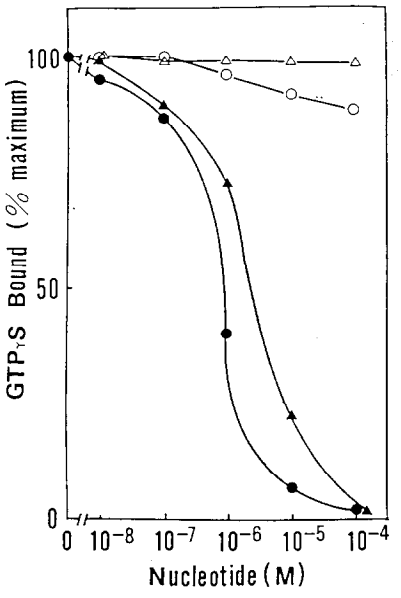

Fig.5. Effects of various nucleotides on the [^{35}S]GTPγS-binding of the major G protein (peak II). Peak II (approx. 62 ng protein) was incubated with 400 nM [^{35}S]GTPγS in the presence of various concentrations of the indicated unlabeled nucleotides for 2 h at 30°C. The radioactivity of [^{35}S]GTPγS bound to the protein in the absence of other nucleotides was about 10000 cpm. (●) GTP, (▲) GDP, (○) ATP, (△) AppNHp. The results shown are representatives of three independent experiments.

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