

# GTP-binding proteins in human platelet membranes serving as the specific substrate of islet-activating protein, pertussis toxin

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Two GTP-binding proteins serving as the specific substrate of islet-activating protein (IAP), pertussis toxin, were purified from human platelet membranes as heterotrimers with an  $\alpha\beta\gamma$ -subunit structure. The  $\alpha$  of the major IAP substrate had a molecular mass of 40 kDa and differed from that of  $G_{i1}$  or  $G_o$  previously purified from brain membranes. The partial amino acid sequences of the 40 kDa  $\alpha$  completely matched with the sequences which were deduced from the nucleotide sequences of the human  $G_{i2}$   $\alpha$  gene. On the other hand, the  $\alpha$  of the minor IAP substrate purified from human platelets was about 41 kDa and cross-reacted with an antibody raised against  $\alpha$  of brain  $G_{i1}$  ( $G_{i1}$   $\alpha$ ). These results indicate that the major IAP substrate present in human platelet membranes is a product of the  $G_{i2}$   $\alpha$  gene.

GTP-binding protein; Islet-activating protein; Pertussis toxin; (Human platelet)

## 1. INTRODUCTION

In a variety of vertebrate cells, GTP-binding proteins function as transducers that carry signals from activated receptors to effectors such as enzymes or ion channels [1]. GTP-binding proteins have been purified from several types of cell membranes as heterotrimers with an  $\alpha\beta\gamma$ -subunit structure. Their  $\alpha$ -subunits which bind GTP or GDP are unique, while the  $\beta\gamma$ -subunits are similar if not identical to each other. Molecular cloning of the  $\alpha$ -subunit genes [2] and cDNAs [3-9] has recently been achieved. The data indicate the existence of at least four genes for the  $\alpha$ -subunits ( $G_{i1}$   $\alpha$ ,  $G_{i2}$   $\alpha$ ,  $G_{i3}$   $\alpha$  and  $G_o$   $\alpha$ ) of GTP-binding proteins serving as the substrate of islet-activating protein (IAP), pertussis toxin, besides two transducin  $\alpha$ -subunits

( $G_{t1}$   $\alpha$  and  $G_{t2}$   $\alpha$ ) [5-8]. More recently, three IAP substrates have been purified from brain membranes [10,11] and compared with predicted amino acid sequences from the  $\alpha$ -subunit genes and cDNAs [12].

Several lines of evidence have suggested that a GTP-binding protein(s) is involved in receptor-coupled phosphoinositide hydrolysis (i.e., phospholipase C activation) in many types of cells [13-16] including human platelets [17,18]. Phosphatidic acid formation and  $Ca^{2+}$  release stimulated by thrombin were inhibited in platelets which had been treated with IAP [17]. These reports suggest that the GTP-binding protein associated with phospholipase C activation may be sensitive to IAP in platelets. However, such a GTP-binding protein present in platelet membranes remains to be isolated and characterized. Here, we report the purification and identification of human platelet GTP-binding proteins serving as the specific substrate of IAP.

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## 2. MATERIALS AND METHODS

### 2.1. Purification of IAP substrates from human platelet membranes

The procedures employed to purify the IAP substrates from human platelet membranes are based on the methods developed to purify  $G_i$  and  $G_o$  from rat [10] or porcine brain [11] with slight modification. After five chromatography steps with Q-Sepharose, heparin-Sepharose, Sephacryl S-300(HR), phenyl-Sepharose and DEAE-Toyopearl 650(S), an aliquot (10 nmol) of the fractions containing IAP substrate activity was diluted with 4 vols Tris-HCl (pH 8.0)/0.1 mM EDTA/1 mM dithiothreitol/100 mM NaCl (TEDN) containing 0.7% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (Chaps) and then applied to a column of hydroxyapatite (HCA-100S;  $1 \times 10$  cm) which had been equilibrated with the above solution. The column was washed and eluted at a flow rate of 1 ml/min with the following series of potassium phosphate gradients using a Pharmacia FPLC system; 0 mM for 10 min; 0–40 mM over 30 min; 40–200 mM over 3 min; 200 mM over 7 min. The eluate was collected in fractions of 1 ml. This chromatography resulted in the separation of two peaks of the IAP-substrate ac-

tivity (fig.1); the first minor peak (II, fractions 22–24 min) and the second major peak (I, fractions 26–28 min). Thus, two IAP substrates were purified to almost homogeneity from the human platelet membranes. The polypeptide composition of the purified proteins was analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and is shown in fig.1A. The radio-labeled bands of IAP-catalyzed [ $^{32}$ P]ADP-ribosylation of the purified proteins are also illustrated in fig.1B.

### 2.2. Assays of activities and immunoblot procedures

The IAP substrate protein was identified by its abilities to be [ $^{32}$ P]ADP-ribosylated in the presence of IAP and [ $\alpha$ - $^{32}$ P]NAD as in [10,11]. Electrophoresis of polypeptides through SDS-polyacrylamide gels and the treatment of the samples with *N*-ethylmaleimide were as described in [19]. Immunoblot analysis was performed essentially as in [11]. Antibodies utilized were: affinity-purified rabbit polyclonal antibodies prepared against the  $\alpha$  of  $G_{i1}$  (anti- $\alpha_{41}$ ), the  $\alpha$  of  $G_o$  (anti- $\alpha_{39}$ ) and the  $\beta\gamma$  of  $G_{i1}$  and  $G_o$  (anti- $\beta$ ) purified from rat brain, which specifically reacted with the  $\alpha_{41}$ ,  $\alpha_{39}$  and  $\beta_{35/36}$ , respectively, as described [11]; a rabbit polyclonal antibody (anti- $\alpha$ ) prepared against the mixture of porcine brain  $\alpha_{41}$ ,  $\alpha_{39}$  and  $\alpha_{40}$ . The latter antibody

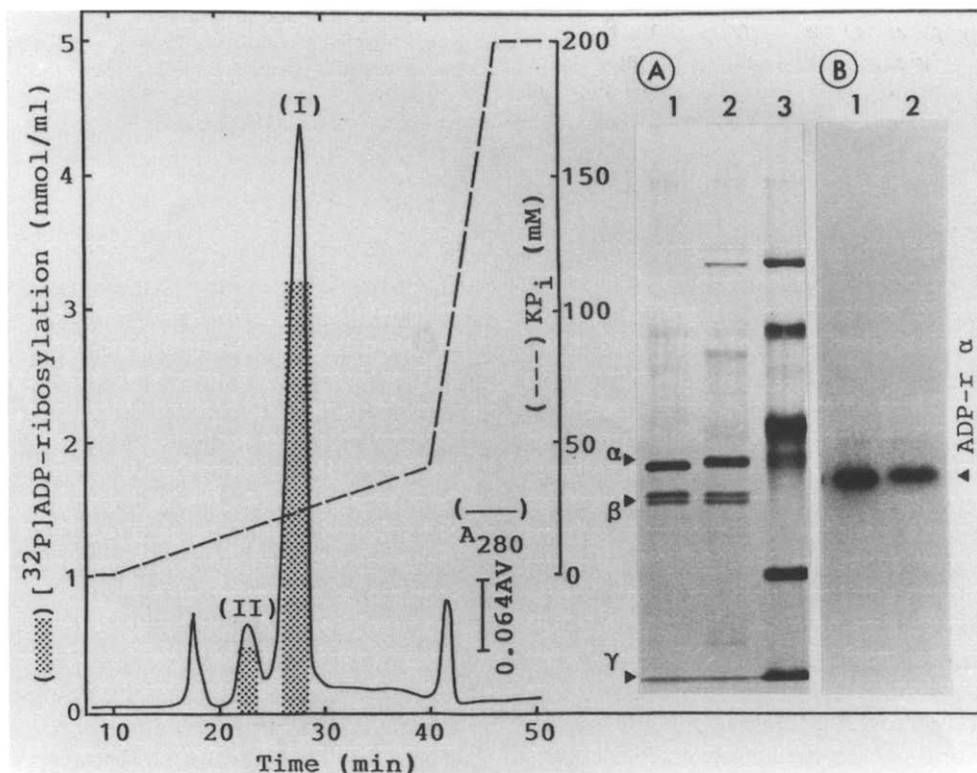


Fig.1. HCA-100(S) chromatography of IAP substrates from human platelet membranes. IAP substrate-rich fractions were applied to HCA-100(S) and eluted as described in section 2. Aliquots (5  $\mu$ l) of the fractions (I; fractions 26–28 min; II, fractions 22–24 min) were assayed for IAP-substrate (stippled areas) activity. (A) SDS-PAGE of fractions I and II. Proteins were subjected to SDS-polyacrylamide gel (10%) electrophoresis and stained with silver. Lanes: 1, peak I; 2, peak II; 3, molecular mass standards (Bio-Rad). The markers were phosphorylase *b* (92.5 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa) and lysozyme (14.4 kDa). (B) [ $^{32}$ P]ADP-ribosylated  $\alpha$ . Lanes: 1, peak I; 2, peak II.

reacted strongly with brain  $\alpha_{39}$ , and poorly with  $\alpha_{41}$  and  $\alpha_{40}$  (see fig.3).

### 2.3. Tryptic digestion of human platelet 40 kDa $\alpha$ -subunit and sequence analysis of the tryptic peptides

Resolution of the major IAP substrate (see fig.1, peak I) to the 40 kDa  $\alpha$  and the  $\beta\gamma$  was performed by phenyl-Sepharose chromatography in the presence of  $\text{Al}^{3+}$ ,  $\text{Mg}^{2+}$  and  $\text{F}^-$  as in [20]. The sample containing approx. 2 nmol of the purified 40 kDa  $\alpha$  was heated at 70°C for 5 min and digested at 37°C for 20 h with tosylphenylalanyl chloromethyl ketone-treated trypsin (TPCK-trypsin) at a protein/trypsin ratio of 50 (w/w). After tryptic digestion, the sample was applied to a column of PepRPC HR5/5 which had been equilibrated with 0.1% trifluoroacetic acid containing 5% acetonitrile. The tryptic peptides were eluted with a linear gradient of 5–40% acetonitrile at a flow rate of 0.7 ml/min using a Pharmacia FPLC system. The absorbance at 214 nm of the eluate was monitored. The eluted fractions were subjected to amino acid sequence analysis with an applied Biosystems model 477A sequencer.

## 3. RESULTS AND DISCUSSION

Platelet responses to agonists are believed to be mediated by phospholipase C activation. Recently, substantial evidence has been accumulated which indicates that phospholipase C activity in platelet is regulated via a GTP-binding protein,  $G_p$  [17,22,23]. This protein is believed to be IAP-sensitive. However, at present, it remains unclear whether regulation of phospholipase C involves a novel GTP-binding protein, or a new role for a previously characterized IAP-sensitive protein. GTP-binding proteins in platelet membranes were detected by IAP-catalyzed ADP-ribosylation or immunoblot analysis, but none have been isolated. Thus, we attempted to purify and identify GTP-binding protein(s) of human platelet membranes.

Proteins obtained by solubilization of human platelet membranes with sodium cholate were applied to columns of Q-Sepharose, heparin-Sepharose, Sephacryl S-300(HR), phenyl-Sepharose and DEAE-Toyopearl 650(S), successively. After these five chromatography steps, IAP substrate-rich fractions were further purified on an HCA-100S column (fig.1). Two peaks of the IAP substrate activity eluting from the column had GTP $\gamma$ S-binding activities. The polypeptide compositions of the major fractions analyzed by SDS-PAGE are also shown in fig.1, together with the autoradiogram of the same fractions radiolabeled by IAP and [ $^{32}\text{P}$ ]NAD. These results indicate that there are two kinds of  $\alpha$  that are the substrates of IAP-catalyzed ADP-ribosylation in human platelet

membranes. The molecular mass of the  $\alpha$  of the main substrate (G(I)) was 40 kDa on SDS gels (fig.2). On the other hand, the  $\alpha$  of the minor IAP-substrate (G(II)) had the same molecular mass as that of  $G_{i1}$  ( $\alpha_{41}\beta\gamma$ ) on SDS gels (fig.2).  $\beta$ -Subunits of 35 and 36 kDa were also observed. It is noteworthy that the content of 36 kDa  $\beta$  is almost equal to that of 35 kDa in human platelet membranes while  $G_{i1}$  ( $\alpha_{41}\beta\gamma$ ),  $G_{i2}$  ( $\alpha_{40}\beta\gamma$ ) or  $G_o$  ( $\alpha_{39}\beta\gamma$ ) has the main  $\beta$  with a molecular mass of 36 kDa (fig.2).

Furthermore, immunochemical cross-reactivities were examined for  $\alpha$  of G(I),  $\alpha$  of G(II),  $G_{i1}$   $\alpha$  and  $G_o$   $\alpha$ .  $G_{i1}$   $\alpha$  and  $G_o$   $\alpha$  resolved from purified rat brain  $G_{i1}$  and  $G_o$  were used to prepare affinity-purified antibodies for immunoblot analysis [11]. As shown in fig.3A, an antibody raised against rat brain  $G_{i1}$   $\alpha$  (anti- $\alpha_{41}$ ) reacted with porcine brain  $G_{i1}$   $\alpha$ , but not with porcine brain  $G_o$   $\alpha$  or  $\alpha$  of platelet G(I). On the other hand, the antibody raised against rat brain  $G_o$   $\alpha$  (anti- $\alpha_{39}$ ) reacted with porcine brain  $G_o$   $\alpha$ , but not with porcine brain  $G_{i1}$   $\alpha$ ,  $G_{i2}$   $\alpha$  or  $\alpha$  of platelet G(I) (fig.3B). Thus, neither anti- $\alpha_{41}$  nor - $\alpha_{39}$  cross-reacted with  $\alpha$  of platelet G(I), suggesting that G(I) was immunologically different from brain  $G_{i1}$  or  $G_o$ . Anti- $\alpha_{41}$  cross-reacted with  $\alpha$  of G(II). However, the homology of the amino acid sequences between  $G_{i1}$   $\alpha$  and  $G_{i3}$   $\alpha$  is more than 90% and it is difficult to identify G(II) as  $G_{i1}$ . The antibody raised against the identical or highly homologous sequences (anti- $\alpha$ ) reacted with these five  $\alpha$  subunits (fig.3C). The  $\beta\gamma$ -subunits resolved from rat brain  $G_{i1}$  and  $G_o$  were also used to prepare an affinity-purified antibody. The antibody thus obtained (anti- $\beta$ ) reacted with all the  $\beta$ -subunits of  $G_{i1}$ ,  $G_{i2}$ ,  $G_o$ , G(I) and G(II), suggesting that the  $\beta$  of the five IAP substrates are the same polypeptide or at least share (a) common antigenic determinant(s) (fig.3D).

For amino acid sequence analysis, platelet G(I) was resolved into the  $\alpha$  and  $\beta\gamma$  as described above. The  $\alpha$  was digested with TPCK-trypsin, and the cleavage products were separated by reverse-phase high-performance liquid chromatography (fig.4). Five fractions (1–5) corresponding to the absorbance peaks were collected and subjected to amino acid sequence analysis. Fractions 3–5 contained a single peptide, while fractions 1 and 2 were mixtures of two and three peptides, respectively. The sequences determined were as follows: 1-1, EIYT-

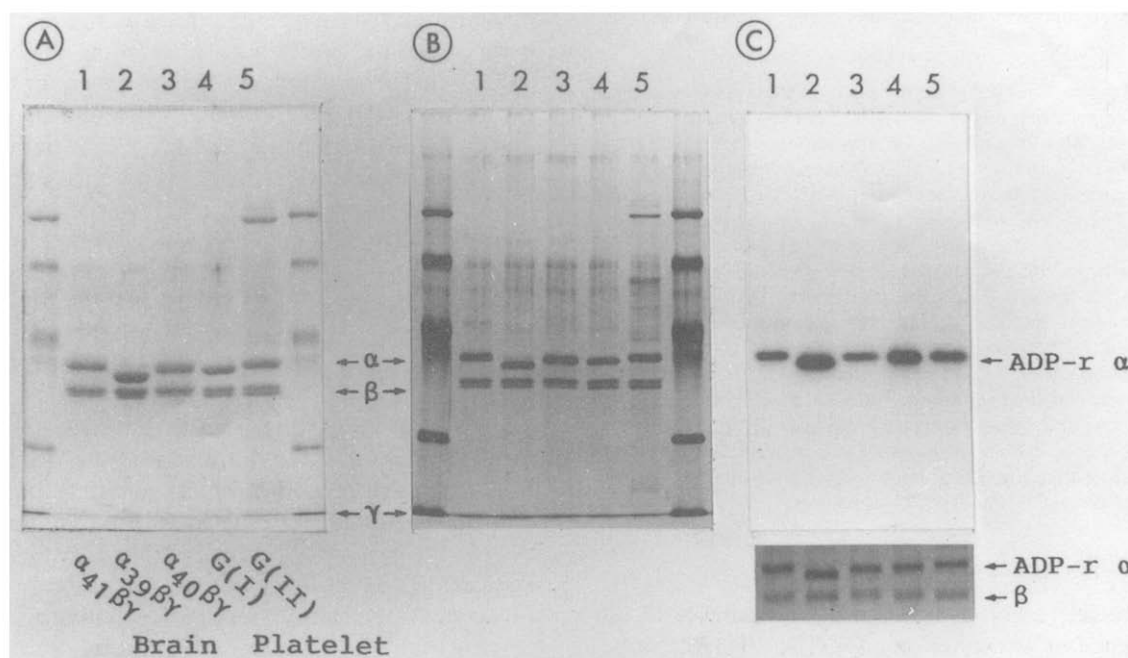


Fig.2. SDS-polyacrylamide gel electrophoresis and IAP-catalyzed ADP-ribosylation of IAP substrates purified from human platelet membranes. Proteins ( $1 \mu\text{g}$ ) were subjected to SDS-polyacrylamide gel (10%) electrophoresis. The gel was then stained with Coomassie blue (A) or silver (B). Proteins which had been [ $^{32}\text{P}$ ]ADP-ribosylated by IAP and [ $^{32}\text{P}$ ]NAD were also subjected to SDS-PAGE followed by silver stain ((C), lower) and autoradiography ((C), upper). Lanes: 1, porcine brain  $G_{i1}$ ; 2, porcine brain  $G_o$ ; 3, porcine brain  $G_{i2}$ ; 4, human platelet  $G(I)$ ; 5, human platelet  $G(II)$ .

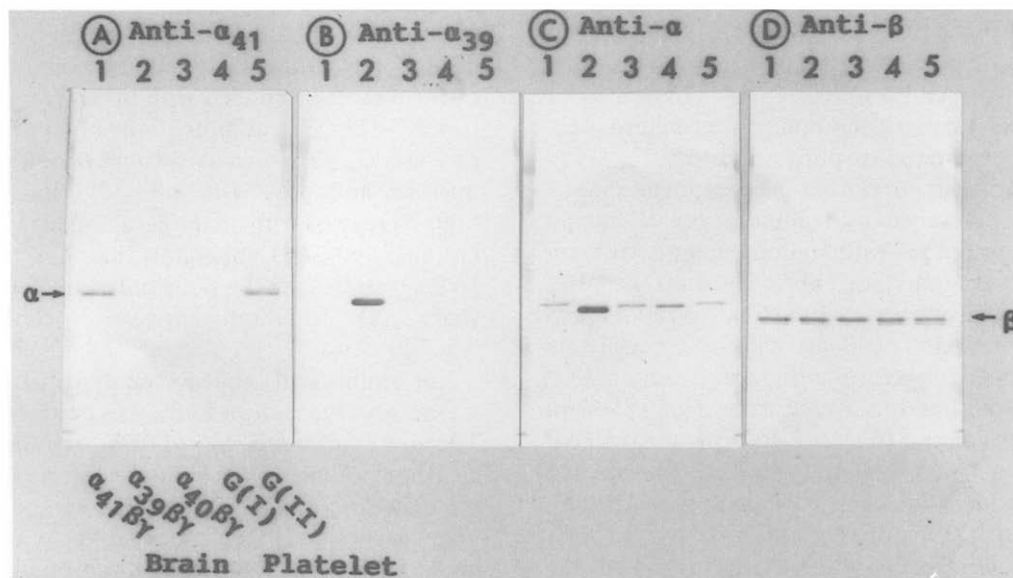


Fig.3. Cross-reaction of human platelet IAP substrates with affinity-purified antibodies specific for the rat brain IAP substrates. Two IAP substrates ( $1 \mu\text{g}$ ) purified from human platelet membranes were subjected to SDS-polyacrylamide gel (10%) electrophoresis and analyzed for immunoblot as described in section 2. Lanes: 1,  $G_{i1}$  ( $\alpha_{41}\beta \gamma$ ); 2,  $G_o$  ( $\alpha_{39}\beta \gamma$ ); 3,  $G_{i2}$  ( $\alpha_{40}\beta \gamma$ ); 4, human platelet  $G(I)$ ; 5, human platelet  $G(II)$ . (A) Anti-rat  $\alpha_{41}$  antibody, (B) anti-rat  $\alpha_{39}$  antibody, (C) anti-porcine  $\alpha$  antibody, (D) anti-rat  $\beta$  antibody.

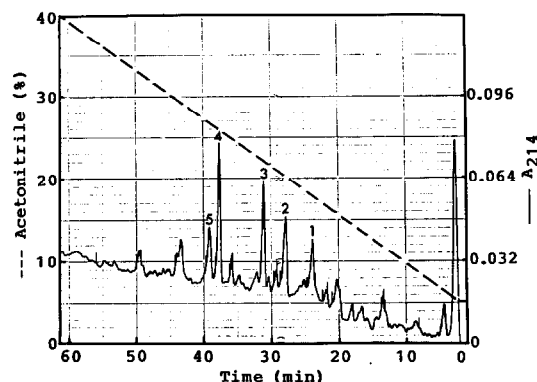


Fig.4. Reverse-phase HPLC analysis of tryptic peptides from human platelet G(I). Tryptic peptides of G(I) were applied to a PepRPC HR5/5 column and eluted as described in section 2. Five peaks (1-5) were subjected to amino acid sequence analysis.

HFTXAT; 1-2, LFDSIXNNK; 2-1, IAQSDYIPT-QQDVLR; 2-2, LLLLGAGESGK; 2-3, DXGLF; 3, LWADHGVQAXFGR; 4, EYQLNDSAAYYL-NDLER; 5, ITHSPLTIXFPEYTGANK. Assignment of the peptides in fraction 1-5 was based on the predicted amino acid sequences from the known GTP-binding protein  $\alpha$ -subunit genes and cDNAs. The partial amino acid sequences of  $\alpha$  of G(I) were completely identical with the predicted sequences of the human  $G_{i2}$   $\alpha$  gene [2], thereby suggesting that the major pertussis toxin substrate in human platelet membranes is  $G_{i2}$ , which has been isolated from porcine brain [11,12].

In this report, we have purified the main IAP substrate, referred to as G(I), from human platelet membranes. The physiological role of G(I) is unknown at present. Since hormonal inhibition of adenylate cyclase activity may be mediated at least in part by the release of  $\beta\gamma$ -subunits [21], G(I) is also thought to have a function, as have  $G_{i1}$  and  $G_o$ , in receptor-linked inhibition of the cyclase. Nevertheless, it is tempting to speculate that  $\alpha$  of G(I) is capable of interacting with an enzyme system other than adenylate cyclase, e.g. phospholipase C, since recent studies have revealed that prior exposure of platelet to IAP attenuates the cell responses to receptor stimulation in a cAMP-independent manner [17,18].

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