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Identification and purification of a novel G protein from neutrophils

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A novel G protein which appears to couple chemotactic peptide receptors to a polyphosphoinositide phospholipase C has been purified from rabbit neutrophils. Neutrophil membranes were solubilized with sodium cholate and fractionated by successive anion exchange, gel filtration and hydrophobic chromatography. Guanosine-5'-(3-*O*-thio)triphosphate binding activity was purified 170-fold from the soluble extract. The α-subunit of the purified G protein was identified by pertussis toxin-catalyzed ADP-ribosylation, and found to have an M_r of 40000. The β-subunit (M_r 36000) comigrated on SDS-polyacrylamide gel electrophoresis with the β-subunits of bovine brain Gi and Go. The neutrophil pertussis toxin substrate is highly unstable in cholate solution unless 30% ethylene glycol is added. Structural and functional analysis of this novel G protein will advance our understanding of the molecular mechanisms of coupling of receptors to phospholipase C.

fMet-Leu-Phe receptor; Guanine nucleotide-binding protein; Phospholipase C; (Rabbit neutrophil)

1. INTRODUCTION

The α-subunits of several of the G proteins (Gi, Go and transducin) are ADP-ribosylated in the presence of pertussis toxin [1–3]. Treatment of neutrophil membranes with pertussis toxin results in the ADP-ribosylation of a single protein (M_r approx. 40000) [4,5] and in the abrogation of phospholipase C-mediated polyphosphoinositide hydrolysis in response to the binding of chemotactic peptides [6,7].

The pertussis toxin substrate in neutrophils has been demonstrated to be immunochemically distinct from Gi (M_r 41000), Go (M_r 39000) or transducin (M_r 39000) [8]. Furthermore, pertussis intoxication of neutrophils does not augment agonist-induced cAMP generation [4,9] as it does in systems in which Gi is known to be present [10,11] indicating that Gi is not functionally present in neutrophils. We report here the purification of this novel pertussis toxin substrate from neutrophils, and propose that it be named Gc for the G protein which appears to mediate the stimulation of polyphosphoinositide hydrolysis by phospholipase C in neutrophils.

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Abbreviations: Gs, Gi, Go, members of a family of GTP-binding membrane proteins including the regulatory components of adenylyl cyclase (Gs and Gi) and transducin; Gc, a new GTP-binding protein from membranes of rabbit neutrophils; GTPγS, guanosine 5'-(3-*O*-thio)triphosphate; NAD, nicotinamide adenine dinucleotide; DEAE, diethylaminoethyl; TED-EG, 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mM dithiothreitol, 30% ethylene glycol

2. MATERIALS AND METHODS

2.1. *Materials*

[^{35}S]GTPγS (1038 Ci/mmol) was from New England Nuclear and [*adenylate*-^{32}P]NAD

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(227 Ci/mmol) was from ICN. Pertussis toxin was obtained from List Biological Laboratories. DEAE-Trisacryl and Ultrogel AcA-34 were from LKB. Phenyl-Sepharose and cholate were purchased from Sigma. Cholate was recrystallized twice. All other materials and chemicals were purchased from commercial sources and were used as obtained.

2.2. *Detergent extraction*

Rabbit neutrophils were obtained from sterile peritoneal exudates as described [12] and erythrocytes were hypotonically lysed. The neutrophils were treated with diisopropylfluorophosphate at 2 μg/ml for 10 min, then pelleted and resuspended in 0.25 M sucrose, 10 mM Tris (pH 7.4), 2 mM phenylmethylsulfonyl fluoride, 2 mM EGTA, 2 mM EDTA and 5 μg/ml leupeptin. Cells were lysed by sonication for 15 s at 40 W. 1 g of membranes was solubilized for 30 min at 4°C in a final volume of 85 ml of 2% cholate in TED-EG. The soluble extract (75 ml) was diluted to 150 ml with TED-EG.

2.3. *DEAE-Trisacryl chromatography (fig.1A)*

The diluted soluble extract was applied to a 100 ml column of DEAE-Trisacryl which had been equilibrated with TED-EG and 1% cholate. The column was eluted at 0.8 ml/min with a 200 ml linear gradient of NaCl (0–225 mM) in TED-EG and 1% cholate. The peak of [^{35}S]GTPγS binding (fractions 57–65) was pooled and concentrated to 2 ml by ultrafiltration through an Amicon PM-30 membrane.

2.4. *Gel-filtration chromatography (fig.1B)*

The concentrated DEAE peak was then fractionated on a column (1 × 52 cm) of Ultrogel AcA-34 in TED-EG, 100 mM NaCl and 1% cholate. The flow rate was 0.2 ml/min and 1 ml fractions were collected.

2.5. *Phenyl-Sepharose chromatography (fig.1C)*

The pooled peak of [^{35}S]GTPγS binding from the AcA-34 column (fractions 23–27) was diluted to 0.25% cholate with TED-EG, and made 500 mM with NaCl. This solution was applied to a 10 ml phenyl-Sepharose column that had been equilibrated with TED-EG, 0.25% cholate and 500 mM NaCl. The [^{35}S]GTPγS binding activity was eluted with a 70 ml linear gradient starting with the equilibration solution and ending with TED-EG, 4% cholate and 50 mM NaCl. Fractions 60–65 were pooled for the calculation of purification and yield (table 1).

2.6. *Assays*

The presence of G proteins was measured by their ability to bind [^{35}S]GTPγS [13]. G protein subunits were separated by electrophoresis of

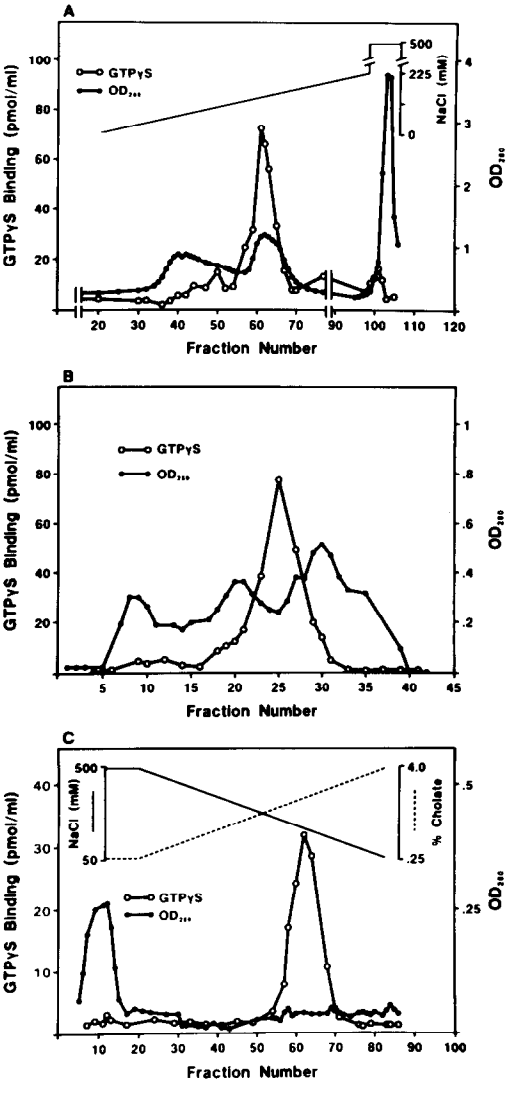

Fig.1. Chromatography of [^{35}S]GTPγS binding activity on sequential columns. (A) DEAE-Trisacryl, (B) Ultrogel AcA-34, (C) phenyl-Sepharose. See section 2 for details.

Table 1

Purification of [^{35}S]GTPγS binding activity from neutrophil membranes

Step	Volume (ml)	Protein (mg)	GTPγS binding (nmol)	Specific activity (nmol/mg)	Recovery (%)
Membranes	60	1068	35.1	0.033	100
Extract	150	338	8.5	0.022	36
DEAE	18	45.2	0.74	0.033	4
Ultrogel	5	3.7	0.29	0.157	1.6
Phenyl-Sepharose	6	0.046	0.086	3.73	0.4

samples through SDS-polyacrylamide gels using the procedure described by Laemmli [14]. Proteins were visualized by silver staining. Identification of the α-subunit of Gc was established by ADP-ribosylation as described [15]. For purposes of comparison, Gi and Go were purified from bovine brain as described [16], except that octyl-Sepharose was used for the hydrophobic chromatography. Protein was determined by the method of Bradford [17].

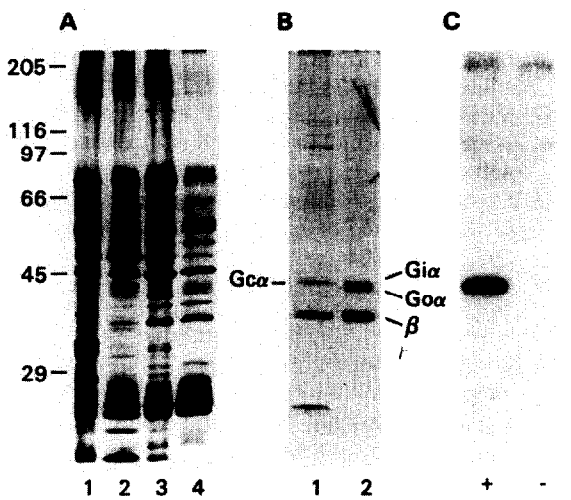

Fig.2. Samples were subjected to electrophoresis through a 1.5 mm slab gel of 10% polyacrylamide and stained with silver. (A) Lanes: 1, crude membranes (18 μg); 2, cholate extract (10 μg); 3, DEAE-Trisacryl [^{35}S]GTPγS binding peak (pooled fractions 57–65) (8.5 μg); 4, Ultrogel AcA-34 binding peak (pooled fractions 23–27) (1.6 μg). (B) Lanes: 1, phenyl-Sepharose [^{35}S]GTPγS binding peak (pooled fractions 60–65) (265 ng); 2, bovine brain Gi and Go (500 ng), purified as described in section 2. (C) Purified Gc was incubated with (+) or without (−) pertussis toxin in the presence of [^{32}P]NAD. Aliquots were electrophoresed through a 10% SDS-polyacrylamide gel, and the autoradiogram was developed overnight. The single band visible is of M_r 40000.

3. RESULTS

We initially based our purification strategy on the methodologies described for the purification of the G proteins Gs, Gi and Go [13,16]. However, in contrast to these previously described G proteins, the [^{35}S]GTPγS binding activity of neutrophil membranes was highly labile after solubilization with cholate. The [^{35}S]GTPγS binding to native membranes showed no appreciable decline after one week at 4°C, but the [^{35}S]GTPγS binding in 1% cholate solution decayed with a $t_{1/2}$ of 1.2 days. The pseudoligand AMF (20 mM $AlCl_6$, 6 mM $MgCl_2$ and 10 mM NaF) only increased the $t_{1/2}$ to 1.5 days. However, the inclusion of 30% ethylene glycol increased the $t_{1/2}$ for decay of [^{35}S]GTPγS binding to 7 days. Ethylene glycol was therefore used throughout the purification to stabilize the G protein.

The detergent solubilized [^{35}S]GTPγS binding activity was fractionated on successive columns as illustrated in fig.1. Octyl-Sepharose was used for the hydrophobic chromatography of Gi and Go from brain, but Gc could not be eluted from this column and phenyl-Sepharose was used instead. SDS-polyacrylamide gel electrophoresis of the crude membranes, the cholate extract, and of the peaks of [^{35}S]GTPγS binding from the DEAE and Ultrogel columns is shown in fig.2A. The major bands visible from the phenyl-Sepharose [^{35}S]-GTPγS binding peak are of M_r 40000, 36000 and approx. 23000 (fig.2B).

Since the pertussis toxin substrate in neutrophils is known to be distinct from Gi, Go or transducin [8], the α-subunit of the neutrophil G protein was identified by ADP-ribosylation using pertussis toxin. A single band of M_r 40000 was ADP-ribosylated in both unsolubilized neutrophil membranes and in purified protein from the phenyl-Sepharose column (fig.2C). The α-subunit of Gc comigrated on SDS-polyacrylamide gel electrophoresis with Giα (fig.2B). The major band with an M_r of 36000 which copurifies with Gcα comigrates on SDS-polyacrylamide gel electrophoresis with the β-subunits of bovine brain Gi and Go (fig.2B), consistent with previous studies which have shown immunologic homology between neutrophil and brain β-subunits [8].

A representative purification of Gc is illustrated in table 1. A 170-fold purification is reported from the cholate extract, but actual purification of the protein is undoubtedly greater due to the decay of solubilized GTPγS binding activity during the 5 day purification.

4. DISCUSSION

Although a phospholipase C-coupled G protein has been implicated in several systems, its isolation has not yet been achieved. In some systems, a convenient assay such as ribosylation is not available [18,19], whereas in other systems it may be a minor G protein. Moreover, at least in the neutrophil, it is a labile protein after solubilization with cholate. The key to our purification was stabilization of the [^{35}S]GTPγS binding activity with ethylene glycol, and the choice of cells. The G protein coupled to phospholipase C in neutrophils is readily identifiable by pertussis toxin-catalyzed ribosylation [4,5], and the neutrophil pertussis toxin substrate is an abundant membrane protein.

An important finding is that Gc is distinct from Gi and Go, in that it is very unstable in an aqueous cholate solution unless ethylene glycol is added, it does not elute from octyl-Sepharose with up to 4% cholate and, as reported by others, it is functionally [4,9] and immunochemically distinct [8].

Identification and purification of this novel G protein from rabbit neutrophils will allow sequencing of this protein for the production of specific anti-peptide antibodies and the molecular cloning of the gene. Furthermore, since this G protein is purified in its 'unactivated' form using ethylene glycol as a stabilizer rather than an activating ligand, the purified protein is suitable for use in functional reconstitution experiments with stimulatory receptors and phospholipase C.

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