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Interaction of α subunit of GTP-binding protein Go with a 20-kDa Triton-insoluble membrane protein in bovine brain

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Heterotrimeric Go bound to the membranes of bovine brain, but Goα remained bound to the membranes even after activation with GTPγS. Furthermore, Goα bound to a Triton X-100-insoluble fraction of the membranes in a saturable manner. However, the 37-kDa Goα eliminated by trypsin at the amino-terminus could not bind to the fraction. Using a blot overlay approach of the Triton-insoluble fraction, only a 20-kDa protein was identified that interacts with Goα. These results indicate that Goα binds to a 20-kDa Triton-insoluble protein in the bovine brain membranes.

GTP-binding protein; Triton-insoluble fraction; Bovine brain

1. INTRODUCTION

GTP-binding proteins (G proteins) function as intermediaries in transmembrane signaling pathways [1] and are composed of a unique α subunit and common βγ subunits. The α subunit appears to specify the function of a particular G protein in receptor-effector coupling. In contrast, the βγ subunits are functionally interchangeable and appear to interact identically with different α subunits [2]. From their hydrophobicity, the βγ subunits have been assumed to serve as an anchor for the α subunit on plasma membranes [3]. G proteins undergo a cycle of subunit dissociation and reassociation according to a cycle of GDP–GTP exchange and hydrolysis. The α subunits of Go and Gi have been observed to remain bound to the plasma membrane even after activation with GTPγS [3]. Therefore, the activated α subunits appear to interact with effectors as the membrane-bound form. However, the association site of α subunits in the membranes is not understood. In this communication, we characterized the association site of Goα in bovine brain membranes. We report here that Goα binds to a 20-kDa Triton-insoluble protein in the membranes.

2. MATERIALS AND METHODS

2.1. *Preparation of the membranes and the Triton X-100-insoluble fraction from bovine brain*

Bovine brains were freshly obtained from a local slaughterhouse. Cerebra were dissected crudely and preparation of the membranes was carried out essentially according to the method of Sternweis and Robishaw [4]. The membranes were extracted with 1% Triton X-100 for 30 min at 0°C. The Triton-insoluble fraction was obtained by centrifugation at 300,000 × *g* for 20 min. The pellet was washed with 1% Triton X-100 and suspended in 20 mM Tris-HCl, pH 7.4, containing 1 mM EDTA and 1 mM dithiothreitol to the protein concentration of 10 mg/ml. Protein concentrations were determined by the method of Lowry et al. [5] with bovine serum albumin as a standard.

2.2. *Binding of Go or Goα to the membranes or the Triton-insoluble fraction*

Go was purified from bovine brain according to the method of Katada et al. [6]. To monitor Goα, Go was [^{32}P]ADP-ribosylated with pertussis toxin (Seikagaku Kogyo, Tokyo, Japan) as described previously [4]. The labeled Goα was separated from βγ subunits on Phenyl–Sepharose CL-4B (Pharmacia LKB Biotechnology Inc.) column as described previously [7]. Limited tryptic digestion of [^{32}P]ADP-ribosylated Goα was performed as reported previously [8]. The membranes (150 μg) or the Triton-insoluble fraction (30 μg), which was derived from the solubilization of 150 μg of the membranes with Triton X-100, was incubated for 30 min with the [^{32}P]ADP-ribosylated Go (5 pmol) at 0°C or the labeled Goα (5 pmol) at 30°C in 30 μl of 20 mM Tris-HCl, pH 7.4, containing 1 mM EDTA, 1 mM dithiothreitol, 10 mM $MgCl_2$ and 0.1 mM phenylmethylsulfonyl fluoride (buffer A), unless otherwise indicated. The Go-incorporated membranes or fractions were washed with buffer A. The incubation mixture was separated into supernatant and particulate fractions by centrifugation at 300,000 × *g* for 20 min. Each fraction was subjected to SDS-10% PAGE followed by autoradiography. The radioactivity of the [^{32}P]ADP-ribosylated protein corresponding to a molecular weight of 39,000 or 37,000 Da was determined by scintillation counting.

2.3. *Blot-overlay system for the labeled Goα*

After the Triton-insoluble fraction (40 μg) had been subjected to SDS-10% PAGE and transferred to a polyvinylidene difluoride membrane (Immobilon, Millipore), the blot was incubated with 10 mM Tris-HCl, pH 7.4, containing 0.15 M NaCl and 1% bovine serum albumin (BSA) and washed with the buffer without BSA. The blot was then incubated for 30 min at 30°C with [^{32}P]ADP-ribosylated Goα (20 pmol) in 20 mM Tris-HCl, pH 7.4, containing 1 mM EDTA, 0.15 M NaCl and 2.5% BSA. After washing with the same buffer without BSA, the blot was air-dried and subjected to autoradiography.

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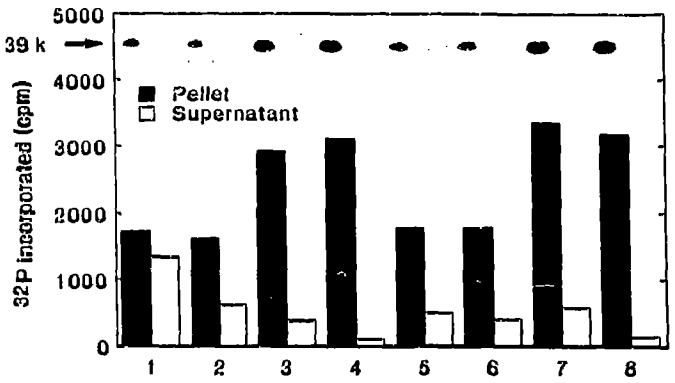

Fig. 1. Interaction of heterotrimeric Go with the bovine brain membranes. [^{32}P]ADP-ribosylated Go (5 pmol) was incorporated into 150 μg of the intact membranes (columns 1,2), 30 μg of the Triton-insoluble fraction (columns 3,4), 30 μg of 30-min trypsin-treated Triton-insoluble fraction (columns 5,6) or 30 μg of 3-min boiled Triton-insoluble fraction (columns 7,8). The Go-bound membranes or fractions were incubated for 30 min at 30°C with (even numbers) or without (odd numbers) 100 μM GTPγS. After the incubation, the amounts of the Goα remained in the pellets (filled column) or released into the supernatants (open column) were determined as described in section 2. The results shown are representative of three independent experiments that yielded similar results.

3. RESULTS AND DISCUSSION

We examined the interaction of heterotrimeric Go with the membranes of bovine brain. As shown in Fig. 1, [^{32}P]ADP-ribosylated Go bound to the membranes, but bound Goα did not release from the membranes in the presence of GTPγS. Go bound more effectively to the Triton-insoluble fraction than the intact membranes, and bound Goα also was not released from the fraction in the presence of GTPγS. The treatment of the Triton- insoluble fraction with trypsin partly reduced the binding activity of Go, but boiling the fraction did not affect the activity. These results suggest that Goα itself binds to the membranes even after activation with GTPγS, and that the binding site of Goα is a Triton-insoluble material that is heat-stable but partly sensitive to trypsin. We further examined the direct interaction of Goα with the Triton-insoluble fraction. As shown in Fig. 2A, both GTPγS- and GDPβS-bound forms of Goα could bind to the Triton-insoluble fraction. GTPγS-bound Goα dose-dependently bound to the fraction, and this binding appeared to be saturable (Fig. 2B). The treatment of the Triton-insoluble fraction with phospholipase A_2 or phospholipase C did not affect the binding of Goα, but the treatment of the fraction with trypsin partly suppressed the binding (data not shown), suggesting that the binding site is of a protein nature but is not a phospholipid.

Previously, it was reported that tryptic digestion of brain and neutrophil membranes cleaved the membrane bound Gα at a site near the amino-terminus and released amino-terminal 2-kDa fragments and the large tryptic fragments, suggesting that α subunits of G proteins are tethered to the plasma membrane via their amino-termini [3]. We next examined the effect of tryptic cleavage of Goα on the binding to the Triton-insoluble fraction. Trypsin cleaved [^{32}P]ADP-ribosylated Goα, producing the labeled digestive 37-kDa fragment

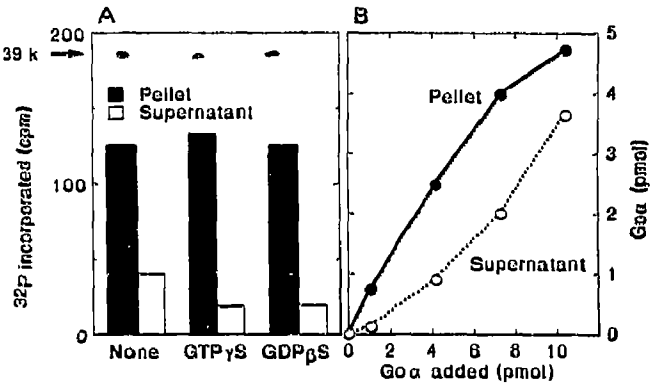

Fig. 2. Interaction of Goα with the Triton-insoluble fraction. (A) After [^{32}P]ADP-ribosylated Goα (5 pmol) had been incubated for 30 min at 30°C with vehicle, 100 μM GTPγS or 100 μM GDPβS, the Goα was further incubated for 30 min at 30°C with the Triton-insoluble fraction (30 μg). (B) After Goα had been labeled with [^{35}S]GTPγS, the indicated amounts of [^{35}S]GTPγS-labeled Goα were incubated with the Triton-insoluble fraction (30 μg). The amounts of the Goα incorporated into the pellets (filled column, ●) or remained in the supernatants (open column, ○) were determined as described in section 2. The results shown are representative of three independent experiments that yielded similar results.

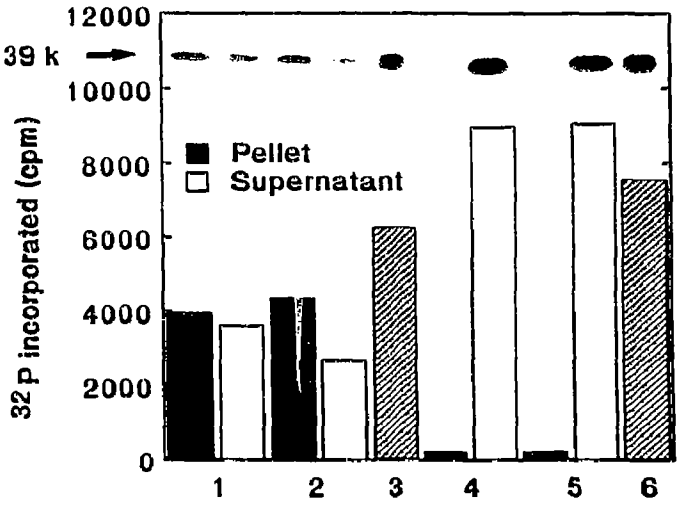

Fig. 3. Effect of limited tryptic digestion of Goα on the interaction of Goα with the Triton-insoluble fraction. After [^{32}P]ADP-ribosylated Goα (5 pmol) had been digested with trypsin (3 μg/ml) in the presence of 100 μM GTPγS, the intact Goα (columns 1,2) or the digested Goα (columns 4,5) were incubated for 30 min at 30°C with 150 μg of the intact membranes (columns 1,4) or 30 μg of the Triton-insoluble fraction (columns 2,5). The amounts of the Goα incorporated into the pellets (filled column) or remained in the supernatants (open column) were determined as described in section 2. Columns 3 and 6 (hatched columns) show the total Goα and the total digested Goα, respectively. The results shown are representative of three independent experiments that yielded similar results.

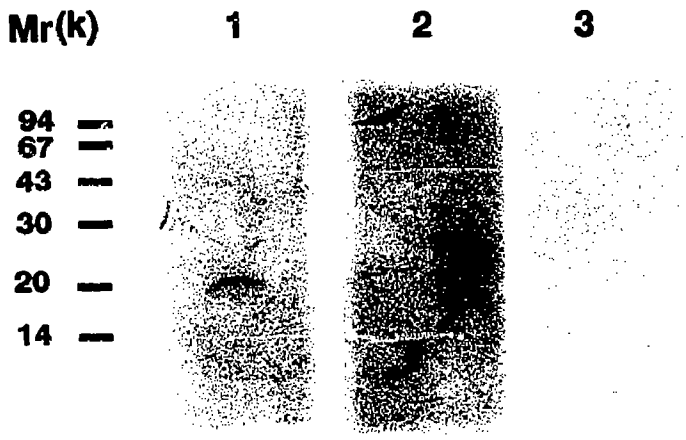

Fig. 4. Interaction of Goα with the immobilized Triton-insoluble protein. A blot of the Triton-insoluble fraction was overlaid with the [^{32}P]ADP-ribosylated Goα (20 pmol) in the presence (lane 2) or absence (lane 1) of unlabeled Goα (20 nmol) or with the labeled 37-kDa Goα digested with trypsin (20 pmol) (lane 3) as described in section 2. The arrows indicate the positions of known protein standards.

without the 2-kDa amino-terminal domain (Fig. 3, columns 3 and 6). As shown in Fig. 3, whereas intact Goα bound to the membranes or the Triton-insoluble fraction, the 37-kDa fragment of Goα could not bind to them. These results indicate that the amino-terminal domain of Goα is necessary for the interaction with the Triton-insoluble fraction. In order to identify the binding site of Goα in the Triton-insoluble fraction, the Triton-insoluble fraction was transferred to a polyvinylidene difluoride membrane and overlaid with the labeled Goα. Fig. 4 shows an autoradiogram of the blot. Goα bound to the protein with an apparent molecular weight of 20,000 Da, and this binding was significantly reduced by the addition of a 10-fold excess of unlabeled Goα, indicating that this binding is specific. On the other hand, the 37-kDa Goα eliminated by trypsin could not bind to the 20-kDa protein, indicating that the 20-kDa protein recognizes the amino-terminal domain of Goα.

Sternweis has already shown that purified Goα showed little interaction with phospholipid vesicles but bound to vesicles containing βγ subunits [2], suggesting that βγ subunits serve as an anchor for Goα but Goα cannot bind to a lipid bilayer. On the other hand, many G protein α subunits including Goα are myristoylated, the site of modification being the amino-terminal glycine [9]. When myristoylated α subunits are expressed in COS cells, α subunits accumulate in substantial excess over βγ subunits, but they are still associated with the membrane [10]. However, removal by site-directed mutagenesis of the *N*-myristoylation consensus sequence from α subunits leads to a loss of membrane localization [10,11]. Therefore, the myristoylation of α subunits is thought to be regulated for the continued association of the α subunits with the membrane following activation from βγ subunits [9]. In this study, we demonstrated that Goα binds to a 20-kDa Triton-insoluble protein in the membranes and this membrane protein may recognize the amino-terminal domain of Goα, including myristate.

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