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Subunit interactions of the Go protein

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The monoclonal antibody, MONO, recognizes an epitope on the G protein αo-subunit [van der Voorn et al., submitted] and readily immunoprecipitates heterotrimeric Go proteins from solubilized, crude bovine brain membranes, as well as from a purified bovine brain G protein preparation. Upon incubation of the immunoprecipitates with GTPγS, all βγ-subunits are released from the αo-subunit. Thus, binding of MONO to the Go protein does not appear to interfere with release of bound GDP, binding of GTPγS or GTPγS-induced subunit dissociation. However, we have been unable to induce a similar dissociation of Go using its physiological activator, GTP. Surprisingly, we did not observe any dissociation of Go (bound to MONO) upon dilution in a range from 500 to 5 nM. Since an apparent K_d of αo-GDP for binding βγ of 340–390 nM has been reported [(1989) J. Biol. Chem. 264, 20688–20696] our results would suggest that binding of MONO to the αo-subunit induces an increased affinity of αo-GDP for βγ. Alternatively, these results could be explained if, under the conditions used, the K_d of αo-GDP for βγ were at least two orders of magnitude lower than estimated previously.

1. INTRODUCTION

Heterotrimeric guanine nucleotide binding regulatory proteins (G proteins) serve to transduce signals from a variety of transmembrane receptors to a heterogeneous group of intracellular effectors. All known G proteins have a common design: they consist of a 39–52 kDa α-subunit that can bind guanine nucleotides and possesses intrinsic GTPase activity, a 35–36 kDa β-subunit and a γ-subunit of about 10 kDa (for recent reviews see [1–4].

G proteins cycle between an inactive, GDP-bound- and an active, GTP-bound form (reviewed in [5,6]. Upon association with GTP, G proteins regulate the activity of appropriate effectors until hydrolysis of GTP to GDP restores the system to its resting state. The dissociation of GDP, the rate limiting step in the basal GTPase cycle, is markedly enhanced by interaction with ligand-occupied receptors. Studies with non-hydrolyzable GTP analogues have indicated that a cycle of subunit dissociation is superimposed on the GTPase cycle: binding of GTPγS to the G protein α-subunit is accompanied by dissociation of the βγ-subunits from the complex [7–9].

Recent experiments in mammalian systems suggest that not only GTP-liganded α-subunits but also free βγ-subunits may directly inhibit or stimulate the activity of specific effectors [10,11]. Dissociation of an activated G protein into its α- and βγ-subunits may thus result in an early bifurcation of the signal transduction pathway. This finding implies that the amounts of free βγ-subunits in the membrane must be tightly regulated. It is presumed that, upon hydrolysis of bound GTP, the GDP-liganded α-subunit again displays high affinity for βγ-subunits. Consequently, hydrolysis of GTP to GDP may lead not only to inactivation of α-subunits, but also to sequestration and concomitant 'inactivation' of free βγ-subunits. In contrast to the free α- and βγ-subunits, the resulting heterotrimeric G protein can be activated efficiently by ligand-occupied receptors [12].

In spite of the postulated, important role of subunit dissociation and re-association, the interactions between G protein α- and βγ-subunits have been difficult to investigate directly. We have described the development and characterization of a monoclonal antibody (MONO) that recognizes an epitope on the Goα-subunit (van der Voorn et al., submitted). Here we show that, using MONO, heterotrimeric Go can be immunoprecipitated from solubilized crude bovine brain membranes, as well as from a purified bovine brain G protein preparation. Making use of this unique property of MONO, we examined the interaction between αo and its βγ-subunits under various conditions.

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2. MATERIALS AND METHODS

2.1. *Materials and miscellaneous methods*

Crude bovine brain membranes were prepared and bovine brain G proteins were purified according [13]. Purified proteins and membranes were aliquoted, quick-frozen in liquid N_2 and stored at −80°C until use. Protein concentrations were determined by the method of

Bradford [14] using bovine serum albumin fraction V (BSA) (Sigma) as a standard. Nucleotides were obtained from Sigma. The purity of several batches of GTP was analyzed by HPLC, showing that >95% of the nucleotide present in these samples was indeed GTP (data not shown).

Mouse monoclonal antibody MONO (IgG1) was generated and characterized as described (van der Voorn et al., submitted): MONO recognizes an epitope located between amino acid 80 and 145 on the bovine αo-subunit. The presence of a proline at position 122 of αo is essential for recognition by MONO. MONO immunoglobulins were crosslinked to prot A-Sepharose beads (Pharmacia) according to [15]. All incubations with MONO-beads were done under constant agitation.

2.2. *Immunoprecipitation*

Immunoprecipitations were carried out in buffer A: 50 mM Na-HEPES, pH 7.6, 1 mM EDTA, 0.1% (v/v) lubrol PX (Sigma) and 2 mM $MgSO_4$, unless stated otherwise. GDP, GTP, GTPγS, purified bovine brain G proteins or crude bovine brain membranes were added as specified in the legends. Crude bovine brain membranes were lyzed in 1 ml buffer A for 30 min at 4°C. Insoluble debris were spun down (12,000 × *g* for 15 min at 4°C) and the supernatant was used in immunoprecipitation experiments. G proteins were immunoprecipitated either with 100 μl (packed volume) MONO-beads (1 h incubation) or with ascites of the monoclonal antibody in a 1:333 dilution. After an incubation for 45 min at 4°C with ascites, 100 μl of a 10% (w/v) suspension of formalin-fixed *Staphylococcus aureus* (Staph A) in buffer A was added and incubated for a further 15 min. Immune complexes were analyzed by SDS-PAGE on a 12% (w/v) gel [16] and stained with Coomassie blue.

3. RESULTS

Previously, we have demonstrated that virtually all Go can be removed from the bovine brain G protein preparation by immunoprecipitation with the monoclonal antibody, MONO (van der Voorn et al., submitted). In addition, MONO can be used to efficiently immunoprecipitate heterotrimeric Go from solubilized, crude bovine brain membranes (Fig. 1, lanes 1). The amount of Go that is recovered from the membranes is consistent with Go protein making up 0.5–1% of total brain membrane protein [13].

Using MONO to monitor Go subunit interactions, we examined the effects of guanine nucleotides on the stability of the complexes between αo and βγ. Upon pre-incubation of either purified bovine brain G proteins or crude bovine brain membranes with GTPγS, αo-subunits devoid of βγ-subunits were immunoprecipitated by MONO (Fig. 1, lanes 3). Similar results were obtained when Go was first immunoprecipitated using MONO–beads and subsequently incubated in the presence of GTPγS: all βγ-subunits were released (Fig. 2, lane 4). From bovine brain membranes, as well as from the purified bovine brain G protein preparation, comparable amounts of αo-subunits were recovered in the absence or in the presence of GTPγS. Apparently, binding of MONO to the αo-subunit does not interfere with release of bound GDP, with binding of GTPγS or with GTPγS-induced subunit dissociation.

However, we have been consistently unable to induce dissociation of Go when using its natural activator GTP

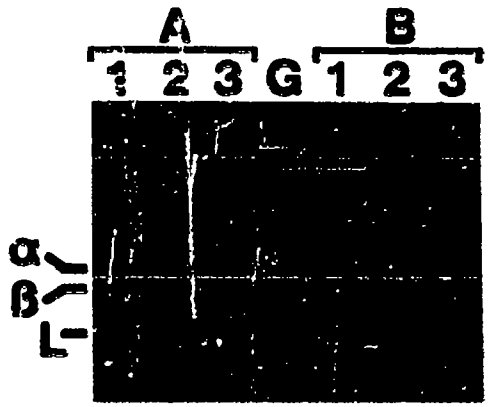

Fig. 1. Effects of guanine nucleotides on Go subunit interactions: incubation of G proteins with guanine nucleotides, followed by immunoprecipitation of Go. 400 μg of solubilized, bovine brain membranes (A) or 2 μg (25 pmol) purified bovine brain G proteins (B) were incubated for 30 min at 20 °C in 2 ml buffer A, supplemented with 0.5 mM ATP and containing either 100 μM GDP (lanes 1), 100 μM GTP (lanes 2) or 100 μM GTPγS (lanes 3). Subsequently, Go was immunoprecipitated using MONO-beads as described in section 2. Immune complexes were analyzed by SDS-PAGE, followed by Coomassie staining. In lane G, 1 μg of purified bovine brain G proteins was loaded. On the left, positions of G protein α- and β-subunits and the position of the immunoglobulin light (L) chain are indicated.

(as opposed to GTPγS) in the presence of physiological Mg^{2+} concentrations (Fig. 1, lanes 2; Fig. 2, lane 5). Comparable results were obtained when using 0.1–10 mM Mg^{2+} or GTP concentrations of 0.5 mM, a value that approximates to intracellular GTP levels [17] (data not shown).

Upon dilution of Go (bound to MONO) in a range from 500 to 5 nM, the amount of αo recovered was constant (Fig. 3), demonstrating that the total Go population originally bound to MONO remained bound during the experiments. At higher dilutions of Go (<5 nM), the affinity of the monoclonal antibody for Go is insufficient, and heterotrimeric Go is lost from the immune complexes (data not shown).

Surprisingly, upon dilution of Go (bound to MONO) in the presence of GDP, and subsequent incubation for 1 h in buffer A, no loss of βγ from the αo-subunit was observed even at the lowest Go concentrations examined (5 nM) (Fig. 3). Similar results were obtained when buffer A was supplemented with 150 mM NaCl (results not shown). As a K_d of αo-GDP for binding βγ of 340–390 nM has been reported [18], one would expect to lose a very substantial proportion of βγ from the αo-subunit upon dilution to 5 nM. As this is apparently not the case, this experiment suggests that, under the conditions used, the affinity of αo-GDP for βγ is much higher than estimated previously.

4. DISCUSSION

In this paper we describe a method to assess Go subunit interactions. The monoclonal antibody,

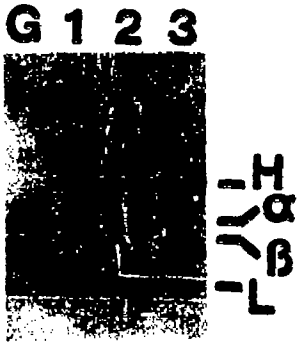

Fig. 2. Effects of guanine nucleotides on Go subunit interactions: immunoprecipitation of Go, followed by incubation with guanine nucleotides. Purified bovine brain G proteins (25 pmol) were immunoprecipitated with ascites of MONO as described in section 2. Subsequently, immunoprecipitates were incubated for 1 h at 20 °C under constant agitation in 2 ml buffer A, containing either 100 μM GDP (lane 1), 100 μM GTPγS (lane 2) or 100 μM GTP (lane 3). After recovery by centrifugation, the immunoprecipitates were analyzed by SDS-PAGE and Coomassie staining. In lane G, 2 μg of the purified bovine brain G protein preparation were loaded. The positions of the α- and β-subunits of Go and the positions of the immunoglobulin heavy (H) and light (L) chains are indicated on the right.

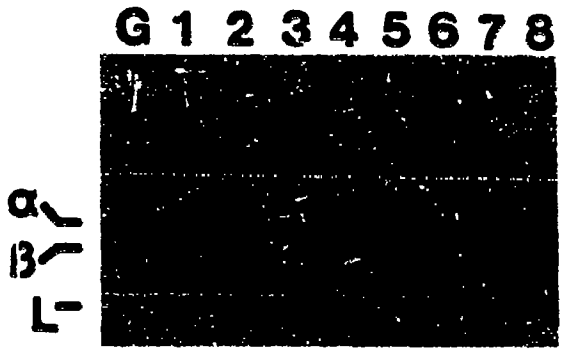

Fig. 3. Dilution does not affect subunit interactions of Go bound to MONO-beads. Using MONO-beads, Go was immunoprecipitated from 100 μl buffer A, containing 50 pmol purified bovine brain G proteins as described in section 2. Next, immune pellets were diluted in the following amounts of buffer A, containing 100 μM GDP, 0.1 ml (lane 1), 0.2 ml (lane 2), 0.5 ml (lane 3), 1 ml (lane 4), 1.66 ml (lane 5) 2.5 ml (lane 6) 5 ml (lane 7), 10 ml (lane 8), and incubated for 1 h at 4 °C. Thus the maximal G protein concentrations in these samples range from 500 to 5 nM. Following the incubation, MONO-beads were recovered by centrifugation and the bound material was analyzed by SDS-PAGE and staining with Coomassie blue. In lane G, 2 μg of the purified bovine brain G proteins were loaded. Positions of the G protein α- and β-subunits and the position of the immunoglobulin light (L) chain are indicated.

MONO, which has been shown to bind exclusively to an epitope on the αo-subunit (van der Voorn et al., submitted), recognizes heterotrimeric Go and free αo equally well. In addition, we show here that MONO can be used to immunoprecipitate Go with high efficiency from crude bovine brain membranes. Under the conditions used, no polypeptides other than βγ-subunits were found to co-immunoprecipitate with the αo-subunits.

Using this monoclonal antibody in immunoprecipitation experiments, the stability of the complexes between αo and βγ (derived either from crude bovine brain membranes or from a purified G protein preparation) can be easily followed. Upon incubation of Go with the non-hydrolyzable GTP analogue, GTPγS, at various Mg^{2+} concentrations, complete dissociation of Go was observed, and αo-subunits devoid of βγ-subunits were immunoprecipitated with MONO. However, we were unable to detect any dissociation of Go when using GTP instead of GTPγS.

Previous studies, in which the interactions between purified αo- and βγ-subunits were investigated, have similarly failed to demonstrate that GTP, the physiological regulator of G protein activation, affects Go-subunit interactions [18–20]. A characteristic shift in the sedimentation coefficient of the αo-subunit occurs upon incubation of Go with GTPγS, as measured by sucrose gradient centrifugation in the continuous presence of excess nucleotide. This shift is due to the dissociation of βγ-subunits. No shift in the sedimentation coefficient of αo was found upon incubation of Go with GTP instead of GTPγS [18,20].

It can be argued that, under the conditions used, Go dissociates into αo-GTP and βγ when GTP is bound, but does so for a very limited timespan only: the relatively fast hydrolysis of GTP by the intrinsic GTPase activity of αo would lead to the formation of αo-GDP, which again has high affinity for βγ, such that they would rapidly re-associate. As the time required to hydrolyze bound GTP is much shorter than the time required to complete the remainder of the basal GTPase cycle [21,22], at steady state, only a minor fraction of Go would be dissociated. Thus, it is possible that the methods employed, including the method presented in this paper, have not been sensitive enough to detect this small proportion of dissociated subunits.

Upon dilution of Go (bound to MONO) in a range from 500 to 5 nM, no loss of βγ from the αo-subunit was observed, a finding which is not consistent with the reported K_d of αo for binding βγ of 340–390 nM [18]. Two explanations for our results could apply: firstly, the high affinity of αo-GDP for βγ we observe in our experiments might result from the binding of MONO to the αo-subunit and thus not necessarily reflect the actual K_d of αo-GDP for binding βγ. If true, this finding would be interesting in itself. We have shown (van der Voorn et al., submitted) that the binding site of MONO on the αo-subunit is located in a large, supposedly cytoplasmic loop (αo amino acids 57–152) [23,24] of unknown function. One could speculate that, analogous to MONO, in vivo, an as yet unidentified cellular factor might interact with this loop and thus increase the affinity of αo-GDP for βγ. Alternatively, the actual affinity of αo-GDP for βγ may be much higher than estimated previously.

Based on our experiments, we can not discriminate between these two possible explanations for our results.

Nevertheless, data reported by other authors [20,22,25] support the latter explanation. Most convincingly, Higashijima et al. [22] report major differences in steady-state GTPase activities between purified heterotrimeric Go and free αo-subunits when measured at various Mg^{2+} concentrations. These experiments were performed at low concentrations (13 nM) of either Go or αo. The observed differences in steady-state GTPase activities are most easily explained by assuming that βγ-subunits can affect the GTPase activity of αo-subunits. However, this assumption implies extensive, direct interactions between αo and βγ during several rounds of the GTPase cycle, which, at the subunit concentrations used (13 nM), would seem inconsistent with the reported K_d of αo-GDP for binding βγ of 340–390 nM [18]. In vivo, a relatively high affinity of α-GDP for βγ-subunits may be required to effectively dampen signal transmission by free α- and βγ-subunits under non-stimulated conditions.

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