Purification of the G-protein G_{13} from rat brain membranes

Rainer HARHAMMER, Bernd NÜRNBERG,* Karsten SPICHER and Günter SCHULTZ

Institut für Pharmakologie, Universitätsklinikum Rudolf Virchow, Freie Universität Berlin, Thielallee 69–73, D-14195 Berlin, Germany

Significant amounts of G_{13} , a member of the recently described G_{12} -subfamily of heterotrimeric G-proteins, have been detected in rat brain membranes by specific antisera. The α -subunits of G_{13} ($G\alpha_{13}$) were purified by using a combination of conventional and subunit-exchange chromatography. Purification was facilitated by the fact that the initial anion-exchange chromatography separated G_{13} from most of the other G-proteins, including $G_{q/11}$. Moreover, $G\alpha_{13}$ -enriched fractions obtained from this chromatographic step were devoid of $\beta\gamma$ -dimers, despite the absence of G-protein-activating agents. Nevertheless, the purified $G\alpha_{13}$ retained its ability to interact with $\beta\gamma$ -dimers under appropriate conditions, i.e. the addition of Lubrol PX instead of

cholate as detergent and the omission of ethylene glycol routinely used as a protecting additive. The interaction was demonstrated by (i) the binding of $G\alpha_{13}$ to immobilized $\beta\gamma$ -complexes and (ii) the formation of stable heterotrimers during sucrose-density-gradient centrifugation. Furthermore, our studies on $G\alpha_{13}$ provide evidence for an extremely slow basal GDP/GTP exchange rate. The purified protein showed negligible binding of guanosine 5'-[γ - ^{35}S]thio]triphosphate (GTP[^{35}S]). Accordingly, dissociation of $G\alpha_{13}$ from immobilized $\beta\gamma$ -complexes was achieved by $\text{AlF}_4^-/\text{Mg}^{2+}$, but not by GTP[S]. These data indicate that G_{13} exhibits properties highly distinct from those of other G-proteins.

INTRODUCTION

Heterotrimeric regulatory guanine-nucleotide-binding proteins (G-proteins) mediate signal transduction between ligand-bound heptahelical membrane receptors and effector systems such as adenylate cyclases, phospholipases C and ion channels [1,2]. They are composed of α -, β - and γ -subunits and are at present classified by the nature of their α -subunit. To date, cDNAs for 21 distinct α -subunits (the products of 17 genes) and of at least 4 β - and 7 γ -subunits have been identified [3,4]. In its inactive form, the α -subunit binds GDP and is associated with the $\beta\gamma$ -dimer. Activation of membrane receptors results in an exchange of GDP for GTP and dissociation of the $\beta\gamma$ -dimer from the α -subunit. This allows the interaction of the α -subunit and/or the $\beta\gamma$ -dimer with effectors. Deactivation follows from hydrolysis of GTP and reassociation of the GDP-bound α -subunit with the $\beta\gamma$ -dimer. On the basis of their amino acid similarity, G-proteins are currently classified in four subfamilies: G_s , G_i , G_q and G_{12} [3]. Members of the G_i -subfamily, except G_{z} , contain a consensus site near the C-terminus for NAD-dependent ADP-ribosylation catalysed by pertussis toxin (PT), whereas members of the G_s -subfamily can be easily modified by cholera toxin (CT). These features have allowed purification of members of the G_i - and G_s -subfamilies from various tissues and facilitated their functional characterization. In contrast, as predicted from the deduced amino acid sequences, members of the G_q - and G_{12} -subfamilies are refractory to ADP-ribosylation by PT, since they lack the C-terminal cysteine-containing motif. Members of the G_q -subfamily were previously purified as PT-insensitive activators of phospholipase C- β isoforms [5–8]. However, only very little information is available about the members of the G_{12} -subfamily, i.e. G_{12} and G_{13} . In particular, at present neither receptors nor effectors are known to couple functionally to these proteins. Hence, for isolation of G_{12} or G_{13} , specific antibodies are necessary. Recently, polypeptides immunoreactive with the α -subunit of G_{13}

($G\alpha_{13}$) have been detected in a broad range of cell types and tissues, including human platelets and rodent brain [9,10], consistent with the widespread distribution of their mRNA [11].

Here, we describe the purification of G_{13} from rat brain membranes, which allowed us to initiate its characterization. We provide evidence for a low basal affinity of purified $G\alpha_{13}$ for GTP. It is also shown that the use of cholate as the detergent decreases the affinity between $G\alpha_{13}$ and the $\beta\gamma$ -complex.

MATERIALS AND METHODS

Membrane preparations

Cell membranes were prepared in accordance with published protocols [12–14] with modifications. Briefly, brains from cattle, mice and rats were collected in ice-cold NaCl (0.9%, w/v) and washed in buffer A (20 mM Tris/HCl, pH 8.0, 1 mM EDTA, 20 mM β -mercaptoethanol) containing 250 mM sucrose. Tissues were homogenized, filtered through two layers of cheesecloth and supplemented with an equal volume of buffer A. Crude membranes were collected by centrifugation at 17000 *g* for 30 min. Membrane pellets, which were resuspended in buffer A by using an Ultra-Turrax, were centrifuged at 25000 *g* for 30 min. This procedure was repeated twice. Washed membranes were shock-frozen in liquid N_2 and stored at -80°C until used. Membrane preparations and all subsequent steps were carried out at 4°C unless otherwise stated.

Purification procedures

The procedure used for purification of $G\alpha_{13}$ -immunoreactive polypeptides was based on protocols developed by numerous laboratories. Specific changes are detailed below. Membranes from 350 rat brains (7.5 g of protein, binding 1.5 μmol of guanosine 5'-[γ -thio]triphosphate (GTP[S])) were thawed and pre-extracted [stirring for 30 min with buffer A containing 0.1 %

Abbreviations used: AMF, mixture of AlCl_3 , MgCl_2 and NaF; CT, cholera toxin, an exotoxin of *Vibrio cholerae*; GTP[S], guanosine 5'-[γ -thio]triphosphate; PT, pertussis toxin, an exotoxin of *Bordetella pertussis*; ECL, enhanced chemiluminescence.

* To whom correspondence should be addressed.

(w/v) sodium cholate, followed by centrifugation at 200000 *g* for 60 min]. The remaining pellet was suspended in buffer A containing 1% sodium cholate and incubated with stirring for 1 h. After centrifugation at 200000 *g* for 60 min, the clear supernatant was removed and supplemented with ethylene glycol to a final concentration of 30% (v/v).

Cholate-extracted proteins (1.1 g, binding 0.7 μ mol of GTP[S]) were applied to a 1.5 litre column of DEAE-Sepharose Fast Flow (Pharmacia, Freiburg, Germany), which was equilibrated with at least 5 litres of buffer A containing 30% ethylene glycol and 0.9% sodium cholate. Proteins were eluted with a linear gradient of NaCl in buffer A (4 litres, 0–500 mM). The eluate was collected in 235 fractions of 17 ml each. Fractions (110–118) containing the major peak of $G\alpha_{13}$ -immunoreactive polypeptides were pooled and concentrated to 22 ml through an Amicon PM 10 filter (Danvers, MA, U.S.A.). The concentrate was loaded on a 1.2-litre column of Ultrogel AcA-34 (Serva, Heidelberg, Germany) and eluted with buffer A containing 30% ethylene glycol, 0.9% sodium cholate, 100 mM NaCl and 10 μ M GDP. Fractions (8 ml each) were collected. Those containing $G\alpha_{13}$ polypeptides (fractions 127–143) were pooled and concentrated through Amicon PM 10 filters. To decrease the ethylene glycol concentration and to change from sodium cholate to Lubrol PX, proteins were diluted with 20 vol. of buffer B [buffer A containing 0.1% (v/v) Lubrol PX, 11 mM CHAPS and 10 μ M GDP].

In the next step, $G\alpha_{13}$ polypeptides were applied to a Mono Q column (1 ml; Pharmacia, Freiburg, Germany) equilibrated with buffer B. The column was washed with buffer B and eluted with three consecutive linear gradients of NaCl in buffer B (0–300 mM, 30 ml; 300–600 mM, 15 ml; 600–1000 mM, 8 ml). Fractions (1 ml) were collected and subjected to immunoblot analysis. Those containing $G\alpha_{13}$ polypeptides were pooled and stored at -80°C .

For final isolation, the pooled Mono Q fractions were mixed with $\beta\gamma$ -agarose in buffer C (100 mM NaCl, 0.1% Lubrol PX, 5 μ M GDP, 3 mM dithiothreitol, 20 mM sodium Hepes, pH 8.0, 1 mM EDTA) and incubated for 1 h at room temperature with stirring. Subsequently, the suspension was washed at least nine times each with 0.3 ml of buffer C and eluted six times each with 0.3 ml of buffer C containing AMF (30 μ M AlCl_3 , 10 mM NaF, 10 mM MgCl_2). Fractions were collected and analysed by SDS/PAGE and immunoblotting.

$G\alpha_o$ mixtures, $G\alpha_{o2}$ - and free $\beta\gamma$ -dimers were purified from bovine or pig brain membranes by methods described previously [13–15]. For synthesis of $\beta\gamma$ -agarose, a protocol described by Pang and Sternweis [16] was followed. The amount of $\beta\gamma$ -dimers bound to the matrix varied from 8.2 to 11.5 nmol/ml between preparations. The viability of the immobilized $\beta\gamma$ -complexes was confirmed by binding of $G\alpha_o$ to the matrix.

Antibodies and immunoblotting

For detection of G-proteins, SDS/PAGE was employed in accordance with Laemmli as detailed previously [10,17]. In order to avoid smeared protein bands, samples obtained from $\beta\gamma$ -agarose affinity chromatography were subjected to sample preparation as described by Schagger and von Jagow [18]. Immunoblotting was performed as described previously [19]. Filter-bound antibodies were detected by peroxidase-coupled anti-rabbit IgG (Sigma, Deisenhofen, Germany) and the enhanced chemiluminescence (ECL) Western-blotting detection system (Amersham, Braunschweig, Germany). Antibodies AS 6 (anti- $\alpha_{o\text{common}}$), AS 8 (anti- $\alpha_{o\text{common}}$) and AS 11 (anti- $\beta_{1/2}$) as well as antibodies to α_{13} (AS 231, AS 272, AS 343), the anti- α_{12} antibody

AS 232 and the anti- $\alpha_{q/11}$ antibody AS 368 have been described previously [10,20,21]. Synthetic peptides corresponding to the C-terminal decapeptides of $G\alpha_{13}$ [(C)HDNLKQLMLQ] or $G\alpha_{12}$ [(C)QENLKDIMLQ] were used for competition experiments.

Sucrose density gradients

Sucrose-density-gradient centrifugation was performed essentially as described previously [22,23]. Linear sucrose gradients (5–20%, w/v) were prepared in buffer C containing 1 mM dithiothreitol and 50 μ M GDP if not stated otherwise. AMF-stabilized $G\alpha_{o2}$ from bovine brain and $G\alpha_{13}$ from rat brain were preincubated with increasing amounts of $\beta\gamma$ -dimer, 50 μ M GDP and 20 mM EDTA for 15 min at 30°C . The amounts of G-protein α -subunits were determined by binding of GTP[^{35}S] ($G\alpha_{o2}$) or estimated from silver-stained polyacrylamide gels ($G\alpha_{13}$). Before loading on top of 5 ml gradients, samples were mixed with BSA (Cohn fraction V; 20 μ g) and cytochrome *c* (40 μ g), which served as markers. Gradients were centrifuged for 15 h at 4°C in a Kontron TST 55.5 rotor at 175000 *g* and collected in 40–45 fractions from the bottom. Fractions were analysed by SDS/PAGE and immunoblotting with antibodies AS 6 and AS 343. Relative intensities of specific bands were determined by laser scanning densitometry (LKB 2202 Ultrosan). The position of cytochrome *c* in the gradient was determined by its absorption at 414 nm. The position of BSA was estimated from nitrocellulose filters stained with Ponceau S. The apparent $s_{20,w}$ values of $G\alpha_{13}$ and $G\alpha_{o2}$ were calculated by comparison of sedimentation rates of G-proteins with BSA ($s_{20,w} = 4.2$ S) and cytochrome *c* ($s_{20,w} = 1.8$ S).

Miscellaneous methods

GTP[^{35}S] (1150–1400 Ci/mmol) and [α - ^{32}P]ATP (800 Ci/mmol) were purchased from DuPont NEN (Bad Homburg, Germany). [^{32}P]NAD $^{+}$ was synthesized from [α - ^{32}P]ATP as described by Cassel and Pfeuffer [24]. The sources of all other reagents used are those described in [14].

GTP[S] binding of proteins was performed as described previously [25]. Briefly, samples were incubated with 500 nM GTP[S] (100000 c.p.m. of GTP[^{35}S]) in a final volume of 60 μ l at 32°C for 30 min unless stated otherwise. Non-specific binding was estimated by addition of 2 mM GTP. CT- and PT-mediated ADP-ribosylation, followed by immunoprecipitation with anti-serum AS 231 or pre-immune serum, was performed as described previously [26–28]. Protein concentrations were determined by the method of Lowry et al. [29] with modifications [30].

RESULTS

Immunoblotting of solubilized membrane proteins of mouse and rat brains with anti- α_{13} antibody AS 231 identified similar quantities of a 42 kDa polypeptide (Figure 1a, lanes 1 and 3). Immunoreactive detection of bands of either species was abolished by preincubation of antiserum with the peptide (10 μ g/ml) used to generate the antiserum (see Figure 1a, lanes 2 and 4), but not with the corresponding peptide (10 μ g/ml) of $G\alpha_{12}$ (see Figure 1a, lane 5). AS 231 was able to immunoprecipitate $G\alpha_{13}$ specifically (see Figure 1c). This allowed examination of ADP-ribosylation of $G\alpha_{13}$ by PT or CT in crude cholate extracts. The $G\alpha_{13}$ -immunoreactive protein failed to be a substrate for ADP-ribosylation when treated with either toxin (see Figure 1b). Interestingly, bovine brain $G\alpha_{13}$ -immunoreactive polypeptides exhibited a slightly changed mobility on SDS/PAGE in comparison with rodent polypeptides (compare Figure 1a,

Figure 1 Identification of $G\alpha_{13}$ polypeptides in cholate extracts of mouse, rat and bovine brain membranes

(a) Comparison of $G\alpha_{13}$ polypeptides from mouse, rat and bovine brain membranes. Cholate extracts (100 μ g of protein/lane) of mouse (lanes 1, 2, 6), rat (lanes 3, 4, 5) and bovine brain (lanes 7, 8) were acetone-precipitated and separated by SDS/PAGE (separating gels with 10% acrylamide). After blotting, nitrocellulose strips were incubated with antiserum AS 231 (lanes 1–5) or AS 343 (lanes 6–8). Both antisera were diluted 1:150. Antisera were preincubated with the peptide (10 μ g/ml) used for their generation (lanes 2, 4, 8) or with the corresponding peptide (10 μ g/ml) of the deduced amino acid sequence of $G\alpha_{12}$ (lane 5). (b) Autoradiography after $[^{32}P]$ ADP-ribosylation of cholate-extracted rat brain membrane proteins (lanes 1, 3) and after immunoprecipitation with AS 231 (lanes 2, 4). Proteins (20 μ g/lane) were incubated with preactivated PT (lanes 1, 2) or CT (lanes 3, 4), separated by SDS/PAGE and exposed to X-ray films. (c) Immunoprecipitation of cholate-extracted rat brain membrane proteins (100 μ g/lane) with AS 231 (lane 2) or pre-immune serum (lane 1). Proteins were resolved on gels, blotted on to nitrocellulose filters and incubated with AS 231 (dilution 1:150). The ECL system was used for detection of filter-bound antibodies. The position of a 43 kDa marker protein is indicated.

lanes 6 and 7). Nevertheless, preincubation of the specific antiserum with the peptide used for its generation resulted in a loss of this immunological signal (see Figure 1a, lane 8).

We chose rat brain membranes as the starting material for purification of G_{13} . As a first step, we loaded cholate extracts supplemented with ethylene glycol on to an anion-exchange column. Figure 2 shows that $G\alpha_{13}$ -immunoreactive polypeptides were eluted in a narrow peak centred at 250 mM NaCl (fractions 110–118). Subsequent fractions exhibited a faint immunoreactive signal. In contrast, most of the G_i/G_o -proteins required higher salt concentrations (360–430 mM NaCl, fractions 170–205) for elution as detected with AS 8 and AS 11. These fractions exhibited a maximum of GTP[S]-binding activity (see Figure 2a), whereas fractions enriched with $G\alpha_{13}$ -immunoreactive polypeptides lacked significant GTP[S]-binding activity. $G\alpha_{q/11}$ -immunoreactive polypeptides were found in the same fractions as G_i/G_o (see Figures 2a and 2c). Interestingly, no β -immunoreactive peptides co-eluted with $G\alpha_{13}$, although activating agents (e.g. GTP, GTP[S], AMF) were omitted. It should be noted that this chromatographic step resulted in a clear separation of $G\alpha_{13}$ polypeptides from most of the other G-proteins. In contrast, $G\alpha_{12}$ -immunoreactive polypeptides were eluted throughout the entire gradient without any enrichment. On gel filtration they were found to be aggregated.

The $G\alpha_{13}$ -containing eluate of the anion-exchange column was subjected to gel filtration on an Ultrogel AcA-34 column. Most of the $G\alpha_{13}$ polypeptides were eluted after the void volume in fractions 127–143 as detected by AS 343 (Figure 3). The pooled

Figure 2 Chromatography of $G\alpha_{13}$ polypeptides on DEAE-Sepharose Fast Flow

Membrane extracts of rat brains were applied to a DEAE-Sepharose Fast Flow column and eluted as described in the Materials and methods section. (a) Protein elution profile (○) and GTP[S]-binding activity (●) are shown. The discontinuous line indicates the gradient from 0 to 400 mM NaCl. GTP[S] binding was performed as follows: portions of the fractions (3 μ l) were incubated at 32 °C for 30 min in 500 nM GTP[S] and GTP[35 S] (100 000 c.p.m.) in a total volume of 60 μ l. Non-specific binding was determined by addition of 2 mM GTP. (b) Portions (100 μ l) of the indicated fractions were acetone-precipitated, separated by SDS/PAGE (10% acrylamide) and immunoblotted with antiserum AS 231 (dilution 1:150). (c) Portions (100 μ l) of the indicated fractions were acetone-precipitated, separated by SDS/PAGE and immunoblotted with antiserum AS 368 (dilution 1:150). The ECL system was used for detection of filter-bound antibodies. The position of a 43 kDa marker protein is indicated.

peak fractions of $G\alpha_{13}$ -immunoreactive polypeptides obtained from the Ultrogel AcA-34 column were concentrated and subjected to f.p.l.c. on a Mono Q column after lowering the ethylene glycol concentration to less than 1.5% and exchange of the detergent from cholate to Lubrol PX. Screening of the eluted fractions with AS 343 revealed a sharp peak of $G\alpha_{13}$ at approx. 200 mM NaCl. Fractions enriched with $G\alpha_{13}$ were free of α_{common} - or $\beta_{1/2}$ -immunoreactive polypeptides, as assessed by AS 8 and AS 11 respectively.

For final purification of $G\alpha_{13}$, we decided to use an affinity matrix of $\beta\gamma$ -complexes immobilized on ω -aminobutyl-agarose. $G\alpha_{13}$ -immunoreactive polypeptides obtained from the Mono Q column were mixed with $\beta\gamma$ -agarose, followed by a washing procedure. Figure 4 shows that elution with an AMF-containing buffer released significant amounts of a single 42 kDa protein, as indicated by silver staining, which was immunoreactive to anti- α_{13} antibodies. In particular, this purified protein was recognized by AS 343, an antibody which is directed against the C-terminus of the deduced amino acid sequence of $G\alpha_{13}$, and by AS 272, an antibody recognizing an internal region of this protein. Although

Figure 3 Ultrogel Aca-34 chromatography of $G\alpha_{13}$ polypeptides

Pooled fractions of DEAE-Sepharose were chromatographed on Ultrogel Aca-34 as described in the Materials and methods section. (a) Fractions were analysed for protein concentration (○). (b) Portions (200 μl) of the indicated fractions were acetone-precipitated, separated by SDS/PAGE (10% acrylamide) and blotted on to nitrocellulose-filters. Filters were incubated with AS 343 (dilution 1:150). The ECL system was used for detection of filter-bound antibodies. The position of a 43 kDa marker protein is indicated.

Figure 4 Isolation of $G\alpha_{13}$ by affinity chromatography on $\beta\gamma$ -agarose

Portions of $G\alpha_{13}$ -immunoreactive material as obtained from the Mono Q column were incubated with 0.5 ml of $\beta\gamma$ -agarose. Procedures of incubation, washes and elution are described in the Materials and methods section. Portions (250 μl) of washes and eluates were supplemented with 10 μg of BSA, precipitated with acetone, resuspended in 50 μl of sample buffer as described in [18] and heated to 40 °C for 30 min. (a) Samples were separated by SDS/PAGE (10% acrylamide), immunoblotted and probed with antibodies AS 343 or AS 272 (dilution 1:150). The spots visible in addition to $G\alpha_{13}$ bands in the immunoblot with AS 272 are non-specific artifacts typical for this antibody [10]. The ECL system was used for detection of filter-bound antibodies. (b) Proteins eluted with AMF were analysed by SDS/PAGE and silver staining. The faint bands visible at 65–67 kDa are artifacts of the silver-staining method. Molecular masses (kDa) of marker proteins are indicated: df, dye front.

significant amounts of $G\alpha_{13}$ were bound to $\beta\gamma$ -agarose, substantial amounts of $G\alpha_{13}$ remained in the flow-through and washes. Indeed, a similar pattern has been described for isolation

Figure 5 Sedimentation coefficients of $G\alpha_{13}$ polypeptides

$G\alpha_{13}$ polypeptides (1 μg) were centrifuged through 5 ml sucrose density gradients in the absence (○) or presence (●) of purified $\beta\gamma$ -dimers (10 μg). The gradients were fractionated, and the fractions were subjected to SDS/PAGE and analysed by immunoblotting with antiserum AS 343 (dilution 1:150). The ECL system was used for detection of filter-bound antibodies. Sedimentation coefficients of $G\alpha_{13}$ were determined by laser scanning densitometry of immunoblots. BSA (20 μg) and cytochrome c (Cyt. c; 50 μg) were added as marker proteins. Their peak positions, determined as described in the Materials and methods section, are denoted by arrowheads.

of $G\alpha_0$ and $G\alpha_8$ from purified preparations by using $\beta\gamma$ -agarose chromatography [16]. The interaction of $G\alpha_{13}$ with immobilized $\beta\gamma$ -dimers confirms that the protein is able to form heterotrimers in the absence of cholate and ethylene glycol. The different purification procedures resulted in low yields of purified $G\alpha_{13}$ (4–8 μg from 7.5 g of membrane protein) as roughly estimated from silver-stained polyacrylamide gels, probably due to low expression of the protein in the membrane.

In order to characterize the purified $G\alpha_{13}$, we examined the binding of GTP[S] to the protein. The GTP[35 S]-binding assay revealed a negligible binding activity. This was unaffected by changing Mg^{2+} concentrations from 50 μM to 30 mM, considering that Mg^{2+} may inhibit GTP binding to certain G-protein α -subunits, e.g. $G\alpha_z$ [31]. Prolongation of incubation times up to 180 min showed no significant binding of GTP[S] to $G\alpha_{13}$. In accordance, $G\alpha_{13}$ was not released from immobilized $\beta\gamma$ -complexes after incubation with GTP[S], but was eluted after AMF activation. These findings point to an apparently poor GTP binding by the purified protein.

It was surprising to obtain $G\alpha_{13}$ dissociated from $\beta\gamma$ -dimers after the initial step of purification, although G-protein-activating agents were avoided. This was consistently observed in all purification procedures performed ($n = 4$). Nevertheless, reversible binding of $G\alpha_{13}$ to immobilized $\beta\gamma$ -complexes demonstrated that the α -subunits retained their ability for heterotrimer formation in the absence of cholate and ethylene glycol. In order to study the interaction between $G\alpha_{13}$ and $\beta\gamma$ -dimers in more detail, we used sucrose-density-gradient centrifugation. Purified $G\alpha_{13}$ monomers migrated with an apparent sedimentation coefficient of 3.1 ± 0.04 S. Addition of $\beta\gamma$ -dimers to $G\alpha_{13}$ in at least 10-fold excess resulted in an increase in the s value to 4.0 ± 0.12 S (Figure 5). This finding indicates formation of heterotrimeric G_{13} , remaining stable at least for the period of centrifugation. In order to compare the affinity of $\beta\gamma$ -dimers for $G\alpha_{13}$ and $G\alpha_{02}$, the α -monomers were incubated with increasing concentrations

Table 1 Sedimentation coefficients of $G\alpha_{13}$ and $G\alpha_0$ after treatment with $\beta\gamma$ -dimers at different molar $\alpha:\beta\gamma$ ratios

All $s_{20,w}$ values were determined as described for Figure 5. The values shown represent means $\pm$ S.D. for the numbers of experiments in parentheses.

$\alpha:\beta\gamma$ ratio	Sedimentation coefficient, $s_{20,w}$ (S)	
	$G\alpha_{13}$	$G\alpha_0$
1:0	3.1 ± 0.04 (3)	3.0 ± 0.04 (2)
1:0.1	3.3 ± 0.13 (2)	3.1 ± 0.08 (2)
1:1	3.6 ± 0.08 (2)	3.5 ± 0.03 (3)
1:10	4.0 ± 0.12 (3)	4.0 ± 0.08 (4)

of $\beta\gamma$ -dimers. Table 1 summarizes these data. The resulting s value of both G-proteins revealed no indications of significant differences in heterotrimer formation.

Interestingly, application of cholate-extracted $G\alpha_{13}$ to sucrose density gradients supplemented with cholate showed an apparent sedimentation velocity of 3.3 ± 0.03 S, whereas G_0 migrated with an s value of 4.0 ± 0.18 S. This confirms the results obtained from the anion-exchange chromatography, indicating that the major fraction of cholate-extracted G_{13} was indeed dissociated into α -monomers and $\beta\gamma$ -dimers, which is in contrast to most other G-proteins.

DISCUSSION

The present paper reports the detection of PT-insensitive $G\alpha_{13}$ -immunoreactive polypeptides in rat brain membranes, their purification and characterization. The purification was significantly facilitated by the fact that the first chromatographic step separated $G\alpha_{13}$ from most of the other G-proteins, including $G_{q/11}$ (see Figure 2). Interestingly, the absence of ethylene glycol completely altered the elution profile of $G\alpha_{13}$, resulting in co-elution with the bulk of G-proteins (B. Nürnberg, R. Harhammer, K. Spicher and G. Schultz, unpublished work). Moreover, although no G-protein-activating agents were used, $G\alpha_{13}$ -containing fractions were devoid of β -immunoreactive polypeptides. Nevertheless, purified $G\alpha_{13}$ retained its ability to interact with $\beta\gamma$ -dimers, as shown by two approaches, (i) the specific and reversible binding of $G\alpha_{13}$ to immobilized $\beta\gamma$ -complexes (see Figure 4), and (ii) the formation of stable heterotrimers during sucrose-density-gradient centrifugation (see Figure 5). However, for formation of heterotrimers it appears to be important to use Lubrol PX as the detergent. The significant spontaneous dissociation of G_{13} in cholate appears unique for G-proteins. Indeed, it should be considered that members of the G_{12} family share less than 45% amino acid identity with other G-proteins [11]. For example, the N-terminus of the $G\alpha_{13}$ -subunit differs markedly from corresponding regions of other G-protein α -subunits. The amino-terminal region was identified to be important for interaction of $G\alpha_i$, $G\alpha_s$, $G\alpha_o$ -subunits with $\beta\gamma$ -dimers [32–34]. Furthermore, amino-terminal truncation of recombinant $G\alpha_{13}$ reduced its affinity for $\beta\gamma$ -dimers [23].

Our studies on purified $G\alpha_{13}$ provide evidence for an apparent poor GTP-binding capability in the absence of an appropriate stimulated receptor: (i) $G\alpha_{13}$ dissociated from immobilized $\beta\gamma$ -complexes after activation with AMF, but not after incubation with GTP[S] as described for $G\alpha_q$ [35], and (ii) none of the $G\alpha_{13}$ preparations showed any GTP[35 S]-binding activity. An expla-

nation may be an extremely slow basal rate of dissociation of GDP from $G\alpha_{13}$. Similarly slow rates of basal nucleotide exchange have been demonstrated for $G\alpha_i$, $G\alpha_s$ and $G\alpha_{q/11}$ [31,35–38]. The lack of basal GTP binding to $G\alpha_{13}$ raises the possibility that critical differences in guanine-nucleotide-binding domains of $G\alpha_{13}$ compared with other G-proteins may exist. For example, a highly conserved domain, Thr-Cys-Ala-Thr-Asp-Thr, is found near the C-terminus in most G-protein α -subunits. This sequence is thought to be directly involved in interaction with the guanine nucleotide [3]. In the corresponding sequence of $G\alpha_{13}$ -subunits the Cys is replaced by Thr and Thr-Asp is replaced by Ile-Asn. Perhaps replacement of moieties, e.g. a thiol by a hydroxyl group or an Asp by an Asn, may influence GTP binding. Slepak et al. [39] reported on $G\alpha_o$ mutants that lost their GTP[S]-binding capability, although only one or two amino acids were replaced.

The slow basal GDP/GTP exchange rate of purified $G\alpha_{13}$ corresponds to results obtained recently by using platelet membranes, which exhibited a negligible basal incorporation of [α - 32 P]GTP azidoanilide into $G\alpha_{13}$ [40,41]. In contrast, incorporation of [α - 32 P]GTP azidoanilide into $G\alpha_{13}$ was significantly enhanced by activation of thrombin and thromboxane A_2 receptors in platelets [41]. Based on these findings, reconstitution experiments with purified $G\alpha_{13}$, $\beta\gamma$ -dimers and receptors appear to be a successful way to clarify further possible physiological functions of these proteins.

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