Roles of Lipid Modifications of Transducin Subunits in Their GDP-Dependent Association and Membrane Binding†

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ABSTRACT: Transducin is an unusually soluble and dissociable heterotrimeric G-protein, although its Tα and T$\beta\gamma$ subunits are N-acylated and farnesylated, respectively. These lipid modifications have been suggested to contribute directly to the GDP-dependent Tα−T$\beta\gamma$ association, through specific lipid recognition sites on both protein subunits. We studied the dependence of subunit association on their bound lipids and on the presence of different lipidic environments. Association of native N-acylated ($_n$Tα) or acyl-free recombinant ($_r$Tα) Tα with farnesylated and carboxymethylated ($_{fc}$T$\beta\gamma$), farnesylated ($_f$T$\beta\gamma$), or farnesyl-free ($_{df}$T$\beta\gamma$) T$\beta\gamma$ was analyzed by gradient centrifugation and gel filtration in the presence of detergent or phospholipid−cholate micelles and by cosedimentation with phospholipid vesicles. Without detergent, $_n$TαGDP and $_{fc}$T$\beta\gamma$ associate only weakly in solution. The loss of Tα acyl or T$\beta\gamma$ farnesyl residues induces total dissociation. With detergent or lipids, isolated $_{fc}$T$\beta\gamma$ binds tightly to micelles or vesicles, while $_{df}$T$\beta\gamma$ does not; $_n$TαGDP binds weakly, while deacylated $_r$TαGDP does not bind at all; and $_n$TαGDP binds cooperatively with $_{fc}$T$\beta\gamma$, while $_r$TαGDP does not. Thus (i) the Tα acyl chain binds weakly, whereas the T$\beta\gamma$ farnesyl chain binds strongly to membrane lipids; (ii) there is no evidence for binding of the Tα acyl chain to a polypeptide site in T$\beta\gamma$, nor for binding of the T$\beta\gamma$ farnesyl chain to a polypeptidic site in Tα, but the Tα acyl chain seems to bind cooperatively with the T$\beta\gamma$ farnesyl chain in the membrane lipids; (iii) the insertion of the two protein-attached lipids into the same membrane could contribute to the association of both subunits by favoring collision coupling of the properly oriented protein moieties on the membrane surface.

Like all heterotrimeric G-proteins that couple to 7 helix membrane receptors, the retinal G-protein transducin comprises a nucleotide-binding subunit, Tα, and a T$\beta\gamma$ subunit made up of two undissociable β and γ polypeptides. Tα cycles between the inactive TαGDP state and the active TαGTP state. Inactive TαGDP is associated with T$\beta\gamma$ and is membrane-bound. TαGDP−T$\beta\gamma$ couples to photoactivated rhodopsin (R*), which catalyzes the release of GDP from Tα and allows the binding of GTP; TαGTP then dissociates from T$\beta\gamma$ and is released in solution. Upon hydrolysis of bound GTP, TαGDP reassociates with T$\beta\gamma$ on the membrane [for reviews, see Stryer (1986) and Chabre and Deterre (1989)].

Covalently bound lipids are found on both the α and $\beta\gamma$ subunits of all heterotrimeric G-proteins [for reviews, see Spiegel *et al.* (1991) and Yamane and Fung (1993)]. The α subunits are generally N-myristoylated on glycine 2, after cleavage of the initial methionine, and/or palmitoylated on cysteine 3 (Linder *et al.*, 1991, 1993). The amino terminus sequences of the α subunits are critical for their interaction with G$\beta\gamma$ (Navon & Fung, 1987; Neer *et al.*, 1988; Journot *et al.*, 1991a) and for their membrane attachment (Journot *et al.*, 1991b), which is most likely due to the attached acyl chain (Kokame *et al.*, 1992). The G$\beta\gamma$ subunits are prenylated, with a C_{15} farnesyl or a C_{20} geranylgeranyl chain on the C-terminus cysteine of the γ subunit. The transducin α subunit lacks cysteine on the N-terminal end and is only N-acylated on glycine 2 (Neubert *et al.*, 1992; Kokame *et al.*, 1992). Tγ is farnesylated and carboxymethylated (Fukada *et al.*, 1990; Ohguro *et al.*, 1991). These lipid modifications contribute to maintain TαGDP associated to T$\beta\gamma$ and membrane-bound. However, in contrast with other G-proteins, both the TαGDP and T$\beta\gamma$ subunits are released from retinal membranes in low-salt buffers devoid of detergent. The TαGTP subunit is even released in cytoplasmic-type saline buffers, while T$\beta\gamma$ is retained on the membrane (Kühn, 1980, 1981) probably via the Tγ farnesyl residue, which is inserted in the membrane lipid layer.

The myristoylation of Tα is in fact an N-acylation by heterogeneous C_{12} or C_{14} fatty acid chains (Kokame *et al.*, 1992; Neubert *et al.*, 1992). The limited binding energy of a single C_{12} or C_{14} acyl chain for the lipid phase of a membrane (Peitzsch & McLaughlin, 1993) may not suffice to anchor permanently an isolated TαGDP subunit into a lipid membrane. TαGDP could be membrane-bound through its association to T$\beta\gamma$. One might then speculate that the acyl chain of Tα interacts directly with a specific protein recognition site in T$\beta\gamma$. In other heterotrimeric G-proteins, the myristoylation of Gα is required for its association with G$\beta\gamma$. This observation led to the implicit or explicit suggestion that the myristoyl chain of GαGDP binds to a polypeptidic site of the membrane-bound G$\beta\gamma$ subunit [see, for example, Hepler and Gilman (1992), Figure 1].

The farnesyl chain of Tγ seems indispensable for the coupling of transducin to illuminated rhodopsin (R*) on photoreceptor membranes and, thus, for the catalysis by R*

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of GDP/GTP exchange on Tα (Ohguro *et al.*, 1990, 1991). The farnesyl chain seems to anchor the Tβγ subunit into the membrane, which then retains TαGDP and presents it to R* (Simond *et al.*, 1991). But Tγ isoprenyl modification also seems required for the catalysis by Tβγ of the ADP-ribosylation of TαGDP by Pertussis toxin (Fukada *et al.*, 1990). This suggests that the farnesyl chain of Tγ plays a direct role in the association of Tβγ with Tα. Oghuro *et al.* (1991, 1994) thus speculated that the Tγ farnesyl might directly recognize polypeptidic sites in Tα and even possibly in R*.

On the basis of these and other observations, models have been proposed for other isoprenylated proteins, such as small G-proteins of the p21ras, p21rho, and p21rac families, where the isoprenyl modification would mediate interactions with specific binding sites on membrane-bound or soluble proteins (Marshall, 1993; Itoh *et al.*, 1993; Kuroda *et al.*, 1993). These models postulate that the isoprenyl chain has low affinity for the membrane lipid layer, supposedly on account of its branched structure, and binds to a specific isoprenyl group recognition site in a membrane receptor protein. It seems unlikely that a lipid-soluble isoprenyl chain would bind to a protein rather than insert within the membrane lipid chains. For the small G-proteins of the ARF family, which are myristoylated at their N terminus like Tα, it has also been proposed that the acyl chain recognizes a binding site on a membrane receptor protein, in addition to interacting with the membrane lipids (Palmer *et al.*, 1993; Helms *et al.*, 1993).

In a previous study, while investigating the possible dissociation of transducin subunits upon cholera toxin-catalyzed ADP-ribosylation (Bornancin *et al.*, 1992), we made the unexpected observation that native GDP-bound transducin, extracted from nonilluminated retinal rod outer segment (ROS) membrane by washing with low ionic strength buffer without GTP, eluted in two separate TαGDP and Tβγ peaks in a gel filtration assay in saline buffer without detergent. Thus, in the absence of membrane or of substituted detergent or lipids, neither N-acylation of TαGDP nor isoprenylation of Tβγ conferred sufficient affinity to the protein to maintain the two subunits associated in solution, even when their concentrations were in the micromolar range. Addition of nonionic detergent or phospholipid−cholate micelles to the elution medium drastically changed the gel filtration elution profiles: TαGDP reassociated with Tβγ, forming a micelle-bound TαGDP−Tβγ complex analogous to the functional membrane-bound heterotrimer. This suggested that the detergent micelle or the phospholipid vesicle contributed to the interaction between the lipid−modified protein subunits. We thus reanalyzed the role of lipid modification of the transducin subunits and the role of membrane lipids in the protein−protein interactions and the protein−lipid interactions that regulate the association of TαGDP−Tβγ heterotrimers and their ability to bind to membranes. We isolated three subtypes of Tβγ from bovine retinal membrane: farnesylated and carboxymethylated ($_{fc}$Tβγ), farnesylated but noncarboxymethylated ($_{f}$Tβγ), and defarnesylated ($_{df}$Tβγ) (Figure 1). We purified native N-acylated Tα ($_{n}$Tα) extracted from bovine retina and a nonacylated recombinant Tα ($_{r}$Tα) produced in Sf9 cells. The solubility of all of these transducin subunits allowed us to test, by gel filtration and sedimentation techniques, the contributions of the Tα acyl and Tβγ farnesyl groups to the Tα−Tβγ interaction, in the absence or presence of detergent micelles or phospholipid vesicles.

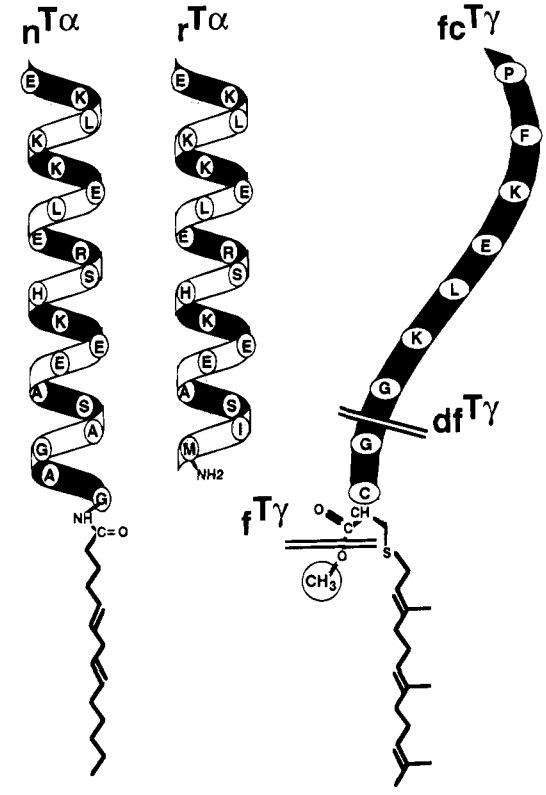

FIGURE 1: Lipid modifications of Tα and Tγ subunits. The N-terminal peptide of native Tα ($_{n}$Tα) is shown with an attached $C_{14:2}$ (Δ5,8), one of the major reported modifications of Tα (Kokame *et al.*, 1992; Neubert *et al.*, 1992). The N-terminal peptide of acyl-free recombinant Tα ($_{r}$Tα) is as previously described by Faurobert *et al.* (1993). Also shown is C-terminal peptide of defarnesylated, farnesylated, and farnesylated and carboxymethylated Tγ as determined by mass spectrometry analysis (see Materials and Methods).

MATERIALS AND METHODS

Aqueous Buffers. Iso buffer: 20 mM Tris-HCl (pH 7.5), 120 mM NaCl, 1 mM $MgCl_2$. Hypo buffer: 5 mM Tris-HCl (pH 7.5), 0.1 mM $MgCl_2$. The buffers were degassed and complemented with 5 mM β-mercaptoethanol and 100 μM PMSF before use.

Detergent Micelles, Phospholipid−Cholate Micelles, and Phospholipid Vesicles. Nonionic detergent micelles: Thesit (Boehringer), a dodecylpoly(ethylene glycol ether), was added to iso buffer at a concentration of 1 g/L (≈2mM), which is 20 times its critical micellar concentration. Mixed phospholipid−cholate micelles were prepared and characterized as previously described (Franco *et al.*, 1993); azolectin (Sigma), a soybean phospholipid mixture containing about 20% phosphatidylcholine, was dissolved at 1 g/L, with 4g/L sodium cholate (Sigma) in iso buffer. Mixed phospholipid−cholate micelles formed, which in gel filtration eluted at a volume corresponding to the size of a 60 kDa protein, as seen by doping the micelles with tritiated dipalmitoylphosphatidylcholine (see, for example, Figure 5). Large unilamellar vesicles were prepared as previously described (Franco *et al.*, 1993). Briefly, 20 mg of azolectin (Sigma) was dissolved in 6 mL of diethyl ether. Aqueous buffer (1 mL) (50 mM Hepes, pH 7.5) was added, and the mixture was sonicated for 3 min at 0 °C. The solvent was removed

under reduced pressure, and the aqueous suspension of vesicles was filtered through a 0.8 μM Millipore filter. The final concentration, determined by dry weight, was 15 ± 3 mg/mL. This stock suspension was stored at 4 °C under nitrogen.

Extraction and Purification of Native Tα and Tβγ from Retinal Membranes. Fresh bovine eyes were obtained from a slaughter house. Retinal rod outer segment (ROS) membranes were prepared under dim red light as usual (Kühn, 1980) and stored at −80 °C. Thawed ROS membrane pellets (50 mg of Rh) were suspended at 12.5 μM Rh concentration in iso buffer and sedimented (5 min at 400000*g* in a TL 100.3 rotor (TL 100, Beckman)). This first supernatant contained a pool of cytosolic Tβγ, which is essentially defarnesylated Tβγ. The pellet was illuminated, twice resuspended (50 μM Rh) in hypo buffer, sedimented to eliminate the cGMP-phosphodiesterase, and resuspended (125 μM Rh) in hypo buffer supplemented with either 500 μM GTP or 200 μM GTPγS (with similar amounts of $MgCl_2$), which simultaneously releases TαGTP or TαGTPγS and the pool of membrane-bound Tβγ. These extracts were concentrated on a Centricon 30 microconcentrator (Amicon), diluted 5-fold in iso buffer to eliminate excess free nucleotide, reconcentrated to 100 μL, and used without further purification in gel filtration and sucrose density gradient experiments. Alternatively, to crudely separate the Tα and Tβγ pools, Tα was first extracted in iso buffer supplemented with either 500 μM GTP or 200 μM GTPγS, and membrane-bound Tβγ subsequently was solubilized by resuspending the membrane pellet in hypo buffer. These crudely separated Tα and Tβγ pools were purified by ion exchange chromatography on a Polyanion SI HR 5/5 column (Pharmacia-LKB), as described before (Deterre *et al.*, 1984): Tα was eluted with a 0-660 mM Na_2SO_4 linear gradient and Tβγ with a 0-660 mM $MgCl_2$ linear gradient, in 20 mM Tris-HCl (pH 7.5), 0.1 mM $MgSO_4$, 5 mM β-mercaptoethanol, and 100 μM PMSF; cytosolic Tβγ, which amounts to about one-tenth of the total pool of Tβγ, was purified from the first iso extract with the same salt gradient. Purified fractions of each protein (TαGDP, TαGTPγS, and Tβγ) were concentrated on a Centricon 30 microconcentrator (Amicon). Purity as controlled by Coomassie-stained SDS−PAGE was about 98%.

Separation of Tβγ Isoforms. Reversed Phase Chromatography and Mass Spectrometry Identification of Tγ Isoforms. The variously modified Tβγ isoforms were separated on a gel filtration column (Superose 12 HR 10/30, Pharmacia) equilibrated with iso buffer without detergent and eluted at a flow rate of 0.5 mL/min. Cytosolic Tβγ eluted in one peak (Figure 4A, f, left), and membrane-bound Tβγ mostly eluted in two other peaks (Figure 4A, c, left). The identification of these Tβγ peaks was adapted from Oghuro *et al.* (1991). Fractions of each peak were injected onto a reversed phase Aquapore $7C_{18}$-OD300 column (250 × 1.0 mm, Applied Biosystem) equilibrated with 0.1% trifluoroacetic acid (TFA) and were eluted with a linear gradient of acetonitrile (0−56%) in 0.1% TFA at a flow rate of 100 μL/min. The peak from cytosolic Tγ eluted at 61 min and those from membrane-bound Tγ eluted at 66 and 67 min, with profiles similar to those described by Ohguro *et al.* (1991). The three isolated Tγ fractions were lyophilized, dissolved in 80% acetonitrile, and submitted to matrix-assisted laser desorption mass spectrometry (LD-MS) on a LASERMAT mass analyzer (Finnigan MAT). The mass determinations were 8330, 8315, and 7957 Da. The two higher masses, obtained from the membrane-bound Tβγ pool, correspond to that of a farnesylated and carboxymethylated γ peptide ($_{fc}$Tγ) and that of a farnesylated but noncarboxymethylated γ peptide, ($_{f}$Tγ); the lowest mass, found mainly in the cytosolic Tβγ pool, corresponds to that of a defarnesylated Tγ ($_{df}$Tγ) that has lost two C-terminal amino acids with the attached farnesyl, as sketched in Figure 1.

Production and Purification of Acyl-Free Recombinant Tα. The acyl-free recombinant transducin α subunit ($_{r}$Tα) was obtained as previously described (Faurobert *et al.*, 1993) by deleting the two N-terminal glycines that can be acyl-binding sites: The N-terminal sequence of Tα, MGAGAS, was changed to MIS (Figure 1). The mutant protein was produced in Sf9 insect cells infected with a recombinant baculovirus and purified. Briefly, frozen cells were thawed in an ice cold lysis buffer containing 20 mM Tris-HCl (pH 7.5), 2 mM $MgCl_2$, 50 μM GDP, 2 μg/mL aprotinin, 1 μg/mL pepstatin, 1 mM iodoacetamide, 5 mM βME, and 1 mM PMSF. The homogenate was centrifuged, and the supernatant was filtered and loaded onto a Pharmacia Blue Sepharose HiTrap column (5 × 1 mL) equilibrated with 20 mM Tris-HCl (pH 7.5), 120 mM KCl, and 2 mM $MgCl_2$ and eluted with a 120−1200 mM KCl linear gradient (1 mL/min). Fractions containing $_{r}$Tα (between 370 and 430 mM KCl) were pooled, concentrated on a Centricon 30 (Amicon), loaded onto a Superose 12 HR 10/30 gel filtration column (Pharmacia-LKB), and eluted with iso buffer. Fractions containing $_{r}$Tα were purified on a Polyanion SI HR 5/5 column (Pharmacia-LKB) as described earlier for native Tα.

Gel Filtration Analysis of Subunit Association in Detergent Solutions. Gel filtration was used as a tool to study subunit association in saline buffer and their interactions in the presence of nonionic detergent micelles or mixed phospholipid−cholate micelles. Crudely separated or purified transducin subunits were incubated in buffer with or without nonionic detergent micelles or phospholipid−cholate micelles. Samples (200 μL) were loaded on a Superose 12 HR 10/30 column (Pharmacia-LKB) that had been equilibrated with a buffer containing the same concentration of detergent or phospholipid−cholate micelles and was eluted at a constant flow rate of 0.5 mL/min. Protein elution was monitored by its UV absorbance at 280 nm. The azolectin−cholate suspension has a relatively high UV absorbance. It introduces a high background level, which is, however, constant as a constant micelle concentration flows through the column. The size of the protein-free phospholipid−cholate micelles was checked by injecting 200 μL of micelle suspension previously labeled by addition of [^{3}H]dipalmitoylphosphatidylcholine ([^{3}H]DPPC), which eluted at a volume corresponding to the size of a 60 kDa protein. The calibration of the column was determined in all buffer conditions by the elution of marker proteins (30 μg of BSA, 30 μg of ovalbumin, 30 μg of carbonic anhydrase, 30 μg of cytochrome C, and 15 μg of aprotinin). The calibration was not modified by the presence of the nonionic detergent micelles (compare Figure 2 left and right), but was markedly changed (with respect to elution volumes) by the presence of phospholipid−cholate micelles. Fractions (300 μL) were collected. The proteins were concentrated by precipitation with chloroform−methanol (Wessel & Flügge, 1984), analyzed by SDS−PAGE, and quantified by densitometric scanning of the Coomassie Blue-stained gels.

Sucrose Density Gradient Sedimentation Analysis of Subunit Association. Linear sucrose gradients (5−20%, 11.2 mL) were prepared in iso buffer with or without nonionic detergent micelles (1g/L Thesit). Marker proteins (25 μg of BSA, 50 μg of ovalbumin, 25 μg of carbonic anhydrase, and 75 μg of cytochrome C) were mixed with about 100 μg of transducin extracts in 200 μL final volume before loading on the top of the gradient. The gradients were centrifuged at 40 000 rpm for 20 h in a Beckman SW 41 Ti rotor at 20 °C and separated in 400 μL fractions. The proteins were concentrated by methanol−chloroform precipitation, analyzed by SDS−PAGE, and quantified by densitometric scanning of the Coomassie Blue-stained gels.

Sedimentation Analysis of Subunits Binding to Phospholipid Vesicles. Purified $_{n}$TαGDP, $_{n}$TαGTPγS, $_{r}$TαGDP, and $_{fc}$Tβγ (5 μM each) and mixtures of these subunits were incubated for 30 min at 30 °C in iso buffer in the presence of 2 mg/mL phospholipid vesicles. The vesicles were then sedimented for 5 min at 4 °C and 400000*g* in a TL 100.3 rotor (TL 100, Beckman). Protein contents of pellet and supernatant were compared by SDS−PAGE.

RESULTS

TαGDP and Tβγ Subunits of Holotransducin Extracted from Retina in the Absence of Detergent Remain Mostly Dissociated in Solution. Inactive, GDP-bound transducin was extracted from nonilluminated retinal membranes by washing with a low ionic strength buffer, in the absence of any detergent and guanine nucleotide. This procedure extracted inactive holotransducin TαGDP−Tβγ, but it also extracted the retinal cGMP-phosphodiesterase (PDE): alternatively, the membranes were illuminated to retain transducin on photoactivated rhodopsin (R*), the PDE was extracted by a low ionic strength wash, and transducin was subsequently released from R* upon the addition of GTP or GTPγS (Kühn, 1980). TαGTP or TαGTPγS subunits were released together with dissociated Tβγ in the low ionic strength buffer. In dissociated TαGTP, GTP hydrolyzes within 20 s (Antonny *et al.*, 1993), after which TαGDP is assumed to reassociate instantly with Tβγ. TαGTPγS is known to remain permanently dissociated from Tβγ.

All of these crude extracts were eluted first on a gel filtration column with an isoosmotic saline buffer without detergent. We expected the elution profile of the inactive holotransducin extract to be characteristic of an associated TαGDP−Tβγ heterotrimer. To our surprise, the profile looked quite similar to that of permanently active transducin extracted in the presence of GTPγS and characteristic of dissociated Tα and Tβγ subunits: TαGDP eluted at the volume expected from its monomeric molecular weight, and Tβγ eluted later in broad, multimodal peaks (Figure 2A,B). Thus, a large fraction, if not all, of TαGDP was dissociated from Tβγ. The other part of TαGDP trailed together with part of Tβγ down to elution volumes corresponding to apparent MW lower than that of either subunit. These TαGDP and Tβγ subunits were retarded on the column, probably via hydrophobic interaction with the Superose gel, and their association state could not be determined from this experiment. In the transducin−GTPγS extract, all of the TαGTPγS eluted at the volume expected from its MW confirming that it remained permanently dissociated from Tβγ; the Tβγ profile was again multimodal, suggesting

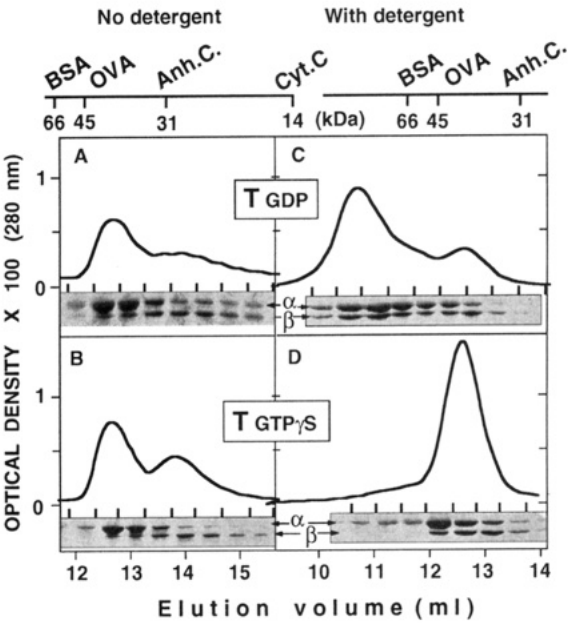

FIGURE 2: Gel filtration elution profiles of crude transducin extracts in the absence or presence of detergent micelles. Transducin extracted from illuminated membranes in the presence of GTP (T_{GDP}), or of GTPγS ($T_{GTP\gamma S}$) was eluted on a Superose 12 gel filtration column (Pharmacia) with buffer containing either (A) no detergent (gel filtration buffer) or (B) detergent micelles (0.1% Thesit in gel filtration buffer), as described in Materials and Methods. The column calibration by marker proteins was not modified by the presence of detergent.

different types of Tβγ with different degrees of hydrophobic interaction with the column.

The addition of nonionic detergent to the sample and elution buffers (Figure 2C,D) dramatically changed the elution profiles. In the inactive holotransducin extracts, most of the TαGDP and Tβγ now eluted in a major peak with an apparent MW corresponding to associated TαGDP−Tβγ; part of TαGDP trailed behind and a minor Tβγ peak subsisted, with an apparent MW of about 40 000. By contrast, in the GTPγS extract, both TαGTPγS and Tβγ eluted with apparent MWs close to 40 000, corresponding to fully dissociated subunits that were not retarded by hydrophobic interaction on the column.

Sucrose gradient sedimentation of the transducin extracts avoided the hydrophobic retardation artifacts of the column support, but this technique had a lower resolving power than gel filtration (Figure 3). Without detergent, the sedimentation profile of the transducin−GDP extract looked similar to that of transducin−GTPγS, which was characteristic of dissociated Tα and Tβγ subunits. When detergent was added, in the gradient as well as in the sample buffer, TαGDP and Tβγ coeluted, presumably as an associated complex. The calibration scale of this gradient was obtained with soluble proteins that do not bind detergent. The apparent MW of the TαGDP−Tβγ complex on this calibration scale was only on the order of 50 000 . This low value probably results from detergent binding to the complex, leading to a higher buyoancy and slowing its sedimentation. In the detergent containing sucrose gradient, TαGTPγS and Tβγ of the GTPγS extract both sedimented in a sharp peak, with lower apparent molecular weights than those of either of the dissociated subunits; this probably corresponds to dissociated TαGTPγS and Tβγ subunits, with each subunit binding a low-density detergent micelle.

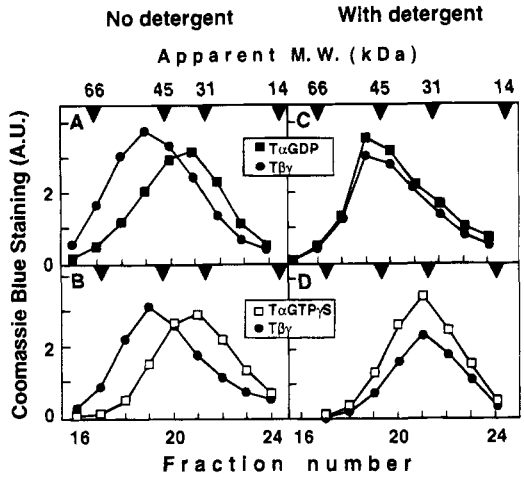

FIGURE 3: Gradient sedimentation of crude transducin extracts in the absence or presence of detergent micelles. Transducin extracts (same as in Figure 2) were loaded on 5−20% linear sucrose gradients containing either no detergent (A and B) or detergent micelles (0.1% Thesit) (C and D). Marker proteins (BSA, ovalbumin, carbonic anhydrase, and cytochrome C) were mixed with each sample before loading on the gradient. The gradients were centrifuged and fractionated as described in Materials and Methods. The protein amount in each fraction was determined by densitometric scanning of Coomassie Blue-stained gels.

Separation of Variously Lipid-Modified Tα and Tβγ Subunits. The preceding studies suggested that transducin−GDP extracted from retinal membrane was heterogeneous in its Tβγ subunit composition. Ohguro *et al.* (1991) recently related the heterogeneity of extracted Tβγ to varying degrees of isoprenylation and carboxymethylation of the Tγ polypeptide. From our extracts, we separated, by gel filtration, three forms of Tβγ that we characterized as farnesylated and carboxymethylated Tβγ ($_{fc}$Tβγ), farnesylated but not methylated Tβγ ($_{f}$Tβγ), and defarnesylated (and C-terminal-cleaved) Tβγ ($_{df}$Tβγ) (see Materials and Methods and Figure 4). Most of the $_{df}$Tβγ was released in solution upon the first resuspension in saline buffer of the stock retinal rod outer segments that had been ruptured by freezing and thawing; $_{f}$Tβγ and $_{fc}$Tβγ were released together with Tα in subsequent extracts by low ionic strength buffer supplemented with GTP or GTPγS. The Tβγ subunit pool was first separated from Tα by ion exchange chromatography. The three forms of Tβγ were then separated from each other by gel filtration and were characterized by reversed phase chromatography and mass spectrometry. Native N-acylated $_{n}$TαGDP and $_{n}$TαGTPγS subunits were purified by ion exchange chromatography. Nonacylated recombinant $_{r}$Tα subunit, with glycine 2 mutated to isoleucine to prevent acylation, was produced and purified from a baculovirus system (Faurobert *et al.*, 1993).

Binding of Lipid-Modified Tα and Tβγ Subunits to Nonionic Detergent Micelles Affects Their Association. The Tα and Tβγ subunits were loaded either separately or together on a gel filtration column and eluted with a buffer with or without Thesit, a nonionic detergent, above its critical micelle concentration (Figure 4).

(1) Isolated Tα Subunits (Figure 4A). In the absence of detergent, the elution volume of $_{n}$TαGDP was slightly larger than that of $_{r}$TαGDP, suggesting a slight hydrophobic interaction of the $_{n}$Tα acyl chain with the column support. The addition of detergent shifted the $_{n}$TαGDP peak toward a lower elution volume, suggesting the release of column interaction and binding of detergent to $_{n}$TαGDP. By contrast, the detergent did not shift the $_{r}$TαGDP peak, in agreement with an absence of interaction of unacylated $_{r}$TαGDP with the column and with the micelles.

(2) Separated Tβγ Subtypes (Figure 4A). $_{fc}$Tβγ, and to a lesser extent $_{f}$Tβγ, was retarded on the column in the absence of detergent, suggesting that they interact with hydrophobic sites on the column. In the presence of detergent, both the $_{fc}$Tβγ and $_{f}$Tβγ peaks shifted toward lower elution volumes, suggesting their release from the hydrophobic sites on the column and their binding to detergent micelles. In the presence of detergent, $_{fc}$Tβγ and $_{f}$Tβγ had distinct elution volumes, suggesting different binding to detergent micelles. Defarnesylated $_{df}$Tβγ eluted at the volume expected from its MW, whether detergent was present or not, suggesting that it interacted neither with the column support nor with the detergent micelles.

(3) Mixtures of Tα and Tβγ (Figure 4B). $_{n}$TαGDP and $_{fc}$Tβγ samples were put together on the column. In the absence of detergent, both subunits eluted partially together in a peak at a larger elution volume than that observed for either of these subunits, when eluted separately under the same conditions (Figure 4B, a). This suggested the formation of $_{n}$TαGDP−$_{fc}$Tβγ complexes retarded by column interactions in the absence of detergent. In the presence of detergent, the mixed $_{n}$TαGDP and $_{fc}$Tβγ subunits eluted partially together in a peak at a smaller volume than that observed for the isolated subunits, suggesting the association of a detergent micelle to the $_{n}$TαGDP−$_{fc}$Tβγ complex. With $_{f}$Tβγ, nTαGDP also formed a $_{n}$TαGDP−$_{f}$Tβγ complex, which was less retarded in the absence of detergent and less accelerated in its presence than the $_{n}$TαGDP−$_{fc}$Tβγ complex (Figure 4B, b). With $_{df}$Tβγ, no association of $_{n}$TαGDP was detected: the elution profiles of mixtures of $_{n}$TαGDP and $_{df}$Tβγ in the absence or presence of detergent coincided with the elution profiles of the separated subunits under the same conditions (Figure 4B, c).

Nonacylated $_{r}$TαGDP did not seem to interact with $_{fc}$Tβγ or with $_{f}$Tβγ, neither in the absence of detergent nor in its presence (Figure 4B, d). In both cases, the subunits in the mixture eluted at the same volumes as when they were eluted separately (Figure 4A, d and e). But $_{r}$TαGDP seemed to interact weakly with $_{df}$Tβγ, independent of the presence of detergent; the elution profiles of the mixed subunits in the absence of detergent were slightly but reproducibly shifted toward smaller elution volumes than those of the corresponding separated subunits (data not shown). Similar shifts were observed in the presence of detergent.

Binding of Lipid-Modified Tα and Tβγ Subunits to Phospholipid−Cholate Micelles. We had observed the influence of nonionic detergent micelles on the subunit elution profiles, but analysis of the binding of the micelles to the proteins was hampered by our ignorance of the elution characteristics of the free detergent micelles in the column. Pulse labeling the micelles with radioactive detergent would not be effective, as detergent molecules rapidly exchange between micelles; the labeled detergent peak would be dilute in the continuous flow of unlabeled micelle suspension. Phospholipid−cholate micelles obtained by dissolving 1 g/L azolectin (phospholipid mixture from soybean) in 4g/L cholate are a better substitute to a phospholipid membrane milieu and can easily be labeled by an admixture of [^{3}H]-dipalmitoylphosphatidylcholine ([^{3}H]DPPC). This insoluble

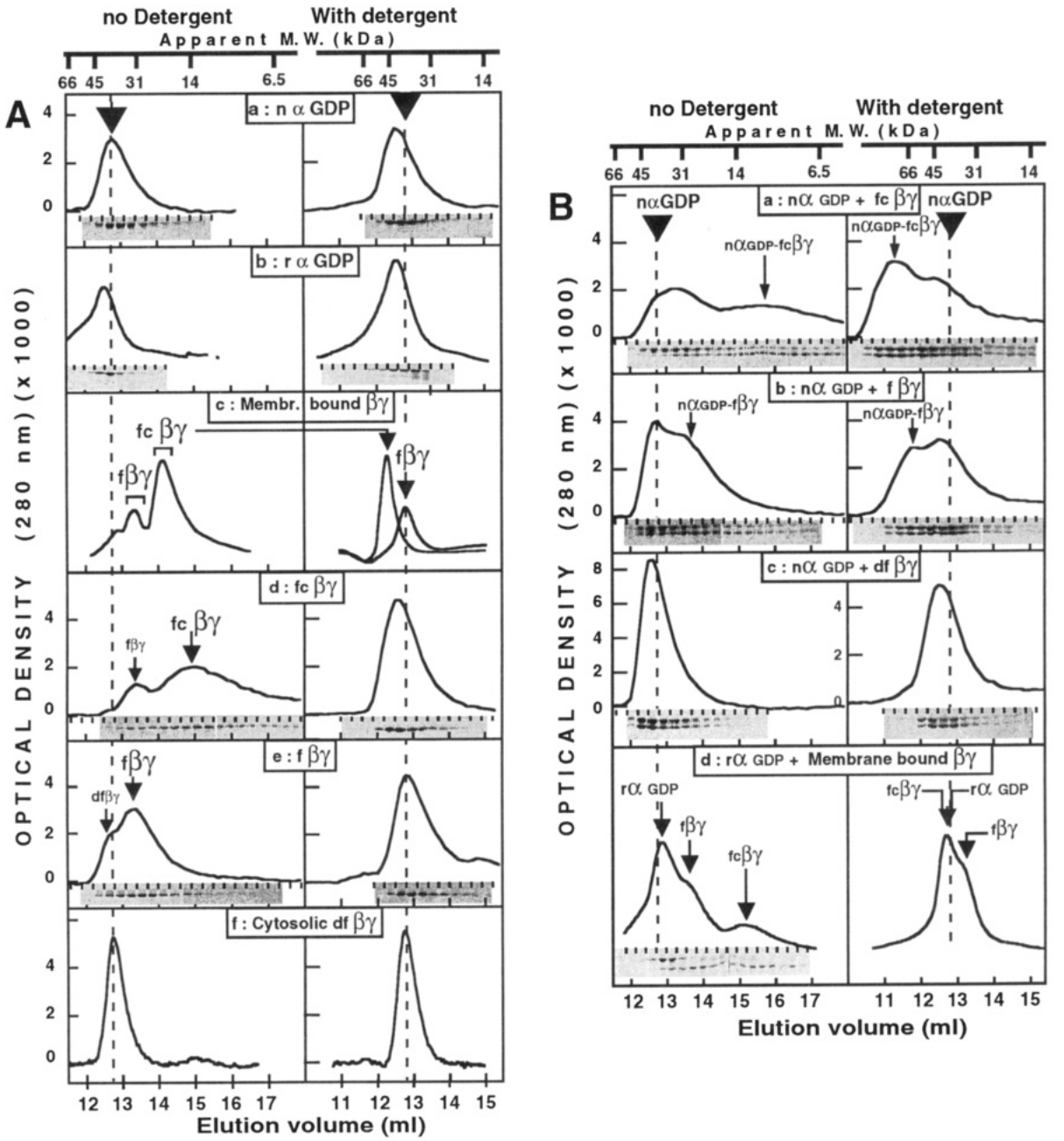

FIGURE 4: Gel filtration elution profiles of transducin subunits in the absence or presence of detergent micelles. Purified transducin subunits were loaded separately or in various combinations on a Superose 12 gel filtration column and eluted with a buffer with or without detergent (see Figure 2). (A) Separated subunits: (a) native ${}_{n}T\alpha GDP$ (15 μg); (b) recombinant ${}_{r}T\alpha GDP$ (15 μg); (c) left column, in the absence of detergent, membrane-bound $T\beta\gamma = {}_{f}T\beta\gamma + {}_{fc}T\beta\gamma$ (100 μg), right column, 5 μg aliquots of the indicated fractions of ${}_{fc}T\beta\gamma$ and ${}_{f}T\beta\gamma$ were eluted separately in the presence of detergent micelles; (d) purified ${}_{fc}T\beta\gamma$ (15 μg) with a small amount of contaminating ${}_{f}T\beta\gamma$; (e) purified ${}_{f}T\beta\gamma$ (15 μg) with a small amount of contaminating ${}_{df}T\beta\gamma$; (f) cytosolic $T\beta\gamma = {}_{df}T\beta\gamma$ (15 μg). (B) Mixture of purified subunits: (a) acylated ${}_{n}T\alpha GDP$ (15 μg) + ${}_{fc}T\beta\gamma$ (15 μg); (b) acylated ${}_{n}T\alpha GDP$ (15 μg) + ${}_{f}T\beta\gamma$ (15 μg); (c) acylated ${}_{n}T\alpha GDP$ (15 μg) + cytosolic ${}_{df}T\beta\gamma$ (15 μg); (d) recombinant, nonacylated ${}_{r}T\alpha GDP$ (15 μg) + membrane-bound $T\beta\gamma$ (unseparated ${}_{fc}T\beta\gamma$ and ${}_{f}T\beta\gamma$, 15 μg). The elution volume of native, acylated ${}_{n}T\alpha GDP$ in the absence of detergent is indicated for reference by the dotted lines throughout all panels.

phospholipid does not exchange rapidly between micelles nor dilute in the aqueous milieu. It thus provides a monitor for the elution characteristics of the micelles. The [^{3}H]-DPPC-labeled phospholipid−cholate suspension was injected on the column and eluted with a cold phospholipid−cholate suspension. The labeled micelles eluted in a sharp peak at a volume corresponding to that of a ≈60 kDa protein, according to calibration with soluble proteins in the same phospholipid−cholate medium (Figure 5A, g).

In this phospholipid−cholate medium, ${}_{n}T\alpha GDP$ eluted with a significantly larger apparent MW than in the absence of detergent (Figure 5A, a); the elution volume was very close to that of [^{3}H]DPPC-labeled phospholipid−cholate micelles. This suggested that ${}_{n}T\alpha$ was bound to a phospholipid−cholate micelle flowing through the column. The elution peak of active ${}_{n}T\alpha GTP\gamma S$ was shifted nearly as much as that of ${}_{n}T\alpha GDP$ (Figure 5A, b), suggesting that the interaction with the micelle did not depend on the protein conformation. In contrast, the elution peak of ${}_{r}T\alpha GDP$ was not shifted (Figure 5A, c), confirming that the interaction with the micelle depended on the acylation of $T\alpha$.

For $T\beta\gamma$ subunits, the elution peak of ${}_{fc}T\beta\gamma$ shifted from its very retarded position in the absence of detergent to a coelution with the [^{3}H]DPPC label in the presence of

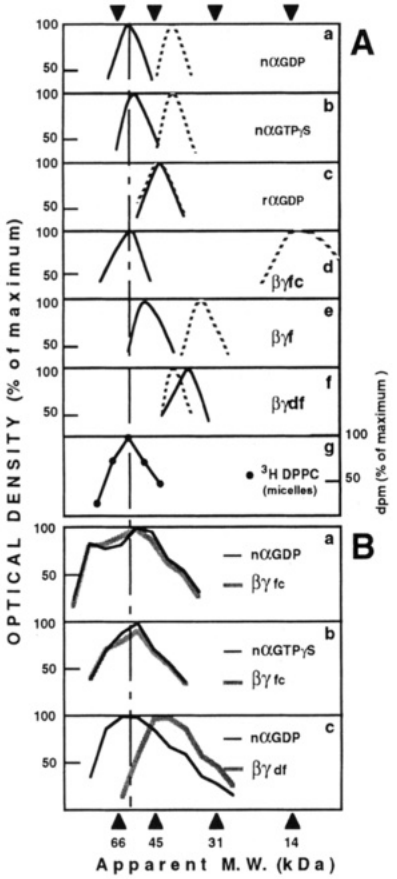

FIGURE 5: Apparent MW shifts of the elution peaks of purified transducin subunits in presence of azolectin−cholate micelles. (A) Elution peaks of the different purified subunits of transducin eluted in presence of azolectin−cholate micelles, as obtained from densitometric scanning of stained gel fractions (bold lines) and drawn on the apparent MW scale obtained from calibration by marker proteins eluted in the same azolectin−cholate micelle suspension. This calibration differs notably, in terms of elution volumes, from that obtained in the absence of detergent. For comparison, the elution peaks of the same transducin subunits, taken from Figure 4, have been redrawn (dotted lines) at the positions corresponding to their apparent MWs in the absence of detergent; panel d shows the elution profile of protein-free phospholipid−cholate micelles as determined by pulse labeling the micelles with [^{3}H]dipalmitoylphosphatidylcholine ([^{3}H]DPPC) (see Materials and Methods). The apparent MW of the micelles is indicated for reference by a dotted line throughout all the panels. (B) Elution peaks of Tα and T$\beta\gamma$ subunits in different mixtures, eluted in presence of azolectin−cholate micelles. (a) $_{n}$TαGDP and $_{fc}$T$\beta\gamma$ partially associate in a high apparent MW peak. (b) $_{n}$TαGTPγS and $_{fc}$T$\beta\gamma$ do not associate, and they elute in the same volume as when they are isolated. (c) $_{n}$TαGDP and $_{df}$T$\beta\gamma$ do not associate, and they elute in the same volume as when they are isolated.

phospholipid−cholate (Figure 5A, d), confirming the strong binding of $_{fc}$T$\beta\gamma$ to a micelle. The $_{f}$T$\beta\gamma$ subunit was also significantly shifted, but did not coelute exactly with the [^{3}H]-DPPC label, suggesting a weaker interaction with the micelles (Figure 5A, e). The elution of $_{df}$T$\beta\gamma$ almost was not affected by the presence of phospholipid−cholate (Figure 5A, f), confirming that a nonfarnesylated subunit does not bind to a phospholipid−cholate micelle.

In the presence of phospholipid−cholate micelles, an association of $_{n}$TαGDP with $_{fc}$T$\beta\gamma$ was revealed by partial coelution of these subunits in a low elution volume peak. As in the presence of nonionic detergent micelles, $_{n}$TαGDP partially associates with $_{fc}$T$\beta\gamma$ (Figure 5B, a), but not with $_{df}$T$\beta\gamma$ (Figure 5B, c). $_{n}$TαGTPγS did not associate with

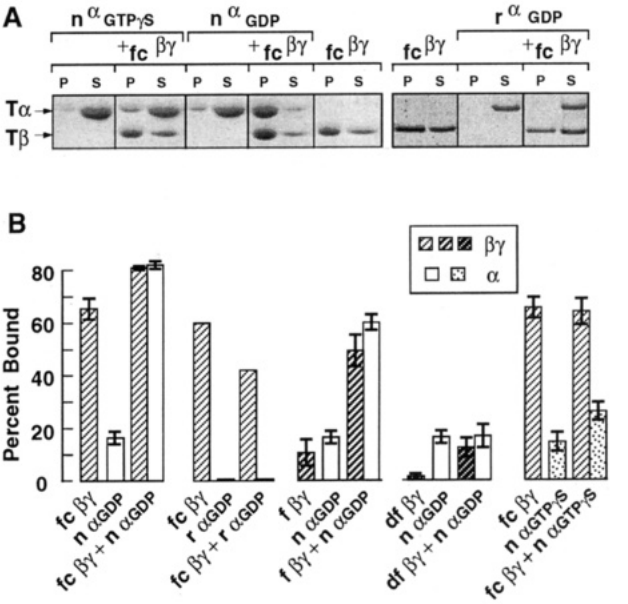

FIGURE 6: Binding of the variously modified transducin subunits to phospholipid vesicles. Purified Tα and T$\beta\gamma$ subunits were incubated with phospholipid vesicles, separately or in various combinations, as indicated (see Materials and Methods) and sedimented. (A) Coomassie Blue-stained SDS−PAGE of aliquot pellet (P) and supernatant (S) fractions. (B) Proportion of vesicle-bound Tα and T$\beta\gamma$ subunits, as quantified by densitometric analysis of pellets and supernatant fractions. These are mean values of three independent experiments and their standard deviations.

$_{fc}$T$\beta\gamma$: both subunits coeluted in the presence of phospholipid−cholate micelles (Figure 5B, b), but in the same elution volume as when either of these subunits was injected separately on the column (Figure 5A, b and d). Thus, $_{n}$TαGTPγS and $_{fc}$T$\beta\gamma$ must bind separately to distinct micelles.

Independent and Cooperative Binding of Lipid-Modified Tα and T$\beta\gamma$ Subunits to Phospholipid Vesicles. Phospholipid vesicles are the best substitute for a protein-depleted membrane. Tα and T$\beta\gamma$ subunits were incubated separately or together with suspensions of large unilamellar phospholipid vesicles and were sedimented. The protein contents of the supernatant solution and vesicle pellet were compared on SDS gels. A typical experiment is shown in Figure 6A, and all of our results are summarized in Figure 6B. The binding of an isolated Tα subunit to a phospholipid vesicle depended on its acylation rather than on its activation state: both acylated $_{n}$TαGDP and $_{n}$TαGTPγS bound weakly, but in comparable amounts to the vesicles, whereas deacylated $_{r}$TαGDP did not bind at all. Similarly, the binding to a phospholipid vesicle of an isolated T$\beta\gamma$ subunit depended on its farnesylation and carboxymethylation: nonfarnesylated dfT$\beta\gamma$ did not cosediment at all with the vesicles, and $_{f}$T$\beta\gamma$ cosedimented only very partially. By contrast, fcT$\beta\gamma$ nearly totally cosedimented with the vesicles. This confirmed the previous indication (Oghuro *et al.*, 1994) that carboxymethylation is an important factor for the interaction of farnesylated T$\beta\gamma$ with lipids.

When acylated $_{n}$TαGDP and various subtypes of T$\beta\gamma$ subunits were incubated together with the vesicles, significant cooperativity was observed for the binding of $_{n}$TαGDP with $_{fc}$T$\beta\gamma$ or $_{f}$T$\beta\gamma$, but not for the binding of $_{n}$TαGDP with $_{df}$T$\beta\gamma$; the binding of $_{n}$TαGDP increased with that of $_{fc}$T$\beta\gamma$ and with that of $_{f}$T$\beta\gamma$. Carboxymethylation of $_{f}$T$\beta\gamma$ does not seem to be essential for its binding to phospholipids in association

with nTαGDP, whereas it is essential for the binding of $_{f}T\beta\gamma$ alone. As for $_{df}T\beta\gamma$, its binding to lipid vesicles was increased only marginally in the presence of $_{n}T\alpha GDP$.

Nonacylated soluble $_{r}T\alpha GDP$ did not associate with vesicle-bound $_{fc}T\beta\gamma$, but rather induced a significant solubilization of $_{fc}T\beta\gamma$ from the vesicles. In contrast, the binding of acylated $_{n}T\alpha GTP\gamma S$ to the lipid vesicles was significantly increased in the presence of vesicle-bound $_{fc}T\beta\gamma$.

DISCUSSION

The interactions responsible for the heterotrimeric association of the G-protein subunits and for their binding to membranes have been strongly debated since the observation first made on transducin (Kühn, 1980, 1981) that α and $\beta\gamma$ subunits could be dissociated and released from the membrane without the help of detergent. The solubility of the α subunits of G_o and G_s was later confirmed by Sternweis (1986), who further observed that, in contrast with $T\beta\gamma$, $G_o\beta\gamma$ and $G_s\beta\gamma$ were not solubilized in the absence of detergent and that the presence of bound $G\beta\gamma$ was required for the association of Gα with phospholipid vesicles. This led to the suggestion that the protein−protein interactions between Gα and $G\beta\gamma$ subunits, as well as between these subunits and the G-protein-coupled receptor and effector molecules, were not occurring within the lipid layer of the membrane, into which the Gα and $G\beta\gamma$ proteins probably were not embedded (Chabre, 1987). This view was soon supported by the discovery that lipid chains were attached to Gα and $G\beta\gamma$. These lipid chains were assumed to insert into the lipid bilayer and anchor the subunits (Spiegel *et al.*, 1990). In this model, the isoprenyl chain of $G\beta\gamma$ and the acyl chain of Gα are viewed as independent membrane anchors for the two subunits, whose association is maintained by specific protein−protein contacts between GαGDP and $G\beta\gamma$, outside of the membrane lipid bilayer. But other models suggested that the $G\beta\gamma$ farnesyl chain associated to a polypeptidic site in Gα (Oghuro *et al.*, 1991) or that the Gα myristyl bound to a polypeptide site in $T\beta\gamma$ [see, for example, Hepler and Gilman (1992), Figure 1). These models were difficult to reconcile with our observation that the TαGDP and $T\beta\gamma$ subunits of transducin, which were assumed to be N-acylated and farnesylated, respectively, and were associated as an inactive heterotrimer on the native retinal membrane, remained mostly dissociated when extracted from their membrane environment in an aqueous solution. We further observed that the solubilized subunits reassociated upon the addition of nonionic detergent micelles, simulating the membrane. Both lipid modifications seemed to be required for subunit association and binding to a micelle. The heterotrimeric association of transducin, its membrane binding, and its high affinity for photoactivated rhodopsin seemed to depend on the integrity of the lipid modifications on Tα and $T\beta\gamma$. Thus, lipid−lipid interactions between the Tα acyl, the $T\beta\gamma$ farnesyl, and the membrane phospholipid chains might control the subunit's association and the heterotrimer membrane binding. A specific lipid−protein interaction between the Tα myristyl chain and $T\beta\gamma$ (or vice versa, between the $T\beta\gamma$ farnesyl chain and Tα) seemed unlikely. To investigate this model, we further analyzed the dependence of transducin subunit association and membrane binding on the integrity of their lipid modifications, in three artificial membrane models: nonionic detergent micelles, mixed phospholipid−cholate micelles, and large unilamellar phospholipidic vesicles. Our models are sketched in Figure 7.

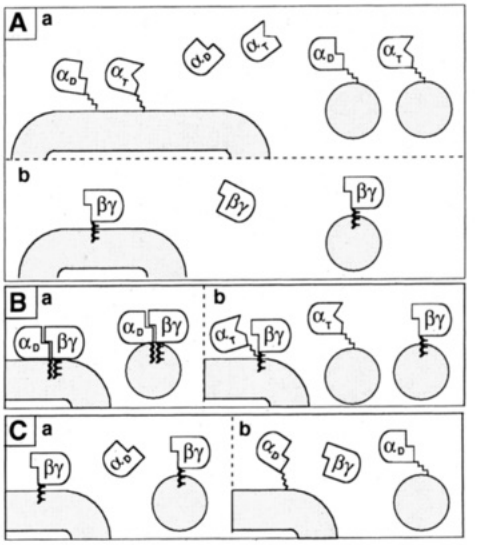

FIGURE 7: Models for the roles of the lipid modifications in transducin subunit association and membrane binding. α_D is the symbol for TαGDP that has a protein-binding site for $T\beta\gamma$. αT is the symbol for TαGTP or $T\alpha GTP\gamma S$ that has no protein-binding site for $T\beta\gamma$. Acylation of Tα and farnesylation and carboxymethylation of $T\beta\gamma$ are also symbolized. The effects of demethylation are not discussed here. (A) Interaction of isolated Tα and $T\beta\gamma$ subunits with phospholipid vesicles and with detergent micelles. (a) Unacylated α_D and α_T do not bind to vesicles or to micelles; the acylated form binds weakly, independent of its GDP or GTP conformation. (b) Defarnesylated $T\beta\gamma$ does not bind to vesicles nor to micelles; farnesylated and carboxymethylated $T\beta\gamma$ binds strongly. (B) Cooperativity of binding of lipid-modified Tα and $T\beta\gamma$ subunits when both lipid modifications are present. (a) The binding of acylated α_D to vesicles and to micelles is highly enhanced by that of farnesylated $\beta\gamma$. (b) The binding of acylated $_{n}T\alpha GTP\gamma S$ to vesicles is slightly enhanced by that of farnesylated $T\beta\gamma$, but no cooperativity in binding was detected in the presence of detergent micelles. (C) Loss of cooperativity in the binding of α_D and $\beta\gamma$ when the lipid modification has been suppressed on either of the subunits.

Our results first suggest that, in the absence of membrane lipids or of the equivalent hydrophobic environment of a detergent micelle, a subunit's lipid chain does not bind to the other subunit's protein moiety, but probably makes a hydrophobic contact with the other subunit's lipid domain. This weak lipid−lipid interaction was detected in the gel filtration assay between $_{n}T\alpha GDP$ and $_{fc}T\beta\gamma$ (Figure 4B, a) and seemed to disappear when either the Tα acyl chain (Figure 4B, d) or the $T\beta\gamma$ farnesyl group (Figure 4B, c) was missing. A protein−protein interaction is also involved in the binding of the subunits in solution; weak binding was detected by the gel filtration assay between acyl-free TαGDP and farnesyl-free $T\beta\gamma$, and it is also observed between acyl-free $_{r}T\alpha GDP$ and farnesylated and carboxymethylated $_{fc}T\beta\gamma$ in the supernatant of the sedimentation assay in the presence of phospholipid vesicles (Figure 6B). As expected, this protein−protein component of the interaction depended on the conformation of Tα and was not observed with $T\alpha GTP\gamma S$.

In the presence of membrane lipids, or of the equivalent hydrophobic environment of a detergent micelle or lipid vesicle, the subunit's lipid chain commands their individual binding to the lipid medium. For the $T\beta\gamma$ subunit, a surprisingly large effect of the carboxymethylation is super-

imposed on that of the farnesylation: $_{fc}T\beta\gamma$ bound efficiently, but $_{f}T\beta\gamma$ did not bind detectably in the sedimentation assay with phospholipid vesicles. Recently, Silvius and l'Heureux (1994) also observed a large difference in the binding affinities of model isoprenylated peptides to lipid vesicles, depending on their C-terminal methylation. The methylation effect was less striking in the gel filtration assays with phospholipid−cholate micelles to which $_{f}T\beta\gamma$ already seems to bind significantly. For TαGDP, N-acylation induces only weak binding to phospholipid vesicles, which subsists in the active TαGTPγS form.

The presence of $_{fc}T\beta\gamma$ on the membrane considerably enhances the binding of $_{n}T\alpha GDP$. This effect depends as much on the farnesyl group of Tβγ as on the acyl group of Tα. When either of the lipid modifications is missing, the cooperativity in the subunit binding to the membrane is totally lost. This lipid-dependent cooperativity in subunit binding to the membrane does not necessarily imply a direct interaction between the lipid moieties of the two subunits: when inserting, even partially and transiently, within the membrane lipids, the acyl and the farnesyl groups considerably increase the local concentrations of the two attached transducin subunits and restrict their relative disorientation (entropic effect). This may suffice to increase the probability of protein−protein contacts with relative positions and orientations of the two subunits that favor their binding. The Tα acyl and Tβγ farnesyl groups might even interact with each other when both insert in the same membrane, as if these peculiar lipid chains segregated together in the membrane lipid phase. This lipid−lipid interaction would further increase protein−protein contacts and association. One might speculate that the protein−binding sites are the terminal peptides to which the lipid chains are attached and would be brought into close proximity to each other at the membrane surface through their lipid attachments.

It has been suggested that the G-protein subunits associate via a three-stranded coiled-coil between the helices of Tα and Tβγ (Lupas *et al.*, 1992). The N-terminal helix of Tα, to which the acyl group is attached, would associate in an antiparallel arrangement with a two-stranded coiled-coil between the N-terminal helices of Tβ and Tγ (Journot *et al.*, 1991; Lupas *et al.*, 1992). The farnesyl group attached to the C terminus of Tγ might not be very close to the interaction site, but an antiparallel arrangement of the N-terminal helix of Tα predicts that the N-terminal myristyl group of Tα projects out from the same end of the three-stranded coiled-coil as the C-terminal farnesyl group of Tβγ. Thus, in the heterotrimer, the two protein-attached lipid chains could be very close to each other and could interact when they are both inserted into the membrane.

With regard to the action of Tβγ on the rate of Tα ADP-ribosylation by pertussis toxin, we confirmed (data not shown) that the catalytic action of farnesylated Tβγ was strongly enhanced in the presence of detergent micelles or lipid vesicles. We did not show or discuss these data, as we noticed that long ago Moss *et al.* (1986) had observed that the ADP-ribosyl transferase activity of the toxin itself was already strongly enhanced by lipids or detergent, in the absence of Tβγ.

In conclusion, none of our results require or support the hypothesis that the Tα acyl chain recognizes a protein site in Tβγ or that the Tγ farnesyl chain recognizes a protein site in Tα. Our data indicate that both the farnesyl and acyl groups insert into the membrane, where they interact with the membrane lipid chains, and possibly with each other. The roles of the lipid modifications in the G-protein subunit heterotrimeric association, heterotrimer membrane binding, and G-protein−receptor interaction can all be understood in terms of lipid−lipid interactions between the Tα acyl, Tβγ farnesyl, and membrane fatty acid chains.

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