A 50 kDa Protein Modulates Guanine Nucleotide Binding of Transglutaminase II[†]

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ABSTRACT: Regulation of cellular response is an important mechanism for controlling cellular functions. The transmembrane signaling of the hormone receptors is regulated by GTP-binding proteins (GTPases) and their associated proteins. Our previous studies demonstrated that the bifunctional GTP-binding protein, $G\alpha_h$ (transglutaminase II), consistently copurified with an ~50 kDa protein ($G\beta_h$) which is dissociated from $G\alpha_h$ upon activation with GTPγS or AlF_4^-. Present immunological and biochemical studies on the regulation of the GTPase cycle of $G\alpha_h$, which involves the α_1-adrenoceptor and 50 kDa $G\beta_h$, reveal that the 50 kDa protein is indeed a $G\alpha_h$-associated protein and down-regulates functions of $G\alpha_h$. Thus, polyclonal antibody against $G\beta_h$ coimmunoprecipitates GDP-bound $G\alpha_h$ but not the GDP−AlF_4^--bound form. The GTPγS binding and GTPase activity of $G\alpha_h$ are inhibited in a $G\beta_h$ concentration dependent manner. Supporting this notion, $G\beta_h$ accelerates GTPγS release from $G\alpha_h$ and changes the affinity of $G\alpha_h$ from GTP to GDP. Moreover, the ternary complex preparation exhibits TGase activity that is inhibited in the presence of the α_1-agonist and GTP. The GTPγS binding by the ternary complex, consisting of the α_1-agonist, the receptor, and G_h, is also inhibited by $G\beta_h$. The inhibition of GTPγS binding with the ternary complex requires a ≥2.7-fold higher concentration of $G\beta_h$ than that for $G\alpha_h$ alone, indicating that the receptor enhances the affinity of $G\alpha_h$ for GTP. In addition, $G\beta_h$ copurifies with an α_1-agonist, adrenoceptor, and $G\alpha_h$ ternary complex, showing that the complex is a heterotetramer. Our data also suggest that $G\beta_h$ does not directly interact with the α_1-adrenoceptor. These findings clearly demonstrate that $G\alpha_h$ associates with a novel protein which modulates the affinity of $G\alpha_h$ for guanine nucleotides and that the GDP-bound G_h is the ground state for the counterpart activator, the α_1-adrenoceptor, in this signaling system.

The involvement of G_h[1] and its family proteins in hormone-mediated transmembrane signaling was first identified by its ability to form a stable ternary complex with the α_1-agonist-bound receptor and by reconstitution studies with purified G_h and α_1-adrenoceptor (Im & Graham, 1990; Im et al., 1990). The effector in the α_1-adrenoceptor signaling was purified by formation of an α_1-receptor-mediated $G\alpha_{h7}$−phospholipase C (PLC) complex from bovine liver membranes. Purification of PLC by dissociation of the $G\alpha_{h7}$−PLC complex revealed that a phosphoinositide-specific 69 kDa PLC was the effector (Das et al., 1993). Total reconstitution studies with α_1-adrenoceptor, G_h as well as G_{h7}, and 69 kDa PLC showed that these three components effectively coupled to each other, resulting in an increased affinity of PLC for calcium [Das et al., 1993; see also Im et al. (1992)].

Our recent studies of peptide sequence and immunological characterization have demonstrated that $G\alpha_h$ and its family proteins were previously identified transglutaminases II (TGase II) (Nakaoka et al., 1994). Coexpression of cDNAs of rat liver TGase II and Chinese hamster α_{1B}-adrenoceptor into various cells showed that both expressed proteins effectively coupled, resulting in phosphoinositide formation. TGase II from guinea pig liver also stimulated 69 kDa PLC (Nakaoka et al., 1994). Consistent with our previous observations with $G\alpha_h$ and its family proteins (Im et al., 1990), classical TGase II was able to bind GTP in a 1:1 ratio (Achyuthan & Greenberg, 1987; Bergamini & Signorini, 1993) and also to hydrolyze it (Lee et al., 1989). These findings implied that $G\alpha_h$ not only possessed two cellular functions, namely GTPase and transglutaminase activity, but also transmitted the receptor signal to the effector. Physiological functions involving the TGase activity of $G\alpha_h$ still remain unclear (Greenberg et al., 1991). Several studies, however, have suggested that TGase II is involved in

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[1] Abbreviations: G-protein, classical GTP-binding regulatory protein consisting of a 39−52 kDa GTP-binding α-subunit, a 35−37 kDa β-subunit, and a 5−10 kDa γ-subunit; G_h, holo enzyme consisting of a ≥74 kDa GTP-binding α-subunit and a 50 kDa protein; $G\alpha_h$, 74 kDa guanine nucleotide-binding regulatory protein and a tissue type transglutaminase, first identified from rat liver membranes by α_1-agonist−receptor−G_h ternary complex formation; $G\alpha_{h7}$, 78 kDa guanine nucleotide-binding protein of the $G\alpha_h$ family purified from bovine heart; GPL TGase II, $G\alpha_h$ family protein which is a GTPase and tissue type transglutaminase purified from guinea pig liver cytosol; $G\alpha_{h7}$-antibody, a polyclonal antibody raised against 78 kDa $G\alpha_{h7}$ and previously designated as $G_{h7\alpha}$-antibody; $G\beta_h$, 50 kDa protein which copurifies with $G\alpha_h$ and $G\alpha_{h7}$; $G\beta_h$-antibody, polyclonal antibody raised against $G\beta_h$ purified from rat liver; GTP, guanosine 5′-triphosphate; GTPγS, guanosine 5′-*O*-3-thiotriphosphate; GDP, guanosine 5′-diphosphate; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; DTT, dithiothreitol.

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regulation of cell growth and differentiation, wound healing, and stabilization of cell−cell interaction (Birckbichler et al., 1981; Murtaugh et al., 1983; Nara et al., 1989; Piacentini et al., 1991; Gentile et al., 1992; van Groningen et al., 1995). It has also been demonstrated that a cytosolic phospholipase A_2 is activated by posttranslational modification by TGase II (Cordella-Miele et al., 1990).

TGase and GTPase activities of $G\alpha_h$ are regulated by two reciprocal activators, Ca^{2+} and GTP (Achyuthan et al., 1987; Lee et al., 1989; Greenberg et al., 1991; Bergamini & Signorini, 1993; Nakaoka et al., 1994). Although TGase II is not known to associate with other proteins, when $G\alpha_h$ was purified from the rat liver membranes, an ~50 kDa protein ($G\beta_h$) consistently copurified with $G\alpha_h$ and subsequently with $G\alpha_{h7}$ from bovine heart membranes under nonactivating conditions (Im et al., 1990; Baek et al., 1993). Moreover, $G\beta_h$ was dissociated from $G\alpha_h$ upon activation of the heterodimeric G_h with a nonhydrolizable GTP analogue, GTPγS, or the G-protein activator, AlF_4^- (Im et al., 1992). These findings indicate that $G\beta_h$ is involved in modulation of the guanine nucleotide binding to $G\alpha_h$. We report herein that $G\alpha_h$ is associated with a novel 50 kDa protein that is involved in regulation of guanine nucleotide binding to $G\alpha_h$ and its family proteins.

EXPERIMENTAL PROCEDURES

Materials. Guinea pig liver transglutaminase II, GTP-agarose, and *N*,*N*′-dimethylated casein were purchased from Sigma. Protein A-agarose, heparin-agarose, and other chromatographic materials were obtained from Pharmacia, and nucleotides were from Boehringer Mannheim. Sucrose monolaurate (SM-1200) was a gift from the Mitsubishi-Kasei Co. (Tokyo, Japan). Dimethyl pimelimidate dihydrochloride was purchased from Pierce, and [^{3}H]putrescine, chemiluminescence reagent, [^{35}S]GTPγS (~1300 Ci/mmol), [γ-^{32}P]GTP (3000 Ci/mmol), [α-^{32}P]GTP (3000 Ci/mmol), and [^{3}H]-prazosin (72.6 Ci/mmol) were from Du Pont New England Nuclear. Other chemical and biochemical materials were of the highest grade commercially available.

Preparations of the α_1-Adrenoceptor and the α_1-Agonist−Receptor−G$\alpha(\beta)_h$ Ternary Complex. The α_1-adrenoceptor was partially purified from the rat liver membranes (Im et al., 1990), except the phentolamine-treated membranes were solubilized with 0.3% sucrose monolaurate (SM) instead of 1% digitonin. The partial purification of the α_1-receptor was achieved by sequential chromatography with heparin-agarose and wheat germ agglutinin-agarose. An α_1-agonist−receptor−$G\alpha(\beta)_h$ ternary complex was prepared from rat liver membranes (1 g) by the method of Im and Graham (1990), except 50 μM GDP and 1 mM $MgCl_2$ were included throughout purification. For the studies, excess salts in the preparations were removed through dried Sephadex G-25 columns (3 mL) equilibrated with the HSDG buffer [20 mM Hepes (pH 7.4), 150 mM NaCl, 1 mM dithiothreitol, and 5% glycerol] containing 50 μM GDP, 1 mM $MgCl_2$, and 0.05% SM. The HSDG buffer was also used for the experiments throughout the study. Changes are specified in the figure legends.

Purification of G_h and Its Family Proteins. G_h from rat liver and G_{h7} from bovine heart membranes were purified as described previously (Im et al., 1990; Baek et al., 1993). $G\alpha_h$, $G\alpha_{h7}$, and guinea pig liver transglutaminase (GPL TGase II) were purified using GTP-agarose [Lee et al., 1989; see also Nakaoka et al. (1994)]. To stabilize the purified proteins, 50 μM GTP and 1 mM $MgCl_2$ were added to the eluted samples after the salt was removed through dried Sephadex G-25 columns equilibrated with the HSDG buffer containing 0.05% SM. In addition, the $\beta\gamma$-subunits of the heterotrimeric G-protein were purified from bovine brain (Sternweis & Robishaw, 1984).

Preparation of [α-^{32}P]GDP−G_h. To radiolabel $G\alpha_h$, G_h (4−5 pmol) was incubated with 5 μM [α-^{32}P]GTP (specific activity, 1.5×10^8 cpm/μM) in the presence of 2 mM $MgCl_2$ at 30 °C for 50 min [see Im et al. (1990) and Baek et al. (1993)]. After incubation, to covalently link the radioligand to $G\alpha_h$, the samples were subjected to UV irradiation for 15 min in an ice bath (Im & Graham, 1990). Free radioligand was removed using a dried Sephadex G-25 column (3 mL) which was pre-equilibrated with 20 μM GDP and 1 mM $MgCl_2$ in the HSDG buffer containing 0.05% SM. The samples were further incubated to hydrolize the remaining [α-^{32}P]GTP and reassociate both proteins at 4 °C overnight and immediately used for the experiments.

Purification of 50 kDa $G\beta_h$. $G\beta_h$ was separated from the purified G_h protein. G_h (200−300 μg) in HSDG buffer containing 0.05% SM was activated with 2 μM GTPγS in the presence of 2 mM $MgCl_2$ at 30 °C for 1 h. The activated G_h was diluted two times with 20 mM Hepes buffer (pH 7.4) containing 1 μM GTPγS, 1 mM $MgCl_2$, and 1 mM DTT and applied onto a Q-Sepharose column (3 mL) equilibrated with 20 mM Hepes (pH 7.4), 50 mM NaCl, 1 μM GTPγS, 1 mM $MgCl_2$, 1 mM DTT, and 0.05% SM. The column was washed with the column equilibration buffer, and the bound proteins were eluted using a step gradient of salt (50 to 500 mM) in the equilibration buffer. $G\beta_h$ and $G\alpha_h$ were eluted at salt concentrations of 70 and 400 mM, respectively [see Im et al. (1992)]. G_h protein was also found between these salt concentrations. The purified $G\beta_h$ was used for both immunization and biochemical studies.

Western Blotting. Polyclonal antibody against 50 kDa $G\beta_h$ was generated in New Zealand white rabbits using the protocol of Baek et al. (1993). $G\beta_h$ (~60 μg in 200 μL) was emulsified with an equal volume of complete Freund's adjuvant and injected subcutaneously into the rabbits. At 3 week intervals, three booster injections were given with 40−50 μg of $G\beta_h$ and incomplete Freund's adjuvant. Rabbit antisera were characterized by immunoblots [Harris et al., 1985; see also Baek et al. (1993)], except antibody cross-reactivity was determined using chemiluminescence. Anti-$G\beta_h$ antibody (designated as $G\beta_h$-antibody) and nonimmune sera were diluted 1:500−1000 in a solution containing 50 mM Tris/HCl (pH 8.0), 80 mM NaCl, 2 mM $CaCl_2$, 0.02% NaN_3, 0.2% Nonidet P-40, and 5% nonfat dry milk. Anti-rabbit Ig, horseradish peroxidase (1:1000 dilution) was used for the secondary antibody. The polyclonal $G\beta_h$-antibody recognized nanogram ranges (≤40 ng) of purified $G\beta_h$ by immunoblotting and enzyme-linked immunosorbent assay (ELISA) (data not shown).

Preparation of Antibody−Protein A-Agarose. For immunoprecipitation studies, immunoaffinity resins of $G\alpha_{h7}$- and $G\beta_h$-antibodies were prepared (Schneider et al., 1982). Briefly, the antisera (1 mL) were mixed with the same amount of protein A-agarose suspended in 10 mL of 0.1 M borate buffer (pH 8.2) for 45 min at room temperature. The antibody-bound protein A-agarose was intensively washed

with 0.1 M borate buffer and further washed three times with 0.2 M triethanolamine (pH 8.2). The resins were incubated with freshly made 20−30 mM dimethyl pimelimidate in 0.2 M triethanolamine (10 mL) at room temperature for 45 min. The agarose was intensively washed with 50 mM ethanolamine (pH 8.2) and then 0.1 M borate buffer containing 0.02% sodium azide. The covalently linked antibody−protein A-agarose was stored at 4 °C until use. Immunoreactivity of the immunoaffinity agarose was determined by immunoblotting, after immunoprecipitation of the corresponding protein. The immunoaffinity resins were stable for more than 2 weeks at 4 °C. Nonimmune sera- or atrial natriuretic factor (ANF)-antibody−protein A-agarose was also prepared using the same protocol. The immunoaffinity agarose was used throughout the immunoprecipitation studies.

Coimmunoprecipitation. Coimmunoprecipitation of the α_1-adrenoceptor in the ternary complex preparations by $G\alpha_{h7}$- and $G\beta_h$-antibodies was performed using the immunoaffinity resins prepared as described above. The ternary complex preparations (100−200 fmol of the α_1-receptor and 120−230 fmol of [^{35}S]GTPγS binding) were incubated with 5 μM (−)-epinephrine, 5 μM (−)-epinephrine plus 5 μM GTPγS, or 5 μM (−)-epinephrine plus 0.1 mM phentolamine in the presence of 0.5 mM $MgCl_2$ in the HSDG buffer containing 0.05% SM at 30 °C for 30 min. The samples were further incubated with the $G\alpha_{h7}$- or $G\beta_h$-antibody affinity resins (40 μL from 500 μL of wet resin in 300 μL of HSDG buffer) with gentle rotation at 4 °C for 2 h. The reaction mixture had a final volume of 250 μL. The samples were centrifuged at 2000 rpm for 10 min. The α_1-receptors were measured in the supernatants after the excess ligands were removed through dried Sephadex G-25 columns (3 mL) equilibrated with the HSDG buffer containing 1 mM $MgCl_2$ and 0.05% SM. Nonspecific binding to the immunoaffinity agarose was determined using ANF-antibody−protein A-agarose under the same conditions as described above.

For the coimmunoprecipitation of $G\alpha_h$ by the $G\beta_h$-antibody, the radiolabeled G_h was prepared as described above. The radiolabeled G_h (1 pmol) was further incubated in the presence of 10 μM GDP and 1 mM $MgCl_2$ with or without AlF_4^- (20 μM $AlCl_3$, 5 mM NaF, and 3 mM $MgCl_2$) at 4 °C for 4−5 h. The samples were then incubated with $G\alpha_{h7}$- and $G\beta_h$-antibody−protein A-agarose (40 μL) with gentle rotation at 4 °C for 2 h. The final sample volume was 250 μL. The pellets were collected by centrifugation at 2000 rpm for 10 min and washed three times with 500 μL of HSDG buffer containing 10 μM GDP plus 1 mM $MgCl_2$ or 10 μM GDP plus AlF_4^-. The pellets suspended in HSDG buffer (30 μL) were denatured in the presence of an equal volume of Laemmli buffer in a boiling water bath for 10 min (Laemmli, 1970). The coimmunoprecipitated proteins were autoradiographed overnight on Kodak XAR-5 X-ray film using double intensifying screens following SDS−PAGE (8−10% gel).

An α_1-Agonist-Mediated Inhibition of the Transglutaminase with the Ternary Complex Preparation. To measure the receptor-mediated TGase activity, the ternary complex preparations were reconstituted into phospholipid vesicles (Im et al., 1992). The vesicles were then preincubated with 5 μM (−)-epinephrine or 5 μM (−)-epinephrine plus 0.1 mM phentolamine in the presence of 2 mM $MgCl_2$ at 4 °C for 2−4 h. The enzyme activity was evaluated in the presence and absence of 100 μM $CaCl_2$ and 2 μM GTPγS by the incorporation of [^{3}H]putrescine (0.1 mM) into *N,N′*-dimethylated casein (1%) at 20 °C for 30 min in the HSDG buffer containing 2 mM DTT in a 100 μL final volume (Achyuthan & Greenberg, 1987; Nakaoka et al., 1994). After incubation, the reaction was stopped in an ice bath by the addition of ice-cold 50% trichloroacetic acid (100 μL) containing 0.1% putrescine. The precipitates were trapped on GF/F glass fiber filters (Whatman), washed six times using 4 mL of 5% ice-cold trichloroacetic acid, and counted.

Other Experiments. Reconstitution of proteins into phospholipid vesicles was achieved by the dilution method (Im et al., 1992). Receptor and G-proteins were quantitated by the ability to bind [^{3}H]prazosin (3 nM) and 1−2 μM [^{35}S]-GTPγS (specific activity, 15−25 cpm/fmol), respectively (Im & Graham, 1990; Im et al., 1990). GTPase activity of $G\alpha_{h7}$ was measured as described (Im et al., 1990). Changes are specified in the figure legends. Protein concentrations were measured by the Bradford method (Bradford, 1976) using a Bio-Rad protein determination kit.

RESULTS

Association of the 50 kDa Protein with $G\alpha_h$. To assess whether the 50 kDa protein is associated with $G\alpha_h$, polyclonal antibody against the 50 kDa protein was raised and tested. The cross-reactivity of polyclonal anti-$G\beta_h$ antibody was evaluated by immunoblotting (Figure 1). As presented in Figure 1A, the $G\beta_h$-antibody recognized the purified $G\beta_h$ from rat liver membranes (lane 1) and a similar molecular mass protein with extract from bovine heart membranes (lane 2). When similar experiments were performed with nonimmune sera or antibody preincubated with the antigen, $G\beta_h$, the results were negative (data not shown). Moreover, the antibody did not recognize any other proteins, showing that $G\beta_h$ is distinct (see lane 2 in Figure 1 A). $G\beta_h$ was widely distributed in tissues and most abundant in rat liver, heart, and spleen as $G\alpha_h$ is (data not shown) [see Baek et al. (1993)]. In addition, immunoblotting studies with the β-subunit antibody (common) of the heterotrimeric G-proteins suggested that the primary structure of $G\beta_h$ probably differs from the $\beta\gamma$-subunits of the heterotrimeric G-proteins. Thus, the $\beta\gamma$-subunits of the heterotrimeric G-proteins purified from bovine brain were not recognized by the $G\beta_h$-antibody. Consistent with this notion, the β-subunit antibody (common) of the heterotrimeric G-proteins did not cross-react with $G\beta_h$.

Since the 50 kDa $G\beta_h$ dissociated from $G\alpha_h$ upon activation of G_h with GTPγS or AlF_4^- [see Experimental Procedures and Im et al. (1992)], using the $G\beta_h$-antibody, association/dissociation of $G\beta_h$ with/from $G\alpha_h$ was reexamined. G_h was radiolabeled using [α-^{32}P]GTP and then incubated with GDP to form GDP-bound G_h. [α-^{32}P]GDP-bound G_h was incubated with immunoaffinity resins of $G\alpha_{h7}$- or $G\beta_h$-antibody in the presence or absence of AlF_4^-, which acts as a γ-phosphate of GTP and activates G-proteins [see Gilman (1987)]. The results are shown in Figure 1B. In the presence of AlF_4^-, the $G\alpha_{h7}$-antibody effectively coimmunoprecipitated 74 kDa GDP-bound $G\alpha_h$ (lane 1) but the $G\beta_h$-antibody did not (lane 2), demonstrating that $G\beta_h$ dissociated from GDP−AlF_4^--bound $G\alpha_h$. On the other hand, in the absence of AlF_4^-, the $G\beta_h$-antibody−protein A-agarose coimmunoprecipitated the GDP-bound form of

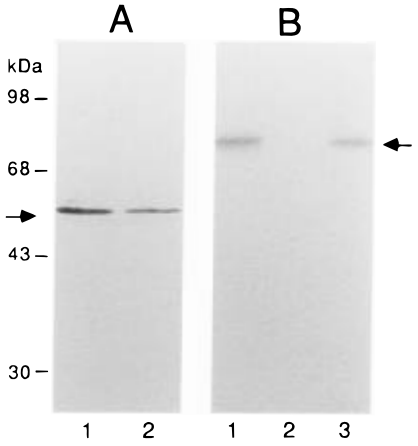

FIGURE 1: Immunological characterization of polyclonal $G\beta_h$-antibody and interaction of $G\beta_h$ with $G\alpha_h$. (A) Cross-reactivity of the polyclonal antibody against 50 kDa $G\beta_h$. The purified $G\beta_h$ (70 ng) and 100 μL extracts of bovine heart membranes were subjected to immunoblotting using $G\beta_h$-antibody. The bovine heart membranes (10 mg/mL) were extracted with 1% cholate in HSDG buffer at 4 °C for 1 h. Immunoblotting was performed as described in Experimental Procedures. The arrow indicates the 50 kDa protein recognized by the antibody with the purified protein (lane 1) and extract from the bovine heart membranes (lane 2). (B) Immunoprecipitation of GDP-bound G_h or GDP−AlF_4^--bound G_h by the $G\alpha_{h7}$- and $G\beta_h$-antibodies. The GDP-bound G_h (1 pmol/tube) was prepared using radiolabeled G_h as detailed in Experimental Procedures. Before being subjected to the immunoprecipitation, G_h was incubated with 20 μM GDP in the presence (lanes 1 and 2) or absence (lane 3) of AlF_4^- at 4 °C for 4 h. The samples were then subjected to immunoprecipitation using $G\alpha_{h7}$- and $G\beta_h$-antibody affinity agarose (40 μL/tube) in a 150 μL final volume, as detailed in Experimental Procedures. The coimmunoprecipitated proteins were autoradiographed overnight following SDS−PAGE (10% gel). Lane 1 shows 74 kDa GTP-binding $G\alpha_h$ which was immunoprecipitated in the presence of AlF_4^- by $G\alpha_{h7}$-antibody. Lanes 2 and 3 are co-immunoprecipitation of $G\alpha_h$ by $G\beta_h$-antibody in the presence (lane 2) and in the absence (lane 3) of AlF_4^-. In the presence of AlF_4^-, a slight 74 kDa band was also observed with the $G\beta_h$-antibody immunoprecipitation (lane 2) after exposure for 72 h. The arrow indicates the radiolabeled 74 kDa $G\alpha_h$.

radiolabeled $G\alpha_h$ (lane 3), showing that $G\beta_h$ was tightly associated with GDP-bound $G\alpha_h$. These results clearly demonstrated that the 50 kDa $G\beta_h$ associates with GDP-bound $G\alpha_h$(TGase II) and indeed dissociates from 74 kDa $G\alpha_h$ upon activation.

Copurification of $G\beta_h$ with the α_1-Agonist−Receptor−$G\alpha_h$ Ternary Complex. Since $G\alpha_h$ is a TGase II (Nakaoka et al., 1994), to observe whether the ternary complex preparations exhibited the TGase activity, the preparations were inserted into the phospholipid vesicles. To optimize experimental conditions, the following biochemical properties of $G\alpha_h$ were considered. The TGase activity of $G\alpha_h$ is inhibited by the binding of GTP or GTPγS and inhibits the GTP binding to $G\alpha_h$ (Achyuthan & Greenberg, 1987; Greenberg et al., 1991). In addition, the GTP binding by the ternary complex is fast and occurs even at low temperatures (Im & Graham, 1990; Im et al., 1990), but optimal temperature for the TGase stimulation is 30−37 °C. Considering these properties of $G\alpha_h$, copurification of TGase II and functional coupling with the α_1-adrenoceptor were examined by determination of α_1-agonist-mediated inhibition of TGase stimulation using 100 μM $CaCl_2$ at 20 °C for 30 min. As shown in Figure 2A, incubation of vesicles containing the ternary complex preparation with GTP resulted in a ≥28% decrease of the Ca^{2+}-activated TGase activity. In the presence of the TGase activator, Ca^{2+}, the TGase activity was greatly increased (≥6-fold), as compared to the activity in absence of the activator. Addition of GTPγS did not significantly change the basal TGase activity. When the ternary complex was induced by incubation of (−)-epinephrine, the GTP-mediated inhibition of TGase activity was further decreased up to the basal level of the TGase activity. Furthermore, in the presence of antagonist, the agonist-mediated inhibition of the TGase activity was reversed up to the level of the GTP-mediated inhibition. These results clearly indicated that $G\alpha_h$ is TGase II that couples to α_1-adrenoceptor, confirming the previous finding (Nakaoka et al., 1994).

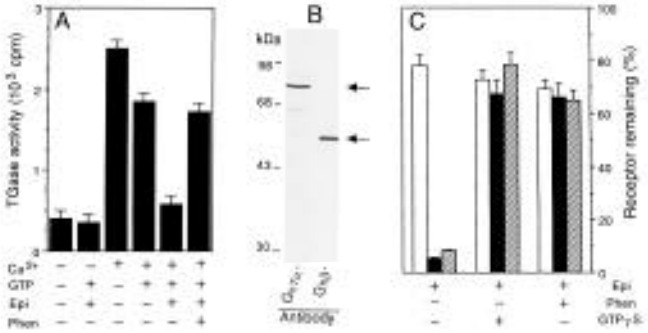

FIGURE 2: Copurification of TGase II and 50 kDa $G\beta_h$ with the α_1-agonist, receptor, and $G\alpha_h$ ternary complex. (A) α_1-Agonist-mediated inhibition of the TGase activity with the ternary complex preparations. The ternary complex preparations, which contained 250 fmol of α_1-receptor and 310 fmol of GTPγS binding, were reconstituted into phospholipid vesicles as described in Experimental Procedures. The vesicles were preincubated under the conditions buffer only, 5 μM (−)-epinephrine, and 5 μM (−)-epinephrine plus 0.1 mM phentolamine as indicated in the figure. After incubation at 4 °C for 3 h, the TGase activity was determined using 100 fmol of the α_1-receptor in the samples (40 μL) with 0.5 mM $MgCl_2$ and 100 μM $CaCl_2$ in the presence or absence of 2 μM GTPγS at 20 °C for 30 min. Basal TGase activity was determined with and without GTPγS in the absence of $CaCl_2$. The results are mean values from three independent experiments in duplicate. Abbreviations are as follows: Epi, epinephrine; Phen, phentolamine. (B) Autoradiogram of the immunoblot of $G\alpha_h$ and $G\beta_h$ with the ternary complex preparation. The ternary complex (100 μg of protein) was subjected to immunoblotting following SDS−PAGE (8% gel). The immunoblotting experiments were performed using $G\alpha_{h7}$-antibody (lane 1) or $G\beta_h$-antibody (lane 2), as detailed in Experimental Procedures. The antibody cross-reactivity was visualized with chemiluminescence, and the film was exposed for 10 s. The arrows indicate 74 kDa $G\alpha_h$ (lane 1) and 50 kDa $G\beta_h$ (lane 2) which were cross-reacted with $G\alpha_{h7}$- and $G\beta_h$-antibody, respectively. (C) Coimmunoprecipitation of the α_1-adrenoceptor in the ternary complex preparations by the $G\alpha_{h7}$- and $G\beta_h$-antibody. The ternary complex preparations (102 fmol of α_1-receptor and 121 fmol of GTPγS binding) were incubated with 5 μM (−)-epinephrine, 5 μM (−)-epinephrine plus 5 μM GTPγS, or 5 μM (−)-epinephrine plus 0.1 mM phentolamine in the presence of 0.5 mM $MgCl_2$ in HSDG buffer containing 0.05% SM at 30 °C for 30 min as indicated in the figure. The samples were further incubated with the $G\alpha_{h7}$- or $G\beta_h$-antibody affinity agarose (40 μL/tube) with gentle rotation at 4 °C for 2 h. The α_1-receptors were measured in the supernatants after the excess ligands were removed through dried Sephadex G-25 columns as detailed in Experimental Procedures. Nonspecific binding to the immunoaffinity agarose was determined using ANF-antibody−protein A-agarose under the same conditions as described above. The data shown are the mean of three independent experiments performed in triplicate. The α_1-receptor density in supernatants are given: ANF-antibody (open bar), $G\alpha_{h7}$-antibody (black bar), and $G\beta_h$-antibody (hatched bar). Abbreviations are as follows: Epi, (−)-epinephrine; Phen, phentolamine.

Copurification of $G\beta_h$ with the ternary complex was then evaluated by immunoblotting with $G\alpha_{h7}$- and $G\beta_h$-antibodies.

As shown in Figure 2B, the ternary complex contained 74 kDa Gα_h and 50 kDa Gβ_h that were specifically recognized by Gα_{h7}- and Gβ_h-antibody, respectively. Furthermore, when the ternary complex preparation was subjected to coimmunoprecipitation, both Gα_{h7}- and Gβ_h-antibody affinity resins effectively coimmunoprecipitated $\geq$87% the α_1-receptor upon activation of the receptor with the α_1-agonist (Figure 2C). On the other hand, when the ternary preparations were incubated with GTPγS or α_1-antagonist, neither antibody affinity resin coprecipitated the α_1-receptor. Thus, the receptors were found to be 65−85% in the supernatants. The ANF-antibody affinity resins did not precipitate the receptors under any conditions. The slight decrease of the receptor in the supernatant with ANF-antibody resins is most likely due to the nonspecific binding of the receptor and/or G$\alpha\beta_h$ to the immunoaffinity resins.

The above results clearly showed that 50 kDa Gβ_h associates with the α_1-agonist, receptor, and Gα_h ternary complex and that Gα_h and Gβ_h dissociated from the receptor upon activation of G_h. These results also indicated that antagonist prevented association of G_h with the α_1-adrenoceptor.

Physical Contact between Proteins. To understand the role of Gβ_h in regulation of Gα_h in the GTPase cycle involving the α_1-adrenoceptor, the partially purified α_1-receptors were reconstituted with GDP-bound Gα_h or Gβ_h in the detergent solution. The reconstituted samples were then subjected to immunoprecipitation under various conditions (Figure 3A). The Gα_{h7}-antibody effectively coimmunoprecipitated the receptors in the presence of agonist but not in the presence of antagonist, consistent with observations from the studies using the ternary complex preparation (Figure 2C). In contrast, the Gβ_h-antibody did not coimmunoprecipitate the α_1-receptor in the presence of either agonist or antagonist. As expected, the control ANF-antibody failed to coimmunoprecipitate the receptor. These findings indicated that Gα_h directly interacts with the receptor upon activation of the receptor but Gβ_h does not. However, failure of the receptor to coimmunoprecipitate by Gβ_h-antibody could be the result of a weak interaction between the α_1-receptor and Gβ_h. This possibility was tested by measuring GTPγS binding following activation of the receptor. Thus, the ternary complex preparation was reconstituted with various concentrations of Gβ_h into phospholipid vesicles. As mentioned above, GTPγS binding by the ternary complex is rapid even at the low temperature (Im & Graham, 1990; Im et al., 1990). Therefore, the GTPγS binding by the vesicles containing the ternary complex was measured at various times at 4 °C, after incubation of the vesicles with (−)-epinephrine or (−)-epinephrine plus phentolamine at 4 °C for 3 h (Figure 3B). The GTPγS binding by the vesicles was fast and reached maximal GTPγS binding within 10 min, and was not significantly changed up to an 8-fold molar excess Gβ_h. However, the vesicles containing a $\geq$12-fold molar excess of Gβ_h showed slower GTPγS binding than the vesicles containing the ternary complex. The vesicles containing a 16-fold molar excess of Gβ_h exhibited only 16% GTPγS binding at 8 min, as compared to the vesicles containing the ternary complex preparation, and did not reach the maximal binding up to 16 min. These data again suggested that Gβ_h does not directly interact with the α_1-receptor and is not involved in activation of Gα_h. These findings also strongly indicated that Gβ_h is likely to play a role in deactivation of Gα_h as a counterpart to the receptor.

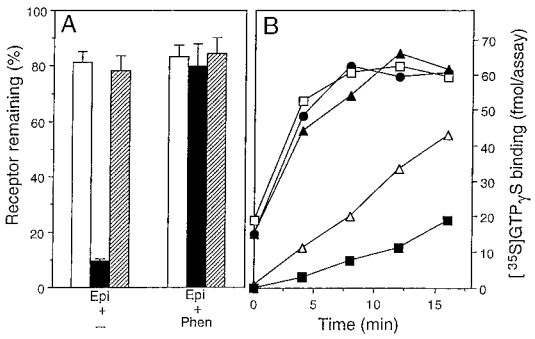

FIGURE 3: Characterization of protein interacting with the α_1-adrenoceptor. (A) Coimmunoprecipitation of the α_1-adrenoceptor with Gα_h or Gβ_h. The partially purified α_1-receptor (150 fmol) from rat liver membranes was reconstituted with either ~25 μg of Gα_h or 17 μg of Gβ_h in HSDG buffer containing 0.05% SM in the presence of 5 μM (−)-epinephrine or 5 μM (−)-epinephrine plus 0.1 mM phentolamine as indicated in the figure. The reconstituted samples were subjected to immunoprecipitation using Gα_{h7}- and Gβ_h-antibody agarose (see also Experimental Procedures). The receptor density was measured in the supernatant after the excess ligands were removed. The data are the mean from three independent experiments performed in triplicate. The α_1-receptor density in supernatants are given: ANF-antibody (open bar), Gα_{h7}-antibody (black bar), and Gβ_h-antibody (hatched bar). Abbreviations are as follows: Epi, (−)-epinephrine; Phen, phentolamine. (B) Effects of Gβ_h on the (−)-epinephrine-stimulated GTPγS binding by the ternary complex. The ternary complex preparations were reconstituted into phospholipid vesicles with Gβ_h. The vesicles were preincubated with 5 μM (−)-epinephrine or 5 μM (−)-epinephrine plus 0.1 mM phentolamine at 4 °C for 3 h. The α_1-receptor was 58 fmol/tube, and the GTPγS binding was 80 fmol/tube. The [^{35}S]-GTPγS binding by the vesicles was measured at various times in the presence of 1 μM [^{35}S]GTPγS (specific activity, 25 cpm/fmol) and 0.5 mM $MgCl_2$ at 4 °C. The samples including phentolamine were taken as nonspecific [^{35}S]GTPγS binding. The data shown are specific [^{35}S]GTPγS binding, and each point is the average of duplicate determinations. Three independent experiments showed similar results. Concentrations of Gβ_h are no addition of Gβ_h (●), 0.32 pmol (□), 0.64 pmol (▲), 0.96 pmol (△), and 1.28 pmol (■).

Effects of Gβ_h on GTPγS Binding and GTPase Activity of Gα_h. The impact of Gβ_h on intrinsic Gα_h activity was then evaluated to understand the exact role of Gβ_h. The effect of Gβ_h on GTP binding by Gα_h was determined by reconstitution of Gα_h with various concentrations of Gβ_h. As shown in Figure 4A, GTPγS binding to Gα_h was slightly increased $\leq$16.7% at low concentrations of Gβ_h (up to a 2-fold molar excess of Gβ_h) as compared to that of Gα_h alone. The reason for the increase in GTPγS binding to Gα_h at low concentrations of Gβ_h is not clear. Up to an ~3.5-fold molar excess addition of Gβ_h, the GTPγS binding by the vesicles was not changed. Further increases in the Gβ_h concentration resulted in gradual inhibition of the GTPγS binding in a concentration dependent manner. Complete inhibition was obtained at a $\geq$5-fold molar excess of Gβ_h. Thermally inactivated Gβ_h did not alter the GTPγS binding by Gα_h, demonstrating a specific effect of Gβ_h on the GTPγS binding.

The question of whether the inhibition of GTPγS binding to Gα_h by Gβ_h affected the intrinsic GTPase activity, GTP hydrolysis by Gα_h, was determined with phospholipid vesicles containing G_{h7} and various concentrations of Gβ_h. The GTPase activity of Gα_{h7} was again gradually decreased with increasing Gβ_h concentrations (Figure 4B). Thus, the GTPase activity was inhibited by ~26% with a 4-fold molar

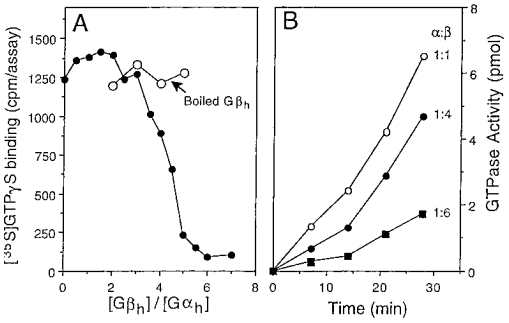

FIGURE 4: . Effect of $G\beta_h$ on GTP binding to $G\alpha_h$. (A) Modulation of GTPγS binding to $G\alpha_h$ by $G\beta_h$. $G\alpha_h$ (100 fmol/tube) was reconstituted with $G\beta_h$ (0−700 fmol) into phospholipid vesicles. The GTPγS binding by the vesicles was measured in the presence of 2 mM $MgCl_2$ using 1 μM [^{35}S]GTPγS (specific activity, 15 cpm/fmol). Nonspecific GTPγS binding was measured in the presence of 500 μM GDPβS. The data shown are the average of duplicated experiments. Similar results were obtained from three independent experiments using different preparations. (B) Effect of $G\beta_h$ on GTPase activity of $G\alpha_{h7}$. G_{h7} which showed an approximately 1:1 molar ratio of $G\alpha_{h7}$ and $G\beta_h$ on the silver-stained gel (data not shown) was reconstituted with two different concentrations of $G\beta_h$, as indicated in the figure, into phospholipid vesicles. The hydrolysis of GTP by $G\alpha_{h7}$ was measured using 0.3 μM [γ-^{32}P]GTP (specific activity, 2000 cpm/pmol) in the presence of 2 mM $MgCl_2$. The amounts of GTPγS binding activity in the vesicles were measured using 1 μM [^{35}S]GTPγS (specific activity, 15 cpm/fmol). GTPγS binding activities by the vesicles were 252 fmol for a 1:1 molar ratio of $G\alpha_h$ and $G\beta_h$, 153 fmol for a 1:4 molar ratio, and 62 fmol for a 1:6 molar ratio. Nonspecific GTP hydrolysis was measured with mock vesicles under the same conditions. The data are the average of triplicate experiments. Similar results were obtained from three independent experiments.

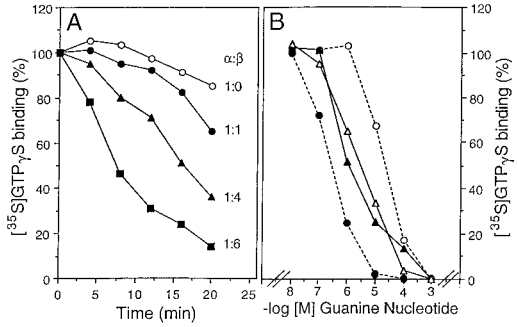

FIGURE 5: Affinity changes of $G\alpha_h$ for guanine nucleotide by $G\beta_h$. (A) Release of [^{35}S]GTPγS from $G\alpha_h$ by $G\beta_h$. $G\alpha_h$ (50 pmol) was incubated with 1 μM [^{35}S]GTPγS (specific activity, 75 cpm/fmol) in the presence of 2 mM $MgCl_2$ at 30 °C for 40 min. The unbound [^{35}S]GTPγS in the samples was removed through a dried Sephadex G-25 column (3 mL) pre-equilibrated with the reaction buffer containing 2 mM $MgCl_2$. The divided samples were reconstituted with $G\beta_h$ in the HSDG buffer containing 0.05% SM and 1 mM $MgCl_2$. The molar ratios of $G\alpha_h$ and $G\beta_h$ are indicated. The [^{35}S]-GTPγS-bound $G\alpha_h$ was measured at various time points in the presence of 10 μM GDP and 2 mM $MgCl_2$ at 30 °C. The data shown above are the average of duplicate determinations. (B) Changes in affinity of $G\alpha_h$ for guanine nucleotides in the presence of $G\beta_h$. $G\alpha_h$ (10 pmol) was reconstituted with either 10 or 50 pmol of $G\beta_h$ into phospholipid vesicles. The [^{35}S]GTPγS binding was measured in the presence of various concentrations of GTP or GDP at 30 °C for 30 min. The concentration of [^{35}S]GTPγS was 150 nM (specific activity, 4100 cpm/pmol). Experimental conditions were as follows: equal molar ratio of $G\alpha_h$ and $G\beta_h$ with GTP (●) and GDP (○); and 5-fold molar excess of $G\beta_h$ over $G\alpha_h$ with GTP (▲) and GDP (△).

excess of $G\beta_h$ and ∼72% with a 6-fold molar excess. However, the turnover of GTP hydrolysis (0.8−1.1 mol mol^{-1} min^{-1}) was not significantly affected, indicating that inhibition of GTP hydrolysis by $G\beta_h$ is due to the inhibition of GTP binding to $G\alpha_h$.

Affinity Changes of $G\alpha_h$ for Guanine Nucleotides by $G\beta_h$. To understand the inhibition mechanism of GTPγS binding to $G\alpha_h$ by $G\beta_h$, the affinity of $G\alpha_h$ for GTPγS was measured in the presence of 10 μM GDP using vesicles containing [^{35}S]GTPγS-bound $G\alpha_h$ and various concentrations of $G\beta_h$. As presented in Figure 5A, the amounts of [^{35}S]GTPγS-bound $G\alpha_h$ were decreased in time and $G\beta_h$ concentration dependent manners. At a 6-fold molar excess of $G\beta_h$, [^{35}S]-GTPγS-bound $G\alpha_h$ was decreased by ≥60% within 10 min, whereas in the absence of $G\beta_h$, [^{35}S]GTPγS-bound $G\alpha_h$ was not significantly changed up to 20 min. These results showed that $G\alpha_h$ has a high affinity for GTPγS and that $G\beta_h$ facilitates the release of GTPγS (GTP) from $G\alpha_h$. Whether the facilitation of GTPγS release from $G\alpha_h$ by $G\beta_h$ leads to a switch of the affinity of $G\alpha_h$ for GDP was evaluated by measuring [^{35}S]GTPγS binding to $G\alpha_h$ in the presence of GTP or GDP using vesicles containing $G\alpha_h$ and two different concentrations of $G\beta_h$ (α:β = 1:1 and 1:5). In these experiments, a limited concentration of [^{35}S]GTPγS (150 nM) was used to allow competition of the ligand with other guanine nucleotides. As shown in Figure 5B, $G\beta_h$ led $G\alpha_h$ to change its affinity toward GDP from GTP (GTPγS). Thus, the half-maximal inhibition of the GTPγS binding of $G\alpha_h$ was ∼0.3 μM with GTP and ∼22 μM with GDP, when the vesicles containing equal molar ratios of $G\alpha_h$ and $G\beta_h$ were used. With the vesicles containing a 5-fold molar excess of $G\beta_h$, GTP became a less potent competitor for GTPγS binding than GDP. The half-maximal inhibition of the GTPγS binding was 1.2 μM GTP and 3 μM GDP. These results clearly demonstrated that $G\beta_h$ modulates the guanine nucleotide binding of $G\alpha_h$ by changing the affinity of $G\alpha_h$ for ligands.

DISCUSSION

We have studied the regulation of the GTPase cycle of $G\alpha_h$ by the receptor and the 50 kDa associated protein, $G\beta_h$. Findings from the study reveal that the 50 kDa protein is indeed the $G\alpha_h$-associated protein that down-regulates the $G\alpha_h$ to the ground state as a counterpart of the receptor. Our data also demonstrate that the receptor activates $G\alpha_h$ by changing the affinity of $G\alpha_h$ from GDP for GTP. The ternary complex is a heterotetramer consisting of α_1-agonist, adrenoceptor, 74 kDa $G\alpha_h$, and 50 kDa $G\beta_h$ in an equimolar stoichiometry. Moreover, upon activation of the receptor, the ternary complex exhibits inhibition of the TGase activity of $G\alpha_h$, showing that this classical transglutaminase II functions as a signal transducer similar to the heterotrimeric G-proteins.

The $\beta\gamma$-subunits of the heterotrimeric G-proteins are primarily involved in the regulation of α-subunit function in its GTPase cycle by a mechanism of dissociation and association (Gilman, 1987; Birnbaumer, 1990). The fundamental role of $G\beta_h$ in the inactivation of $G\alpha_h$ is similar to that of the $\beta\gamma$-subunits of the heterotrimeric G-proteins; i.e., the $\beta\gamma$-subunits promote the dissociation of GTPγS and increase the affinity of the α-subunits for GDP (Northup et al., 1983; Higashijima et al., 1987a,b). Several laboratories have also demonstrated that the $\beta\gamma$-subunits of the heterotrimeric G-proteins are involved in activation of the α-subunit by directly interacting with the receptor in the presence of

agonist. These receptors include β-adrenoceptor (Hekman et al., 1987; Im et al., 1988; Kurstjens et al., 1991), serotonin (Law et al., 1991), and rhodopsin (Halpern et al., 1987). As mentioned above, since 50 kDa $G\beta_h$ does not accelerate the α_1-receptor-mediated GTPγS binding, $G\beta_h$ probably is not involved in activation of $G\alpha_h$ by the α_1-receptor. Supporting this notion, $G\beta_h$-antibody fails to coimmunoprecipitate the α_1-receptor in the presence of either agonist or antagonist when the receptor and $G\beta_h$ are reconstituted (Figure 3A). Furthermore, inhibitory effects of $G\beta_h$ on GTPγS binding by the α_1-agonist, receptor, and $G\alpha\beta_h$ ternary complex were significantly less sensitive than with $G\alpha_h$ alone (compare Figures 3B and 4A), indicating that activation of the receptor leads $G\alpha_h$ to the low-affinity state for $G\beta_h$. These observations also showed that the α_1-receptor activation induces a conformation of $G\alpha_h$ which exhibits high affinity for GTP.

The $\beta\gamma$-subunits of the heterotrimeric G-proteins are involved in a variety of cellular functions via the regulation of signal transduction, such as down-regulation of receptors, promotion of phosphorylation by receptor-specific kinases (β-adrenoceptor kinase and rhodopsin kinase) (Inglese et al., 1993; Palczewski & Benovic, 1991), stimulation or inhibition of adenylyl cyclases (Tang & Gilman, 1991; Federman et al., 1992) and PLC-β (Smrcka & Sternweis, 1993; Boyer et al., 1994), and activation of channels (Logothetis et al., 1987; Ito et al., 1992). In these regards, the functions of 50 kDa $G\beta_h$ should be further studied. Finally, since $G\alpha_h$ possesses two enzyme activities, GTPase and TGase, it is of interest to study whether $G\beta_h$ is also involved in regulation of the TGase activity.

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