α_{1B}-Adrenoceptor Interacts with Multiple Sites of Transglutaminase II: Characteristics of the Interaction in Binding and Activation†

Jian-Fang Feng, Caroline D. Gray, and Mie-Jae Im*

Department of Molecular Cardiology, The Lerner Research Institute, The Cleveland Clinic Foundation, Cleveland, Ohio 44195

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ABSTRACT: We previously reported that a novel GTP binding protein ($G\alpha_h$) is tissue type transglutaminase (TGII) and transmits the α_{1B}-adrenoceptor (AR) signal to phospholipase C (PLC) through its GTPase function. We have also shown that PLC-δ1 is the effector in TGII-mediated signaling. In this study, interaction sites on TGII for the α_{1B}-AR were identified using a peptide approach and site-directed mutagenesis, including in vivo reconstitution of TGIIs with the α_{1B}-AR and PLC-δ1. To identify the interaction sites, 11 synthetic peptides covering ~132 amino acid residues of the C-terminal domain of TGII were tested. The studies with the peptides revealed that three peptides, $L^{547}-I^{561}$, $R^{564}-D^{581}$, and $Q^{633}-E^{646}$, disrupted formation of an α_1-agonist−α_{1B}-AR−TGII complex and blocked α_{1B}-AR-mediated TGase inhibition in a dose-dependent manner, indicating that these peptide regions are involved in recognition and activation of TGII by the α_{1B}-AR. These three regions were further evaluated with full-length TGIIs by constructing and coexpressing each site-directed mutant with the α_{1B}-AR and PLC-δ1 in COS-1 cells. Supporting the findings with these peptides, these TGII mutants lost 56−82% the receptor binding ability and reduced by 29−68% the level of α_{1B}-AR-mediated IP_3 production via PLC-δ1 as compared to those with wild-type TGII. The results also revealed that the regions of $R^{564}-D^{581}$ and $Q^{633}-E^{646}$ were the high-affinity binding sites of TGII for the receptor and critical for the activation of TGII by the receptor. Taken together, the studies demonstrate that multiple regions of TGII interact with the α_{1B}-AR and that the α_{1B}-AR stimulates PLC-δ1 via TGII.

Transglutaminases (TGases) are Ca^{2+}- and thiol-dependent enzymes that catalyze the formation of an ϵ-(γ-glutamyl)-lysine isopeptide bond between peptide-bound glutamyl residues and the lysyl group of polypeptides (see refs *1* and *2* for reviews). Five TGases have been identified and are distributed in a specific tissue, except tissue type TGase which is ubiquitously expressed in mammalian tissues. The TGase family includes keratinocyte TGase (TGI), tissue-type TGase (TGII[1], $G\alpha_h$), epidermal TGase (TGIII), prostate TGase (TGIV), and plasma TGase, a subunit of factor XIII (FXIIIa). Unlike other TGases, TGII possesses an extra enzyme activity, namely GTPase that binds and hydrolyzes one molecule of GTP per TGII molecule (*3*−*5*). The two enzyme activities of TGII, GTPase and TGase, regulate each other via the counter inhibitors, GTP and Ca^{2+} (*3*). GTP binding to the TGII inhibits the TGase activity, and Ca^{2+} binding to TGII inhibits the GTP binding. Accumulating evidence has indicated that the GTPase function of TGII transmits a hormone receptor signal to an effector enzyme, playing a similar role of the "classical" heterotrimeric G-proteins (see ref *6* for review). To date, known TGII-coupled receptors include the α_{1B}-AR, α_{1D}-AR, and oxytocin receptor (*7*−*13*). Coupling of TGII to the α_1-AR has been demonstrated in different tissues, including rat liver (*7*, *13*) and hearts from various species (*8*, *13*), and in vivo reconstitution in COS-1 and buffalo rat liver cells (*9*, *10*). Coupling of oxytocin receptor with TGII has been observed in human myometrium (*11*, *12*). The effectors in TGII-mediated signaling are shown to be PLC-δ1 (*12*, *14*) and calcium-mediated K^+ channel (*15*). Activation of PLC via TGII has been observed in HeLa cells (*16*) and macrophages (*17*). The identity of PLC(s) remains unknown in these cells.

The primary structure of all TGases exhibits a high degree of homology in the middle portions of the polypeptides, where the TGase active site and the putative calcium binding site are located (*18*, *19*). The N- and C-terminal regions of TGII, however, differ substantially from those of other TGases, suggesting that both domains are involved in unique functions of TGII, e.g., the receptor-mediated signaling. In fact, our laboratory has demonstrated that a PLC-δ1 interaction site is located very near the C-terminus of TGII and that eight amino acid residues ($V^{665}-K^{672}$) are critical for recognition and stimulation of PLC-δ1 (*14*, *20*). Although it appears that TGII does not contain common amino acid motifs of the GTP binding site (*9*), recent studies have

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* To whom correspondence should be addressed: Mie-Jae Im, Ph.D., Department of Molecular Cardiology (FF-30), The Lerner Research Institute, The Cleveland Clinic Foundation, 9500 Euclid Ave., Cleveland, OH 44195. Telephone: (216) 444-8860. Fax: (216) 444-9263. E-mail: imm@cesmtp.ccf.org.

[1] Abbreviations: AR, adrenoceptor; TGII, tissue-type transglutaminase which is also a GTPase; G-protein, GTP binding regulatory protein; GTP, guanosine 5′-triphosphate; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); GDP, guanosine 5′-diphosphate; ATP, adenosine 5′-triphosphate; AppNHp, adenyl-5′-yl imidodiphosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; IP_3, inositol 1,4,5-triphosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; DTT, 1,4-dithiothreitol.

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indicated that the GTPase active site is located at the beginning of the TGase active site domain (*21*−*23*). A 5.5 kDa region (residues 139−185) has been defined as the GTPase domain (*21*). These studies with the fusion TGII protein have also shown that TGII binds and hydrolyzes ATP (*21*, *22*). However, Lai et al. (*24*) have proposed that the GTP binding site is separate from the ATP binding site, on the basis of the observations that TGase activity is inhibited in the presence of GTP or GDP, but not in the presence of ATP, and that GTP binding to TGII induces a conformation change, but ATP binding does not. Along the same lines, [α-^{32}P]GTP photoaffinity labeling of TGII protein is not inhibited in the presence of AppNHp, a nonhydrolyzable analogue of ATP (*21*). We have also previously reported similar findings with G_h (TGII associated with a 50 kDa regulatory protein) purified from rat liver or bovine heart; GTP or GTPγS binding by G_h is not affected by treatment with AppNHp (*5*, *13*). In addition, a fibronectin binding site is located within seven amino acid residues at the N-terminus of TGII (*25*). This secreted TGII has been implicated in cell stabilization by modification of extracellular matrix proteins, vitronectin, fibronectin, and osteonectin (*1*, *2*).

Recently, it has been demonstrated that α_{1B}-AR specifically interacts with TGII via its third cytoplasmic loop (*10*). On the other hand, the interaction site(s) on TGII for the receptor has not been defined. In this study, the α_{1B}-AR interaction sites on TGII have been identified by applying a synthetic peptide approach. The regions identified by the peptide studies have been characterized further with full-length TGII by constructing and coexpressing a site-directed mutant of each region with the α_{1B}-AR and PLC-δ1. The results revealed that the α_{1B}-AR interacts with multiple regions within the C-terminal domain of TGII and that the α_{1B}-AR couples to PLC-δ1 through TGII.

EXPERIMENTAL PROCEDURES

Materials. An inositol 1,4,5-triphosphate [^{3}H]radioreceptor assay kit, [^{3}H]phosphatidyl 4,5-bisphosphate (30 Ci/mmol), [^{3}H]putrescine (35.74 Ci/mmol), and [^{3}H]prazosin (79.8 Ci/mmol) were from DuPont NEN (Boston, MA). Nucleotides were purchased from Boehringer Mannheim. Sucrose monolaurate (SM-1200) was obtained from the Mitsubishi-Kasei Co. (Tokyo, Japan). Monoclonal anti-guinea pig liver TGII antibodies, Ab-1 and Ab-2 (clones CUB 7402 and TG100), were obtained from NeoMarkers (Fremont, CA), and polyclonal anti-bovine $G_{h7\alpha}$ (bovine heart TGII) antibody was raised by us (*13*). Monoclonal anti-bovine PLC-δ1 antibody was from Upstate Biotechnology Inc. (Lake Placid, NY). Monoclonal ID4 antibody was obtained from National Cell Culture (Washington, DC). Guinea pig liver TGII, protein A−agarose, and wheat germ agglutinin (WGA)−agarose were obtained from Sigma. CNBr-activated Sepharose 4B was from Pharmacia. Other chemicals and biochemical materials were of the highest grade available (*5*).

Design of Peptides. We compared the C-terminal domain of TGII to coagulation factor XIIIa and other TGases (see refs *18* and *19*) and then selected unique regions to synthesize peptides considering the following properties (Figure 1A): (i) regions clustered with charged amino acid residues, because protein−protein interaction sites are likely to be exposed to the surface of a protein, e.g., the GDP-bound

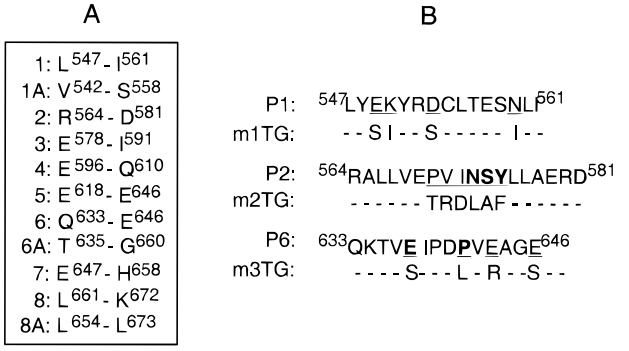

FIGURE 1: (A) Synthesized peptides derived from human heart TGII. The synthesized peptides are numbered with Arabic numerals in consecutive order from the N-terminus to the C-terminus of TGII. The amino acid residues are denoted with one-letter abbreviations. (B) Substituted amino acid residues within the regions of peptides (P) 1, 2, and 6 for the mutation. The amino acid residues, which were mutated within the corresponding regions of P1, P2, or P6, are underlined. The substituted amino acids are shown in the same line with the generated mutants of TGII, indicated as m1TG, m2TG, and m3TG. Unique residues inserted into TGII among TGases are shown in bold; these were replaced with hydrophobic residues having approximately the same space-filling volume.

form of TGII interacts with the α_{1B}-AR; (ii) regions clustered with unique polar amino acid residues; and (iii) unique hydrophobic regions, but regions recruiting neighboring hydrophilic residues to increase the overall hydrophilicity. The peptides were synthesized at the Structural Biology Core of the Lerner Research Institute at the Cleveland Clinic Foundation. The purity of the synthesized peptides was determined by HPLC and mass spectroscopy.

Construction of the Site-Directed TGII Mutant. On the basis of the results obtained from the peptide competition studies on the inhibition of the interaction of TGII with the α_{1B}-AR, three site-directed mutants of the identified regions were constructed from human heart TGII cDNA by polymerase chain reaction (PCR), as shown in Figure 1B. For each mutant, two overlapping internal primers containing the amino acid changes were synthesized: sense-1, CGTAGTGCCTTACGGAGTCCATCCTCA, and antisense-1, TCCGTAAGGCAGCTACGGTAGATGCTATAGA for L^{547}−I^{561}; sense-2, GACCCGCGACCTGGCCTTCCTGCTGGCTAGAGGGACCTC, and antisense-2, GAAGGCCAGGTCGCGGGTCTCCACGAGGAGGGCCCGCAC for R^{564}−D^{581}; and sense-3, CCTGGTGAGGGCAGGGAGCGAAGTTAAGGTGAGAATGGAC, and antisense-3, GCTCCCTGCCCTCACCAGGTCTGGGATCAGCACCGTCTTCTG for Q^{633}−E^{646} (underline residues are the changed nucleotides). Two external primers that span unique restriction sites within the TGII cDNA were also synthesized: 5′-primer, GGCAGTGACTTTGACGTCTTTG; and 3′-primer, GGAGCAGGGGTC*CCTTAGG*CGG (single underline indicates the *Aat*II restriction site and italics the *Aoc*I restriction site). Two sets of PCRs for each mutation were performed using the 5′-primer and antisense primer for the first set, and the sense primer and 3′-primer for the second set. The two resulting PCR products were annealed and used as template DNA in the third PCR with the 5′-primer and 3′-primer. Each PCR product was ligated into pCRII (Invitrogen) and identified by restriction analysis and complete DNA sequencing of both strands. The correct mutagenized cassette was used to replace the corresponding region of wild-type TGII (wtTGII) DNA

via the restriction enzymes *Aat*II and *Aoc*I. The resulting gene was subcloned into a modified eukaryotic expression vector, pMT2′ (*9*, *20*), and verified by DNA sequencing.

Expression and Characterization of the Expressed Proteins. COS-1 cells were transiently transfected with various cDNAs (α_{1B}-AR-ID4, wtTGII or its mutants, and PLC-δ1 that had been inserted into a PMT2′ vector). Chinese hamster α_{1B}-AR cDNA was tagged with ID4 at the C-terminal end, and the engineered cDNA (3 μg per 5×10^6 cells per 60 mm dish) was cotransfected with or without wtTGII or its mutant cDNAs (10 μg per 5×10^6 cells per 60 mm dish) and rat brain PLC-δ1 cDNA (3 μg per 5×10^6 cells per 60 mm dish) by the DEAE-dextran method (*20*). After the transfected cells were grown in culture dishes for 60−72 h, some of the dishes were used to prepare the membranes or cell lysates, and others were used to determine functional coupling of the expressed proteins. The preparation of membranes or lysates was achieved as described previously (*20*) and stored in an HDGD buffer [20 mM HEPES (pH 7.4), 1 mM EDTA, 1 mM EGTA, and 1 mM DTT] containing 10% glycerol at −80 °C. The level of the expressed α_{1B}-AR was determined using a specific α_1-antagonist [^{3}H]prazosin (*9*, *20*). The expression level and functional activity of the expressed wild-type and mutant TGII proteins were measured by Western blot analysis using monoclonal guinea pig liver TGII antibody and by evaluating the TGase and GTP binding activities, respectively (*20*). Western blot analysis was performed to determine the level of PLC-δ1 expression (*14*). To determine the increase in the PLC activity in expression of PLC-δ1, membranes were treated with 1% sodium deoxycholate in an HSD buffer [20 mM HEPES (pH 7.4), 150 mM NaCl, and 1 mM dithiothreitol] at 4 °C for 1 h. Total PLC activity was determined in the presence of 200 μM Ca^{2+} and 50 μM [^{3}H]PIP_2 (specific activity of 1000 cpm/nmol) at 30 °C for 10 min (*14*). The level of the expressed proteins was estimated with autoradiography of the immunoblots by densitometry (Fotodyne Inc. Vari Quest 100 apparatus). The protein concentration was determined by the method of Bradford (*26*) using bovine serum albumin as a standard. Changes in methods are described in detail in the figure legends.

Synthesis of Antibody and Peptide Affinity Resins. For immunoprecipitation studies, the $G_{h7\alpha}$ or ID4 antibody was covalently cross-linked to protein A−agarose by the method of Schneider et al. (*27*). The antibody−protein A−agarose was stored at 4 °C until it was used. The immunoreactivity and binding capacity of immunoaffinity resins were determined by immunoblotting after subjecting the antibody affinity resins to immunoprecipitation using guinea pig liver TGII or membrane extracts from COS-1 cells expressing α_{1B}-AR-ID4. The estimated binding capacity of the antibody affinity resins was ∼20 μg of TGII/mL and ∼10 pmol of α_{1B}-AR-ID4/mL. The peptide affinity resins were prepared with CNBr-activated Sepharose 4B using a protocol provided by the manufacturer (Pharmacia).

α_1-Agonist−Receptor−TGII Complex Preparation. The formation of the ternary complex consisting of (−)-epinephrine, α_{1B}-AR, and TGII was induced in rat liver membranes, and the complex was prepared with a slight modification of the method described by Im and Graham (*7*). Briefly, rat liver membranes (2 g of protein) were preincubated with 5×10^{-5} M (−)-epinephrine, 10^{-6} M (±)-propranolol, and 10^{-7} M rawolscine at 4 °C for 4 h. The complex was solubilized with 0.2% sucrose monolaurate (SM) in HDGD buffer containing 200 mM NaCl and protease inhibitors (*7*) and isolated using heparin−agarose and WGA−agarose columns. The ternary complex bound to WGA−agarose was washed with HDGD buffer containing 100 mM NaCl and 0.02% SM with the α_1-agonist and eluted with 300 mM *N*-acetylglucosamine in HDGD buffer containing 10^{-4} M phentolamine, 10% glycerol, and 300 mM NaCl. For the studies, the excess of the ligand in the ternary complex preparations was removed through a dried Spephadex G-25 column (*13*). The ternary complex preparations (yield of 1 pmol of α_{1B}-AR/mL) were stored at −80 °C until they were used.

Determination of Peptide Effects. To determine the effects of the peptides on the interaction of TGII with the α_{1B}-AR, the α_{1B}-AR−TGII complex preparations (50 fmol of receptor per tube) were incubated with various peptides in the presence of 5×10^{-5} M (−)-epinephrine or 10^{-4} M phentolamine at 4 °C for 4 h while the mixture was gently rotated, followed by addition of the $G_{h7\alpha}$ antibody affinity resins (50 μL). After incubation, the supernatants and the antibody affinity resins were separated by centrifugation at 1000*g* for 5 min. The amount of unbound receptors was determined in the supernatants using [^{3}H]prazosin (4 nM final concentration) after removing the excess of the ligand through a dried Sephadex G-25 column. Recovery of the dried Sephadex G-25 column was 50−60%. To determine the level of direct binding of the peptide to the α_{1B}-AR, the receptor was highly expressed in COS-1 cells and extracted from the COS-1 cell membranes (1−2 pmol of receptor/mg) with HDGD buffer containing 200 mM NaCl, 5% glycerol, and 0.2% SM at 4 °C for 1 h. The extracts (30 fmol of receptor per tube) were incubated with the peptide affinity resins (50 μL) in the presence of 5×10^{-5} M (−)-epinephrine or 10^{-4} M phentolamine overnight at 4 °C. The level of unbound receptors was measured in the supernatant using [^{3}H]prazosin (4 nM final concentration) after removing the excess of the ligand through the dried Sephadex G-25 columns. Throughout the study with peptides, the levels of the receptors in α_1-antagonist-treated samples were used as a control for each peptide, since the antagonist-bound α_{1B}-AR did not interact with TGII (*13*).

Co-Immunoprecipitation. For the binding of wtTGII and its mutants to the α_{1B}-AR, the membrane preparations (20−30 mg of protein) from COS-1 cells expressing the α_{1B}-AR alone or with wtTGII or each TGII mutant were extracted with 0.2% SM in HDGD buffer containing 200 mM NaCl and 5×10^{-5} M (−)-epinephrine or 5×10^{-5} M (−)-epinephrine with 4 nM prazosin at 4 °C for 1 h. To observe the ability of the TGII mutants to bind to the receptor, the extracts (0.25 pmol of receptor per tube) were incubated with ID4 antibody affinity resins (50 μL) in the presence of 5×10^{-5} M (−)-epinephrine or 5×10^{-5} M (−)-epinephrine with 4 nM prazosin at 4 °C for 2 h in a 500 μL final volume. After the supernatant was removed, the resins were washed three times with HDGD buffer (1 mL per wash) containing 5×10^{-5} M (−)-epinephrine or 4 nM prazosin, 100 mM NaCl, and 0.02% SM. Binding of the TGII proteins to the α_{1B}-AR was analyzed by immunoblotting using the TGII antibody following SDS−PAGE (10% gel). For the interaction of PLC-δ1 with wtTGII and its mutants, the membranes

from COS-1 cells expressing wtTGII or its mutants with PLC-δ1 were extracted with 0.2% SM in HDGD buffer containing 200 mM NaCl, 5×10^{-5} M GTPγS, and 2 mM $MgCl_2$ at 4 °C for 1 h. The extracts (200 μg of protein per tube) were incubated with TGII antibody (2 μg, clone CUB 7402) and protein A−agarose (50 μL) in the presence of 5×10^{-5} M GTPγS and 2 mM $MgCl_2$ at 4 °C overnight in a 500 μL final volume. The resins were washed three times with HDGD buffer (1 mL per wash) containing 5×10^{-5} M GTPγS, 2 mM $MgCl_2$, 100 mM NaCl, and 0.02% SM. Co-immunoprecipitation of PLC-δ1 was analyzed by immunoblotting using a mixture of monoclonal PLC-δ1 and TGII antibodies following SDS−PAGE (7% gel).

TGase Activity. The Ca^{2+}-stimulated TGase activity of the expressed wtTGII and its mutants was determined using the membranes in the presence and absence of 0.5 mM $CaCl_2$ using [^{3}H]putrescine (1 μCi) and *N*,*N*′-dimethylcasein (1%) in HSD buffer (*3*). For the GTPγS-mediated inhibition of the TGase activity, the membranes were preincubated in the presence and absence of 5×10^{-6} M GTPγS and 2 mM $MgCl_2$ in HSD buffer at 30 °C for 20 min, and the TGase activity was determined at 30 °C for 30 min using the same conditions described above. The level of α_{1B}-AR-activated TGase inhibition was determined using rat liver membranes which contain the homogeneous α_{1B}-AR subtype (*7*). Thus, rat liver membranes (50 μg of protein per tube) were preincubated with 5×10^{-5} M (−)-epinephrine or 5×10^{-5} M (−)-epinephrine with 4 nM prazosin in the presence of various peptides at 4 °C for 4 h while the mixture was gently rotated. The TGase activity was determined in the presence of 0.3 mM $CaCl_2$, 5×10^{-6} M GTPγS, and 1 mM $MgCl_2$ at 30 °C for 15 min in a 100 μL final volume (*28*).

Production of Inositol 1,4,5-Triphosphate in Intact Cells. The level of both basal and α_{1B}-AR-activated production of inositol 1,4,5-triphosphate (IP_3) was determined in the intact COS-1 cells expressing proteins of interest using the IP_3 [^{3}H]-radioreceptor assay kit (see also ref *29*). Throughout the study, after the transfected cells were grown for 60−72 h, the cells were preincubated with 20 mM LiCl at 37 °C for 30 min. IP_3 formation in the cells was stopped at 8 min by adding ice-cold 20% TCA (final) after removing the media, as recommended by the manufacturer. Before the experiments were performed, the time course of IP_3 formation was determined in the intact COS-1 cells coexpressing the α_{1B}-AR, wtTGII, and PLC-δ1. We found that the level of α_{1B}-AR-mediated IP_3 production was increased in a time-dependent manner up to 10 min (data not shown).

RESULTS

Identification of α_{1B}-AR Interaction Sites on TGII Using Synthetic Peptides. Localization of the α_{1B}-AR interaction sites on TGII was determined by utilizing the ability of the peptides to block the formation of an α_1-agonist−receptor−TGII complex. To evaluate the blocking potency of each peptide, the agonist- or antagonist-treated ternary complex preparations were incubated with a peptide and the $G_{h7\alpha}$ antibody resins. The level of the unbound receptors was determined (Figure 2A). The specificity of the antibody was evaluated using the preimmune sera resins. The results showed that preimmune sera did not immunoprecipitate the α_{1B}-AR (PRE). On the other hand, $G_{h7\alpha}$ antibody specifically co-immunoprecipitated ~90% the α_{1B}-AR (BUFFER). Among

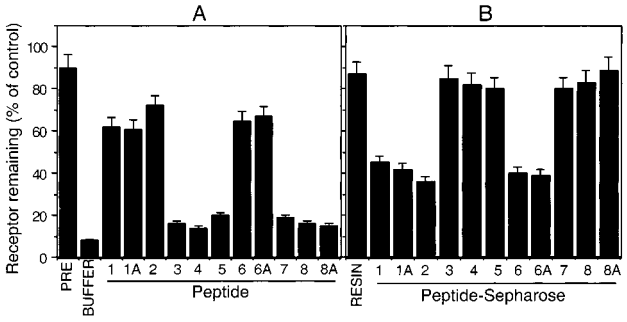

FIGURE 2: Effects of peptides on the interaction of TGII with the α_{1B}-AR. (A) Blocking the ability of the peptides to co-immunoprecipitate the α_{1B}-AR by $G_{h7\alpha}$ antibody. The ternary complex preparations (50 fmol per tube) were incubated with 50 μM peptides in the presence of 5×10^{-5} M (−)-epinephrine or 1×10^{-4} M phentolamine, as described in detail in Experimental Procedures. The levels of unbound receptors in the supernatant of the agonist-treated samples were compared with those in the antagonist-treated samples (control) for each peptide. PRE represents the case where preimmune sera cross-linked protein A−agarose was included. BUFFER represents the case where $G_{h7\alpha}$ antibody−protein A−agarose was included without addition of peptide. The numbered columns indicate the peptides shown in Figure 1A. The data depicted here are the mean of three independent experiments, each performed in duplicate. (B) Binding of the α_{1B}-AR to the peptide affinity Sepharose 4B. The membrane extracts (30 fmol per tube) from COS-1 cells expressing the α_{1B}-AR were incubated with the peptide affinity resins (50 μL) in the presence of 5×10^{-5} M (−)-epinephrine or 5×10^{-5} M (−)-epinephrine with 10^{-4} M phentolamine. The unbound receptor density in agonist-treated samples was compared with those in the antagonist-treated sample (control) for each peptide. RESIN represents the case where the ethanolamine cross-linked Sepharose 4B was included. The Arabic numbers indicate the peptides shown in Figure 1A that were cross-linked to the CNBr−Sepharose 4B. The data depicted here are the mean of three independent experiments performed in duplicate.

11 peptides tested, three peptides (P1, P2, and P6) as well as the overlapping peptides (P1A and P6A) inhibited 62−74% the co-immunoprecipitation of the α_{1B}-AR compared to that with other peptides (14−21% inhibition), indicating that these three peptide regions are the interaction sites of the receptor on TGII. The levels of unbound receptors were ~62% with P1 and P1A, ~74% with P2, and ~64% with P6 and P6A. Moreover, the inhibition of the co-immunoprecipitation of the α_{1B}-AR by the peptides was a result of the direct binding of these peptides to the α_{1B}-AR. As shown in Figure 2B, when the binding ability of the peptides to the receptor was evaluated using peptide affinity resins, these peptides (P1, P1A, P2, P6, and P6A) were able to bind ~2-fold more of the α_{1B}-AR than the other peptides did.

Effects of Peptides on α_1-AR-Mediated GTPγS Binding to TGII with Rat Liver Membranes. We have then determined whether interaction of the α_{1B}-AR with these peptide regions is involved in activation of GTP binding by TGII. The binding of GTP (or its analogue, GTPγS) to TGII is facilitated by activation of the receptor, whereas Ca^{2+}-mediated TGase stimulation is inhibited upon GTP (or GTPγS) binding to TGII (*9*, *28*). Thus, the stimulation of GTPγS binding to TGII can be specifically evaluated by determining the level of α_{1B}-AR-mediated inhibition of TGase activity. Figure 3A shows the typical course of α_1-AR-mediated TGase inhibition with rat liver membranes. Ca^{2+}-mediated TGase activation was inhibited in the presence of GTPγS. This GTPγS-mediated inhibition was further enhanced by activation of the α_{1B}-AR with (−)-epinephrine.

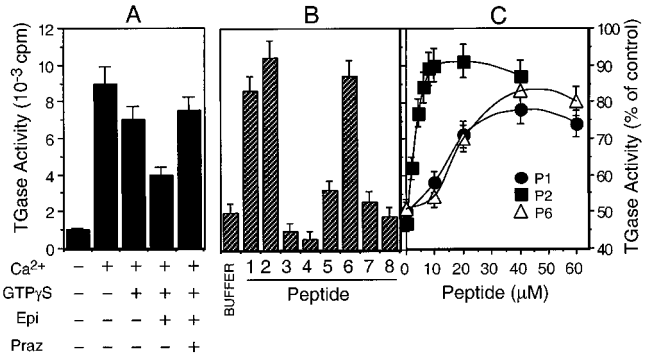

FIGURE 3: Reversal of α_{1B}-AR-mediated inhibition of TGase activity by the peptides. (A) The α_1-agonist-mediated inhibition of TGase activity in rat liver membranes. Rat liver membranes (50 μg per tube) were preincubated with or without 5×10^{-5} M (−)-epinephrine or 5×10^{-5} M (−)-epinephrine with 4 nM prazosin. After incubation at 30 °C for 20 min, the level of TGase activity was determined, as described in Experimental Procedures. The data depicted here are the mean of three independent experiments, each performed in duplicate. Abbreviations: Epi, (−)-epinephrine; Praz, prazosin. (B) Blocking of the α_1-receptor-mediated TGase inhibition by the peptides. Rat liver membranes (50 μg per tube) were preincubated with the various peptides (40 μM) in the presence of 5×10^{-5} M (−)-epinephrine or 5×10^{-5} M (−)-epinephrine with 4 nM prazosin. After incubation at 4 °C for 4 h, the TGase activity was measured, as described in detail in Experimental Procedures. The level of TGase activity in the presence of the agonist was compared with that in the presence of the antagonist (control) for each peptide. BUFFER represents the case where no peptide was added. The numbers represent the peptides shown in Figure 1A. (C) Peptide dose-dependent reversal of α_1-receptor-mediated TGase inhibition. Membranes were preincubated with various concentrations of peptides under the same conditions, as described in detail for panel B. The levels of TGase activity were then measured, as described in Experimental Procedures.

On the other hand, the α_1-agonist-mediated inhibition of TGase activity was blocked in the presence of the α_1-antagonist prazosin. These data demonstrated that inhibition of the TGase activity was the result of stimulation of GTPγS binding to TGII by the activated α_{1B}-AR. To evaluate the blocking ability of each peptide on receptor-mediated TGase inhibition, rat liver membranes were incubated with each peptide in the presence of the α_1-agonist or the α_1-antagonist. As shown in Figure 3B, the three peptides (P1, P2, and P6) again blocked α_1-agonist-mediated TGase inhibition, increasing 35−45% of the enzyme activity. Moreover, the TGase inhibition was blocked in a dose-dependent manner by these peptides (Figure 3C). The half-inhibitory concentration (IC_{50}) of the peptides was ~20 μM with P1, ~3 μM with P2, and ~22 μM with P6. These results clearly indicated that interaction of the α_{1B}-AR with these three sites of TGII directly leads to the stimulation of GTPγS binding by TGII.

Activity and Level of the Expressed Proteins. To further evaluate the findings with peptides P1, P2, and P6 in an expression system, a site-directed mutant of each region was constructed. The constructs of wtTGII and the mutants of TGII were transiently coexpressed with the α_{1B}-AR-ID4 and PLC-δ1 in COS-1 cells. The expression level of the receptors was 200−250 fmol/mg of membrane proteins. The expression levels of PLC-δ1 were high and similar to each other (Figure 4B). Expression of PLC-δ1 resulted in an ~44% increase in total PLC activity as compared to that with the α_{1B}-AR alone (Figure 4D). The levels of wtTGII and its mutants were similar to each other and ~12-fold higher than

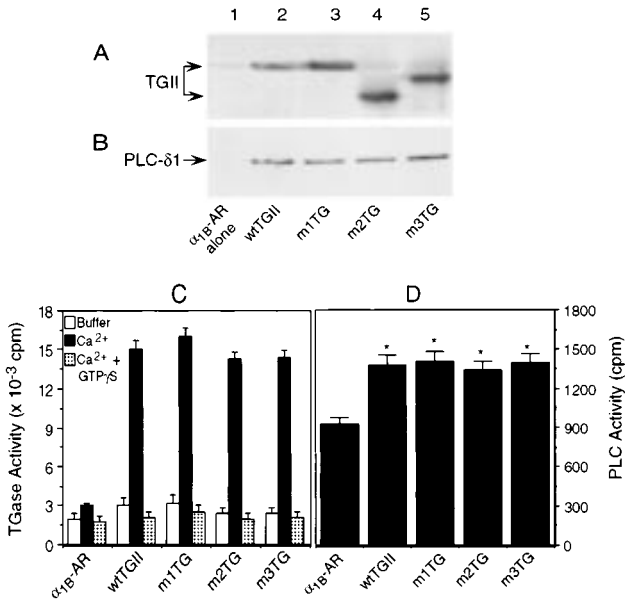

FIGURE 4: Expression levels of the proteins in COS-1 cells transfected with the α_{1B}-AR, TGII or its mutants, and PLC-δ1. The expression levels of TGII and its mutants are shown in panel A, and the expression level of PLC-δ1 is shown in panel B. The membranes (150 μg per lane) of COS-1 cells expressing the α_{1B}-AR alone or coexpressing the α_{1B}-AR with TGII or its mutants and PLC-δ1 were extracted using 1.0% sodium cholate and 250 mM NaCl at 4 °C for 1 h. The extracts were subjected to immunoblotting using monoclonal TGII or PLC-δ1 antibody, following SDS−PAGE (10% gel). (C) TGase activity and inhibition of TGase activity by GTPγS with membranes containing the expressed TGII proteins. The membranes (10 μg per tube) were preincubated with and without 5×10^{-5} M GTPγS in the presence of 2 mM $MgCl_2$ at 30 °C for 20 min. The level of TGase activity was measured in the presence of 0.5 mM $CaCl_2$ at 30 °C for 30 min in a final volume of 100 μL. The results shown are the mean of two independent experiments, each carried out in duplicate. (D) Increase in PLC activity in expression of PLC-δ1. The PLC activity was determined with the membranes (50 μg of protein per tube) in the presence of 200 μM Ca^{2+}, as described in Experimental Procedures. The results are means ± SEM of three independent experiments performed in duplicate, and statistical differences determined by analysis of variance for multiple comparisons. The asterisks indicate that $P < 0.05$ vs values for membranes expressing the α_{1B}-AR alone.

that of endogenous TGII (Figure 4A). The TGase activity of wtTGII and its mutants was ~11-fold higher than that of the α_{1B}-AR expressed alone (Figure 4C). The Ca^{2+}-mediated TGase activation of TGII proteins was completely inhibited in the presence of GTPγS, showing that both TGase and GTP binding activities of the mutants were not altered. It should be noted that the expressed TGII mutants (m2TG and m3TG) were mobilized faster than wtTGII and m1TG on a SDS−PAGE gel (Figure 4A). Although this aberrant shift of the mutants was not clearly understood, when the affinity of the expressed TGII proteins for Ca^{2+} and GTPγS binding was evaluated, the Ca^{2+}-dependent activation and GTPγS-dependent inhibition of the TGase activity of the mutants were similar to those of wtTGII, indicating that the overall structure of these mutants was intact. Changes in mobility have also been observed with the native TGII proteins on the SDS−PAGE gel despite their similar primary structures in all species cloned and examined so far (*9*, *13*).

Interaction of the TGII Mutant Proteins with the α_{1B}-AR. To evaluate the ability of each mutant to bind to the α_{1B}-

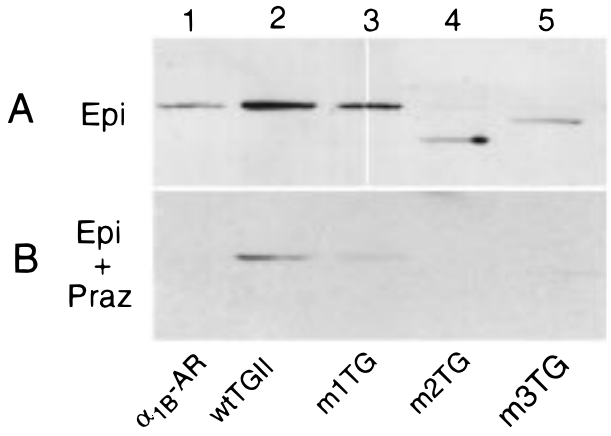

FIGURE 5: Interaction of TGII and its mutants with the α_{1B}-AR. The extracts prepared from membranes of COS-1 cells expressing the α_{1B}-AR alone or the α_{1B}-AR with TGII or its mutants were incubated with ID4 antibody affinity resins (50 μL) in the presence of 5×10^{-5} M (−)-epinephrine (A) or 5×10^{-5} M (−)-epinephrine with 4 nM prazosin (B) at 4 °C for 2 h. The level of binding of TGII and its mutants to the α_{1B}-AR was determined by Western blotting with the TGII antibody, following SDS−PAGE (10% gel). Here m1TG, m2TG, and m3TG indicate the TGII mutants shown in Figure 1B. Abbreviations: Epi, (−)-epinephrine; Praz, prazosin.

AR, the membrane extracts prepared from the COS-1 cells coexpressing the α_{1B}-AR-ID4 with wtTGII or its mutants were incubated with ID4 antibody resins in the presence of the α_1-agonist or the α_1-antagonist. The level of binding of TGII proteins to the α_{1B}-AR was determined by immunoblotting with the TGII antibody. As presented in Figure 5A, co-immunoprecipitation of endogenous TGII was observed in membranes containing the receptor alone (lane 1), but the level was lower than those of the membranes containing the α_{1B}-AR and wtTGII (lane 2). These observations suggested that high-level expression of wtTGII enhanced the interaction of both proteins. Moreover, the co-immunoprecipitation of the TGIIs via the α_{1B}-AR was specific. Thus, co-immunoprecipitation of the TGIIs via the activated α_{1B}-AR was substantially blocked in the presence of the α_1-antagonist (Figure 5B). The interaction of the TGII mutants with the activated α_{1B}-AR was significantly decreased compared to that of wtTGII (lanes 3−5 in Figure 5A). The level of the TGII proteins bound to the α_{1B}-AR was as follows: wtTGII (100%) > m1TG (∼44%) > m2TG (∼21%) > m3TG (∼18%). These results clearly demonstrated that all three sites on TGII identified by the peptide experiments are important for the interaction with the activated α_{1B}-AR and that the mutated amino acids within the TGII mutants are the critical residues for the recognition of the receptor.

Interaction of TGII Proteins with the Expressed PLC-δ1. The membrane extracts from the cells coexpressing wtTGII and its mutants with PLC-δ1 were incubated with $G_{h7\alpha}$ antibody resins in the presence of GTPγS. Co-immunoprecipitation of PLC-δ1 was evaluated by immunoblotting using both TGII and PLC-δ1 antibodies (Figure 6A). The results revealed that both proteins effectively interacted each other and that the levels of PLC-δ1 that co-immunoprecipitated with the TGII proteins were comparable to each other, indicating that the ability of the mutants to couple with PLC-δ1 was intact. Moreover, co-immunoprecipitation of PLC-δ1 with TGII was also observed in the rat liver membrane extracts (lane a), demonstrating that interaction of PLC-δ1

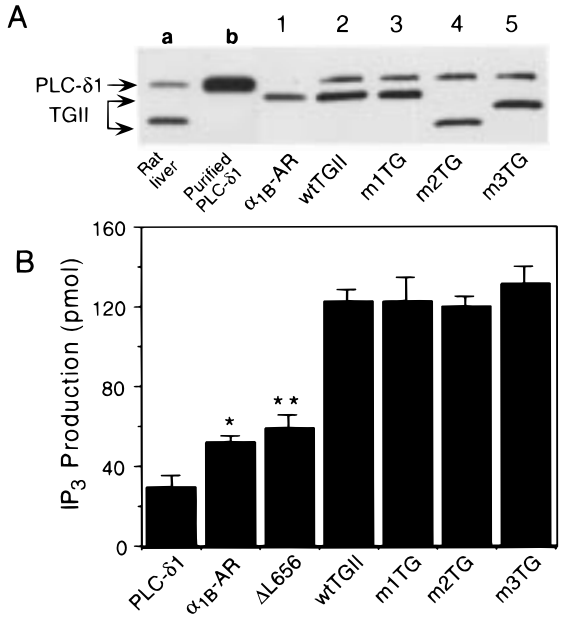

FIGURE 6: Interaction of PLC-δ1 with the expressed TGII proteins. (A) Co-immunoprecipitation of PLC-δ1 with wtTGII and TGII mutants by the TGII antibody. In these experiments, an TGII antibody (NeoMarkers, clone CUB 7402) that recognized the middle portion of TGII was used. Membrane extracts prepared from COS-1 cells expressing PLC-δ1 with TGII proteins were incubated with the antibody in the presence of 5×10^{-5} M GTPγS and 2 mM $MgCl_2$. Co-immunoprecipitation of PLC-δ1 with TGII proteins was achieved, as described in detail in Experimental Procedures. The levels of immunoprecipitated PLC-δ1 and TGII proteins were determined by Western blotting with both monoclonal TGII and PLC-δ1 antibodies, following SDS−PAGE (7% gel). An experiment with extracts of the rat liver membranes was also performed under the same conditions (lane a). The recombinant PLC-δ1 purified from DH5α cells expressing PLC-δ1 (*14*) was also applied as a control (lane b). With longer exposures, a faint band of PLC-δ1 was also observed with the membrane extract of cells expressing the α_{1B}-AR alone. (B) An increase in basal PLC activity in coexpression of TGII proteins and PLC-δ1. COS-1 cells expressing PLC-δ1 or the α_{1B}-AR alone or with TGII proteins were used to determine the basal level of IP_3 formation, as described in Experimental Procedures. The level of IP_3 formed (picomoles per milligram of protein) was normalized using the protein concentration of the cell lysates. In these experiments, a TGII mutant (ΔL656) was also coexpressed with PLC-δ1. The TGII mutant has been shown to lack a PLC interaction site (*20*). The results are means ± SEM of three independent experiments performed in duplicate, and statistical differences determined by analysis of variance for multiple comparison. The single asterisk indicates that $P < 0.05$ vs values for cells transfected with PLC-δ1 alone. The double asterisk indicates that $P < 0.05$ vs values for cells cotransfected with PLC-δ1 with wtTGII.

with the expressed TGII proteins was not due to the overexpression of both proteins. When the basal IP_3 formation was determined in the intact COS-1 cells coexpressing both proteins (Figure 6B), the levels of IP_3 production with the mutants were similar to that with wtTGII, consisting of the co-immunoprecipitation of PLC-δ1(Figure 6A). The basal level of IP_3 production was also significantly increased with coexpression of TGII proteins with PLC-δ1. The data showed that coexpression of both proteins resulted in ∼4- and ∼2.4-fold increases compared to those of the cells expressing PLC-δ1 alone (lane 1) or the α_{1B}-AR alone (lane 2), respectively. Moreover, the increase in the basal level of IP_3 formation was the result of interaction of these TGII proteins with PLC-δ1. Thus, cells coexpressing PLC-δ1 with a deletion mutant

(ΔL656 in lane 3) of TGII, which lacks the PLC interaction site (*20*), exhibited ~50% less IP_3 formation than that with the cells coexpressing wtTGII or the other mutants.

Role of TGII Proteins in α_{1B}-AR-Mediated PLC-δ1 Stimulation. We have previously shown that GTPγS-bound TGII stimulates fusion PLC-δ1 (*14*). To assess whether the α_{1B}-AR couples to PLC-δ1 via TGII, the receptor-mediated stimulation of PLC-δ1 was evaluated by in vivo reconstitution in COS-1 cells expressing α_{1B}-AR, wtTGII, and PLC-δ1 (Figure 7A). To observe the specific coupling between TGII and PLC-δ1, the TGII mutant (ΔL656) was also coexpressed. With cells expressing the α_{1B}-AR alone, activation of the receptor resulted in an ~6-fold increase in the level of IP_3 formation. When the receptor was coexpressed with wtTGII, the level of receptor-mediated IP_3 formation was further increased ~1.7-fold compared to that of the cells expressing the receptor alone. Coexpression of the α_{1B}-AR with TGII and PLC-δ1 yielded the highest levels of IP_3 production, ~3.8- and ~1.6-fold higher than those with cells expressing the α_{1B}-AR alone or the α_{1B}-AR with wtTGII, respectively. The α_{1B}-AR-mediated PLC-δ1 stimulation was completely blocked to the basal level when the cells were incubated with the α_1-antagonist. Furthermore, in cells coexpressing the TGII mutant (ΔL656) with α_{1B}-AR and PLC-δ1, the levels of both basal and receptor-mediated IP_3 production were reduced ~2.5- and ~3-fold, respectively, compared to those with the cells coexpressing wtTGII with α_{1B}-AR and PLC-δ1. These results clearly indicated that the increase in the level of IP_3 formation is mediated by the coupling of these three signaling molecules.

When the TGII mutants were coexpressed with α_{1B}-AR and PLC-δ1, a substantial reduction of the level of α_1-agonist-mediated IP_3 production was observed (Figure 7B). At the (−)-epinephrine concentration of 10^{-4} M, the order of the level of receptor-mediated PLC-δ1 stimulation by the TGII proteins was as follows: wtTGII (100%) > m1TG (~71%) > m2TG (~49%) > m3TG (~32%). These results demonstrated again that the three sites of TGII identified by the peptides interacted with the α_{1B}-AR and that the interaction is involved in the subsequent activation of TGII by the receptor.

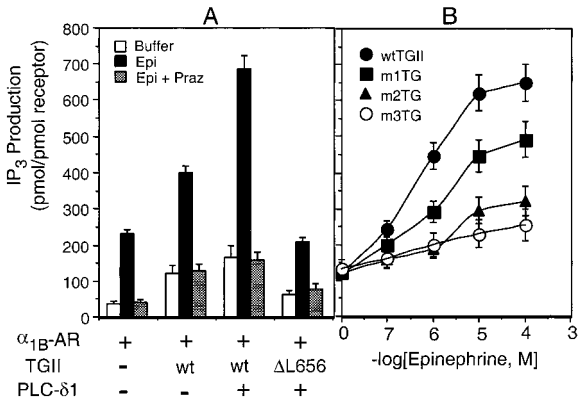

FIGURE 7: α_{1B}-AR-mediated PLC-δ1 stimulation through TGII proteins in intact COS-1 cells. (A) Production of IP_3 in COS-1 cells coexpressing α_{1B}-AR, wtTGII, and PLC-δ1. The COS-1 cells were cotransfected with various combinations of cDNAs, as indicated. After the transfected cells were grown for 60 h, the cells were preincubated with 20 mM LiCl at 37 °C for 30 min. Then, the ligands [5×10^{-5} M (−)-epinephrine or 5×10^{-5} M (−)-epinephrine with 4 nM prazosin] were added. The level of IP_3 production was determined, as described in Experimental Procedures. The results depicted here are the mean of three experiments, each performed in duplicate. Abbreviations: Epi, (−)-epinephrine; Praz, prazosin; wt, wild-type TGII; ΔL656, deletion TGII mutant lacking a PLC interaction site. (B) Production of IP_3 in COS-1 cells coexpressing the TGII mutants with α_{1B}-AR and PLC-δ1. The cells were incubated with various concentrations of the α_1-agonist (−)-epinephrine, as indicated. The level of formation of IP_3 in the cells was determined under the same conditions as described for panel A (see also Experimental Procedures). Here m1TG, m2TG, and m3TG indicate the TGII mutants generated by mutagenesis, as shown in Figure 1B.

DISCUSSION

Involvement of TGII in hormone receptor-mediated signaling was first demonstrated by isolation and characterization of an α_1-agonist−α_{1B}-AR−TGII complex from rat liver membranes (*7*). Subsequently, TGII was found to associate with an ~50 kDa protein which regulated the GTPase function (*5*, *13*, *30*) and to stimulate PLC (*6*, *31*, *32*). Recent structure−function studies with TGII have revealed the location of an interaction site of PLC-δ1 (*14*, *20*) and GTPase active site (*21*−*23*). In this study, taking advantage of the synthetic peptide approach (*33*), we identified recognition sites of the α_{1B}-AR on TGII using synthetic peptides derived from the C-terminal domain of TGII. To further understand the ability of TGII to couple with the α_{1B}-AR and PLC-δ1 and to refine the findings with peptides, site-directed TGII mutants were constructed and evaluated in an expression system.

Our studies with the combination of peptides and site-directed mutants have revealed that three peptide regions, P1 (L^{547}−I^{561}), P2 (R^{564}−D^{581}), and P6 (Q^{633}−E^{646}), are the α_{1B}-AR interaction sites on TGII that directly lead to the activation of GTP binding to TGII. Thus, these peptides were able to inhibit the formation of an α_1-agonist−α_{1B}-AR−TGII ternary complex by directly binding to the receptor (Figure 2A,B) and to block α_{1B}-AR-mediated TGase inhibition in a dose-dependent manner (Figure 3B,C). Consistent with the finding, the TGII mutants of these peptide regions significantly lost the ability to interact with the α_{1B}-AR (Figure 5A) and to stimulate PLC-δ1 via the α_{1B}-AR (Figure 7B).

The results with the TGII mutants have also indicated that the P2 and P6 regions of TGII are the critical sites for the interaction and activation of TGII by the α_{1B}-AR. Thus, the mutants (m2TG and m3TG) exhibited a significantly low binding affinity (22 and 18% of that of the wild type, respectively) for the receptor and a substantially reduced level of α_{1B}-AR-mediated PLC-δ1 stimulation (see Figures 5A and 7B). On the other hand, the P1 region of TGII is most likely to be a low-affinity binding site, since the mutant showed an ability to bind to the receptor that was ~2-fold higher than that with the other mutants (lane 3 in Figure 5A). Although incomplete mutation in this mutant should be considered, it may not be the case because the mutants of the P2 and P6 regions have substantially lost the ability (79−82%) to bind to the receptor. The studies with the mutants also suggest that the coupling of the α_{1B}-AR with TGII occurs in a cooperative manner, since mutation of a single interaction site of the receptor on TGII results in a 56−82% decrease in the level of binding of the mutants to the α_{1B}-AR (Figure 5A). In addition, it is interesting to observe that

the binding affinity of the mutants for the α_{1B}-AR somewhat correlates with the level of reduction of the receptor-mediated PLC-δ1 stimulation via the mutants (see Figures 5A and 7B). These findings indicate that the binding affinity of each region of TGII for the receptor contributes to the degree of TGII activation.

Consistent with our findings that the α_{1B}-AR interacts with three regions of TGII, at least three regions of the α_{1B}-AR are shown to contact with a G-protein (*34*, *35*). Thus, the studies with chimeric α_{1B}-AR−β_2-AR have demonstrated that substitution of the N- and C-terminal portions of the third cytoplasmic loop and the N-terminal region of the cytoplasmic tail of the α_{1B}-AR with the corresponding region of other receptor substantially abolished the PLC stimulation. In the case of the heterotrimeric G-proteins, three regions on the α subunits are proposed to interact with the receptors that have been determined by various approaches, including peptides, mutagenesis, site-directed antibodies, and chemical modification (*36*, *37*). These regions include extreme N- and C-terminal ends, an ADP−ribose binding site, and a portion of the C-terminal domain. The findings suggest that despite extensive primary structural differences between TGII and the α subunits of the heterotrimeric G-proteins, coupling of TGII with the receptor seems to share these common structural features.

Finally, we have clearly demonstrated that the α_{1B}-AR is able to stimulate PLC-δ1 via TGII (Figure 7A). In α_{1B}-AR-mediated transmembrane signaling, including the α_{1A}- and α_{1D}-AR, the coupling event of the signaling molecules is diverse. The α_{1B}-AR is known to stimulate primarily PLC via pertussis toxin insensitive G-proteins in various tissues (*38*; see refs *39* and *40* for reviews). However, after reconstitution of the receptor with or without G-proteins in an in vivo system, the receptor is capable of coupling with several G-proteins, including Gq family, pertussis toxin sensitive G-protein, and TGII, resulting in activation of multiple effectors PLA_2 and PLCs. For example, expression of the α_{1B}-AR in COS-1 cells increases the level of inositol phosphate formation via pertussis toxin insensitive G-protein, while expression of the receptor in CHO cells also stimulates arachidonic acid release via pertussis toxin sensitive G-protein (*41*). Coupling of the α_{1B}-AR with Gαq, Gα_{11}, Gα_{14}, and Gα_{16} was also demonstrated in COS-7 cells (*42*). On the other hand, the α_{1A}-AR has been shown to activate PLA_2 through a pertussis toxin sensitive G-protein in various tissues (*43*, *44*). However, transfection of the α_{1A}-AR in COS-7 stimulates PLC better than the α_{1B}-AR in the same expression system (*45*). Similarly, coexpression of the α_{1B}-AR with Gαq stimulates PLC better than coexpression of the receptor with TGII in COS-1 cells (*9*, *10*). The studies by Chen et al. (*10*) have demonstrated that TGII couples only to the α_{1B}-AR and the α_{1D}-AR, while Gαq couples to all three subtypes of α_1-ARs. All these observations suggest that the receptor uses multiple signaling pathways through which they can initiate signals in their target cells. Supporting this notion, the expression and cellular response of the α_1-AR subtypes differ among cell types and species (*39*, *46*). In this regard, the TGII mutants which significantly impair the interaction with the α_{1B}-AR will help to elucidate the TGII-mediated signaling pathway specifically, including the PLC-δ1-involved signaling.

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