The GTP binding $G_\alpha_h$ (transglutaminase II (TGase II)) is unique in that the enzyme exhibits two distinct enzyme activities, namely guanosine triphosphatase (GTPase) and TGase, with a signal transfer role (Ref. 1; see also Refs. 2 and 3). The GTPase function of $G_\alpha_h$ differs from other TGases, coagulation factor XIIIa (4), which is guanosine 5'-triphosphate (GTP) binding site of $G_\alpha_h$, and a chimera in which residues Val665-Lys672 of hhG$_h$ and human coagulation factor XIIIa, human heart FXIIIa (5–9). Thus, eight amino acid residues near the C terminus of hhG$_h$, are critical for recognition and stimulation of PLC.

The amino acid sequences of all TGases including G$_h$ are critical for recognition and stimulation of PLC.

EXPERIMENTAL PROCEDURES

Isolation and Mutagenesis of hhG$_h$, cDNA—Full-length cDNA of human heart G$_h$ (hhG$_h$) was isolated from a human heart cDNA library by polymerase chain reaction (PCR) using two oligonucleotides, CCGCCGACCATGGCCGAGGAGCTGGTCAT (5'-primer) and TGGGCCCAAGGGCACATTTCACATTTC (3'-primer), synthesized from the known nucleotide sequences of human endothelial TGase II (14). After the PCR product (~2 kilobases) was cloned into the pCRsTMII without purification, the insert was digested with KpnI and NotI and cloned into the modified eukaryotic expression vector pMT2. Four amino acid truncated mutants of hhG$_h$ were generated by introducing a TAA stop codon using the following oligonucleotides: 1) CTCGACGGCTTTACGTTGGCCT, 2) GTTACCCAGCACTTTGTTGAGGCCGC, 3) GAGGGCAGAAGGTTCTTTACCA, and 4) TCTCCTGGTGTGGACGGGTC. The hhG$_h$/human FXIIIa chimera was constructed by ligation of PCR products generated from two different sets of primers containing nucleotide sequences of human FXIIIa (5': 5'-GTGATCCAGATTCGCCAGAGGAGCTGGTC-TTC-3' and 3'-primer 5'-GTCGGCTATCAGCTTGACCAC-5'-primer). Each PCR fragment was ligated and cloned into the eukaryotic expression vector pMT2. Orientation of the constructs was confirmed by restriction enzyme mapping and DNA sequencing.

Expression of hhG$_h$, Proteins and Preparation of Membranes—Transfection and membrane preparation were performed using the method of Nakaoka et al. (1). COS-1 cells (5 x 10$^5$ per 100-mm dish) were cotransfected with plasmids containing $G_\alpha_h$-adrenoreceptor cDNA (4-5 $\mu$g of cDNA) and hhG$_h$, or its mutants (8-10 $\mu$g of cDNA) using the DEAE-dextran method. The cells were grown for 48-72 h after transfection. The membranes prepared from the transfected COS-1 cells were suspended in a buffer (20 mM Hepes, pH 7.4, 1 mM EDTA, 1 mM EGTA, 0.5 mM dithiothreitol, 10% glycerol, and protease inhibitors) and stored at 8°C until use.

Analysis of Expressed hhG$_h$, Protein and Its Mutants—All expressed proteins were estimated by immunoblotting with guinea pig liver TGase II and G$_\alpha_h$, antibodies (1, 6). Membrane proteins (100 $\mu$g) were solubilized with 1% sodium cholate and subjected to SDS-polyacrylamide gel electrophoresis (10% gel). The proteins were transferred to a nitrocellulose membrane (Millipore) and probed with the antibodies by the methods of Baek et al. (6). Antibody cross-reactivity of proteins was visualized with chemiluminescence reagent (DuPont NEN) using Kodak XAR-5 film. GTP binding ability of the partially purified expressed proteins (50 ng/tube) was measured by GTP-mediated inhibition of the TGase activity in the presence and absence of GTP. Partial purification of the expressed proteins was achieved by Q-Sepharose chromatography. The lysates (50 mg of protein) of transfected COS-1 cells were solubilized with 0.4% succrose monolaurate (SM) at 4°C for 1 h. The extracts were applied to a Q-Sepharose column (0.4 ml) equilibrated with a buffer containing 20 mM Hepes, pH 7.4, 1 mM EDTA, 0.5 mM dithiothreitol, 70 mM NaCl, and 0.05% SM. The columns were washed with 3-5 ml of 170 mM NaCl in the same buffer. The hhG$_h$, and its mutant proteins were eluted with 500 mM NaCl in the same buffer. The yields were analyzed.
by immunoblotting using G_{h/Ga} antibody and Coomassie Blue staining following SDS-polyacrylamide gel electrophoresis. The TGase activity of the purified proteins was determined in the presence of 0.5 mM CaCl$_2$ and 1 mM dithiothreitol by evaluating $[^3H]$putrescine incorporation into N,N-dimethyl casein. Coimmunoprecipitation—For coimmunoadsorption of PLC or the $\alpha_1$-adrenoreceptor with $hG_{Ga}$, and its mutant proteins, G$_{h/Ga}$ antibody-protein A-agarose was prepared according to the method of Schneider et al. (15). The membranes (150 $\mu$g/sample) in HSD buffer (20 mM Hepes, pH 7.4, 150 mM NaCl, and 0.5 mM dithiothreitol) were solubilized with 0.4% SM in the presence of 50 mM GTP-$\gamma$S and 2 mM MgCl$_2$ at 4°C for 1 h. The extracts were incubated with the antibody-agarose or preimmune-agarose (30 $\mu$g/sample) in HSD buffer (20 mM Hepes, pH 7.4) at 4°C for 3 h. The membranes (150 $\mu$g/sample) in HSD buffer (20 mM Hepes, pH 7.4, 150 mM NaCl, 0.5 mM dithiothreitol, and 0.05% SM) were preincubated with $[^3H]$putrescine in the presence of 0.5 mM CaCl$_2$ for 20 min at 30°C in a 100-$\mu$l final volume. The PLC activity absorbed to preimmune-protein A-agarose was negligible and taken as a nonspecific binding. Inhibition of the coimmunoprecipitation of PLC by peptides was determined using the membrane extracts, which were preincubated with peptides. The samples were incubated with the antibody-agarose (30 $\mu$l/tube) in the presence of 50 mM GTP-$\gamma$S and 2 mM MgCl$_2$ at 4°C for 2 h. The resin-bound PLC activity was determined using 50 $\mu$Ci $[^3H]$putrescine in the presence of 0.5 mM CaCl$_2$ for 20 min at 30°C in a 100-$\mu$l final volume. The PLC activity absorbed to preimmune-protein A-agarose was negligible and taken as a nonspecific binding. Inhibition of the coimmunoprecipitation of PLC by peptides was determined using the membrane extracts, which were preincubated with peptides. The samples were incubated with the antibody-agarose (30 $\mu$l/tube) in the presence of 50 mM GTP-$\gamma$S and 2 mM MgCl$_2$ at 4°C for 2 h. The resin-bound PLC activity was determined under the same conditions as described above. The phosphatidylinositol 4,5-bisphosphate was 100 $\mu$M (1200 cpm/nmol) in the assay. For the coimmunoprecipitation of the $\alpha_1$-adrenoreceptor with $hG_{Fa}$, and its mutants, the membranes that coexpressed $hG_{Fa}$, and its mutants with $\alpha_1$-adrenoreceptor or $\alpha_1$-receptor alone were incubated with $5 \times 10^{-6}$ M (--)epinephrine at 4°C for 3 h. The membranes were then solubilized with 0.2% SM in HSD buffer in the presence of $5 \times 10^{-6}$ M (--)epinephrine at 4°C for 1 h. The recovery of proteins in the extracts usually reached ~40% for the receptor and 40–50% for $hG_{Fa}$, and its mutants (see also Ref. 5). Since the $hG_{Fa}$, and its mutants were overexpressed 3–5-fold as compared to the $\alpha_1$-adrenoreceptor, the membrane extracts (100 fmol of the $\alpha_1$-receptor) were incubated with 100 $\mu$l of $hG_{Fa}$, antibody-agarose or nonimmune sera-agarose in 300 $\mu$l (final volume) with gentle rotation at 4°C for 2 h. After centrifugation at 2000 rpm, the $\alpha_1$-receptor density (50 $\mu$Ci [3H]prazosin) was measured using 3 nm $[^3H]$prazosin (final) in the presence or absence of $10^{-4}$ M phentolamine and 200 $\mu$l (final volume) after removing excess (--)epinephrine through a dried 3-ml Sephadex G-25 column. The preimmune serum-agarose-treated samples were used as controls to calculate the amounts of the receptor immunoquantitated for each sample. Other Assays—PLC activity was evaluated by the method of Im et al. (8). The $\alpha_1$-adrenoreceptor density was determined using the radiolabeled $\alpha_1$-specific antagonist $[^3H]$prazosin (5). The $\alpha_1$-adrenoreceptor-activated PLC stimulation was determined after normalizing receptor number. The protein concentration was determined by the method of Bradford (16).

RESULTS AND DISCUSSION

The isolated full-length $hG_{Fa}$ cDNA was an exact match in the nucleotide and deduced amino acid sequences with the human endothelial TGase II (14). To identify interaction sites of $hG_{Fa}$ with the $\alpha_1$-adrenoreceptor and PLC, systematic 10-amino acid-deleted mutants of $hG_{Fa}$ cDNA(s) were generated from the C-terminal end (Fig. 1). The full-length $hG_{Fa}$ cDNA and truncated $hG_{Fa}$ cDNA(s) were cotransfected into COS-1 cells with $\alpha_{1B}$-adrenoreceptor cDNA. The expressed proteins were recognized by the G$_{h/Ga}$ antibody as well as guinea pig TGase II antibody and were of the expected sizes with ~80 kDa for full-length $hG_{Fa}$, and a decrease in size as the length of nucleotide deletion increased (Fig. 2A). The expression level was ~4–10-fold higher than the endogenous G$_{alpha}$ (TGase II) is seen in the membranes from expressed $\alpha_{1B}$-receptor ($\alpha_{1B}$-AR) alone and mutants. The protein expression level was ~4–10-fold higher than the endogenous G$_{alpha}$, B, the GTP-mediated inhibition of TGase activity of the expressed $hG_{Fa}$, and mutants. The inhibition of TGase activity (50 ng/tube) was determined with GTP in the presence of 0.1 mM $[^3H]$putrescine, 1% N,N-dimethyl casein, 0.5 mM CaCl$_2$, and 2 mM MgCl$_2$ in HSD buffer at 30°C for 30 min. The inset shows the stimulation of TGase (50 ng/tube) in the presence of 0.5 mM CaCl$_2$ without GTP under the same conditions. The results are the mean of three independent experiments performed in duplicate.

Fig. 2. A, an autoradiogram of an immunoblot of the expressed membrane proteins from COS-1 cells transfected with $hG_{Fa}$, or mutant $hG_{Fa}$ polypeptides using polyclonal G$_{h/Ga}$ antibody. Molecular sizes (in kilodaltons) are shown on the left. Endogenous G$_{alpha}$ (TGase II) is seen in the membranes from expressed $\alpha_{1B}$-receptor ($\alpha_{1B}$-AR) alone and mutants. The protein expression level was ~4–10-fold higher than the endogenous G$_{alpha}$, B, the GTP-mediated inhibition of TGase activity of the expressed $hG_{Fa}$, and mutants. The inhibition of TGase activity (50 ng/tube) was determined with GTP in the presence of 0.1 mM $[^3H]$putrescine, 1% N,N-dimethyl casein, 0.5 mM CaCl$_2$, and 2 mM MgCl$_2$ in HSD buffer at 30°C for 30 min. The inset shows the stimulation of TGase (50 ng/tube) in the presence of 0.5 mM CaCl$_2$ without GTP under the same conditions. The results are the mean of three independent experiments performed in duplicate.
Fig. 3. Coupling ability of expressed hhGα1h and its mutants. A, epinephrine-stimulated inositol 1,4,5-triphosphate (IP3) accumulation in membranes from COS-1 cells coexpressed with α1B-receptor and hhGα1h, or its mutants. The α1-receptor-mediated PLC stimulation was determined after normalizing receptor number (100 fmol/tube) in a 100-μl final volume. Receptor number was normalized, since the receptor number is the determinant in signal manipulation, not G-protein number (17), and the expression level of hhGα1h and its mutants was also 3-5-fold higher than the receptor level. The results are the mean ± S.E. of three independent experiments performed in duplicate. α1B-AR, α1B-adrenoreceptor; Ep, (−)-epinephrine; Ph, phentolamine. B, remaining α1B-adrenoreceptor in the supernatants after coimmunoprecipitation with hhGα1h and its mutants using Gα1h antibody-protein A-agarose. The α1-adrenoreceptor absorbed to preimmune protein A-agarose was less than 5% as compared to the resin-untrated samples. The preimmune-resin-treated samples were taken as 100% for each sample. C, immunoabsorption of a complex of PLC with hhGα1h, and its mutants by Gα1h antibody-protein A-agarose. The PLC activity absorbed to preimmune protein A-agarose was negligible and taken as nonspecific binding. The data shown are the mean ± S.E. of three independent experiments in triplicate.

and ΔE646 mutants lost the agonist-mediated PLC stimulation, exhibiting a similar level to that of the α1B-receptor alone. The ΔN666 mutant stimulated PLC upon activation of the α1-receptor, but to a lesser extent than wild type. These data suggested that a region comprising 20 amino acids between Leu661 and Val666 contains a PLC interaction site. The deletion size increased (Fig. 4A). In the presence of GTP, the expressed hhGα1h and the mutant ΔK676 increased PLC stimulation 2-fold (Fig. 4B). Increases in deletion size also resulted in a gradual decrease of GTP-mediated PLC stimulation. Mutants ΔL656 and ΔE646 lost ability to stimulate PLC in response to GTP. These results were consistent with the finding from the coimmunoprecipitation studies and strongly suggested that a region between His657 and Lys677 on hhGα1h contained a PLC interaction site.

To further define this putative PLC interaction site, four overlapping peptides corresponding to the deleted regions of hhGα1h, were synthesized and tested for their ability to inhibit coimmunoprecipitation of PLC (Fig. 5A). Peptide 4 (Leu661-Lys672), among the four peptides, was able to inhibit coimmunoprecipitation of PLC (Fig. 5B). Coimmunoprecipitation of PLC was inhibited in a concentration-dependent manner, and at 100–200 μM of the peptide, the inhibition reached ~80%, suggesting that other interaction site(s) probably exist (Fig. 5C). Table 1 summarizes binding characteristics of the interaction site for Gα1h with hhGα1h, and PLC was ~20 μM.

The findings that a region of 12 amino acids between Leu661 and Lys672 in hhGα1h contains a PLC interaction site were

and the receptor interaction site on these mutants was intact (Fig. 3B). However, the mutant, ΔE646, consistently coimmunoprecipitated less receptor (~80%) than other mutants but more than the receptor alone (~35%). Although less coimmunoprecipitation of the receptor with this mutant suggested that this region on hhGα1h might contain the receptor interaction site, this point should be further investigated. Coimmunoprecipitation of the receptor with membrane extract from the expressed α1B-receptor alone was probably due to complex formation between the internal Gα1h and the receptor.

The loss of PLC interaction site was then assessed by coimmunoprecipitation (Fig. 3C). The results revealed that the ΔK676 mutant coimmunoprecipitated PLC as effectively as the wild type, whereas the ΔL656 and ΔE646 mutants failed to coimmunoprecipitate PLC, showing a similar level to that of the α1B-receptor alone. The ΔL666 mutant again showed lower coimmunoprecipitation of PLC than hhGα1h, but higher than the ΔL656 and ΔE646 mutants. The loss of the PLC interaction site was further confirmed by determining PLC stimulation in response to GTP (Fig. 4, A and B). As expected, the basal levels of PLC in membranes expressing hhGα1h and mutants were increased 3-6-fold compared to the α1B-receptor alone. Within these increases, the PLC basal activity gradually decreased as the deletion size increased (Fig. 4A). In the presence of GTP, the expressed hhGα1h and the mutant ΔK676 increased PLC stimulation 2-fold (Fig. 4B). Increases in deletion size also resulted in a gradual decrease of GTP-mediated PLC stimulation. Mutants ΔL656 and ΔE646 lost ability to stimulate PLC in response to GTP. These results were consistent with the finding from the coimmunoprecipitation studies and strongly suggested that a region between His657 and Lys677 on hhGα1h contained a PLC interaction site.
refined by a chimera, hhGαh/FXIIIa, in which eight amino acid residues Val665-Lys672 of hhGαh were substituted with the corresponding region (Ile707-Ser714) of human factor XIIIa (see Fig. 1) (13). This region of FXIIIa was chosen because FXIIIa does not interact with or stimulate PLC or bind GTP (4), and this region of FXIIIa is distinct among TGases (13). The chimera was expressed ~7-fold higher than endogenous Gαh and to the similar level of hhGαh (Fig. 6A). The chimera protein exhibited GTP binding and TGase activity at the same levels as the wild type (Fig. 6B). In addition, using the partially purified chimera when the GTP-mediated inhibition of TGase activity was titrated, the inhibition was similar to ΔK676 and wild type, indicating that substitution of this region did not change GTP binding affinity (data not shown). The chimera also failed to stimulate PLC in response to GTP (Fig. 6C) and upon activation of the α1B-receptor (data not shown). The Gαh antibody did not coimmunoprecipitate PLC but effectively coimmunoprecipitated the receptor (data not shown). These findings clearly demonstrate that the C-terminal region of hhGαh, from Val665 to Lys672 is a critical site for interaction and stimulation of PLC.

The substituted region of the chimera has a significant change in properties of the amino acids (Fig. 1). Thus, four charged amino acids (Asp667, Glu669, Asp671, and Lys672) were substituted for serine, except Asp671. Hydrophobic amino acids (Val665 and Val666) were also changed to smaller amino acids in peptides 3 and 4 are indicated with asterisks. B, effect of peptides of hhGαh on coimmunoprecipitation of PLC by Gαh, antibody-protein A-agarose. The membrane extracts (100 μg/tube) were preincubated with 100 μM peptide and subjected to coimmunoprecipitation. PLC activity was determined as detailed under “Experimental Procedures.” The results are the mean ± S.E. of three independent experiments performed in duplicate. C, competition of peptide 4 with hhGαh to coimmunoprecipitate PLC by Gαh, antibody-protein A-agarose. The experiments were performed with various concentrations of peptide 4 under the same conditions as detailed under “Experimental Procedures.” The data presented are a mean of the duplicated experiments using three independently expressed proteins.

Fig. 5. A, map of the synthesized peptides of hhGαh. Overlapping amino acids in peptides 3 and 4 are indicated with asterisks. B, effect of peptides of hhGαh on coimmunoprecipitation of PLC by Gαh, antibody-protein A-agarose. The membrane extracts (100 μg/tube) were preincubated with 100 μM peptide and subjected to coimmunoprecipitation. PLC activity was determined as detailed under “Experimental Procedures.” The results are the mean ± S.E. of three independent experiments performed in duplicate. C, competition of peptide 4 with hhGαh to coimmunoprecipitate PLC by Gαh, antibody-protein A-agarose. The experiments were performed with various concentrations of peptide 4 under the same conditions as detailed under “Experimental Procedures.” The data presented are a mean of the duplicated experiments using three independently expressed proteins.

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α₁-Adrenoreceptor, Transglutaminase II, and Phospholipase C

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