GTP Binding and Signaling by G\textsubscript{h}/Transglutaminase II Involves Distinct Residues in a Unique GTP-binding Pocket*

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G\textsubscript{h} is a dual function protein. It has receptor signaling activity that requires GTP binding and Ca\textsuperscript{2+}-activated transglutaminase (TGase) activity that is inhibited by GTP binding. G\textsubscript{h} shows no homology with other GTP-binding proteins, and its GTP-binding site has not been defined. Based on sequence analysis of [\alpha-\textsuperscript{32}P]GTP-photolabeled and proteolytically released internal peptide fragments, we report localization of GTP binding to a 15-residue segment (159YVLTQQGFIYQGSVK173) of the G\textsubscript{h} core domain. This was confirmed by site-directed mutagenesis; a G\textsubscript{h}/XIIIa chimera (in which residues 162–179 of G\textsubscript{h} were substituted with the equivalent but nonhomologous region of the non-GTP-binding TGase factor XIIIa) and a G\textsubscript{h} point mutant, S171E, retained TGase activity but failed to bind and hydrolyze GTP and did not support \alpha\textsubscript{1B}-adrenergic receptor signaling. Slight impairment of GTP binding (1.5-fold) and hydrolysis (10-fold) in the absence of altered TGase activity did not affect signaling by the mutant K173N. However, greater impairment of GTP binding (6-fold) and hydrolysis (50-fold) abolished signaling by the mutant K173L. Mutant S171C exhibited enhanced GTP binding and signaling. Thus, residues Ser\textsubscript{172} and Lys\textsubscript{174} are critical for both GTP binding and signaling but not TGase activity. Mutagenesis of residues N-terminal to Gly\textsubscript{170} impaired both GTP binding and TGase activity. From computer modeling of G\textsubscript{h}, it is evident that the GTP-binding region identified here is distinct from, but interacts with, the TGase active site. Together with structural considerations of G\textsubscript{h} versus other GTP-binding proteins, these findings indicate that G\textsubscript{h} has a unique GTP-binding pocket and provide for the first time a mechanism for GTP-mediated regulation of the TGase activity of G\textsubscript{h}.

G\textsubscript{h} is a dual function protein with transglutaminase (TGase)\textsuperscript{1} and receptor signaling activities. The Ca\textsuperscript{2+}-activated TGase activity catalyzes post-translational, covalent protein modification, either by formation of an isopeptide bond between glutamine residues and the \epsilon-amino group of intrachain lysines or modification of glutamine residues by polyamines (1). Of the six structurally related TGase proteins (2–4), G\textsubscript{h} (tissue TGase, TGase II) is the only one reported to bind and hydrolyze GTP (5, 6). It is located extracellularly at the cell surface (7) and intracellularly, where it is both membrane-associated and cytosolic. G\textsubscript{h}/TGase II has been implicated in a wide variety of processes including apoptosis, bone ossification, wound repair (8), and signal transduction (9). The biological significance of the signaling activity of G\textsubscript{h} remains poorly defined. In transfected cell-based studies, G\textsubscript{h} stimulates phospholipid C (PLC)-mediated inositol phosphate (IP) production in response to agonist activation of \alpha\textsubscript{1B}- and \alpha\textsubscript{1A}-adrenergic receptors but not \alpha\textsubscript{1A}-adrenergic receptors (ARs) (9, 10) and of TP\textsubscript{1} (11) thromboxane A\textsubscript{2} receptors (12). G\textsubscript{h} also couples the oxytocin receptor to PLC activation (12). G\textsubscript{h} activates the \delta isoform of PLC (13), although the mechanism is controversial. One model suggests that GTP-bound G\textsubscript{h} activates PLC\textsubscript{61} (13), whereas recent work indicates that PLC\textsubscript{61} may be negatively regulated by interaction with empty or GDP-bound G\textsubscript{h}. According to this latter model, PLC\textsubscript{61} is activated as a result of receptor-stimulated GTP/GDP exchange, which allows G\textsubscript{h} to dissociate from PLC\textsubscript{61} (14). Data from transgenic mice overexpressing G\textsubscript{h} in the heart (15) as well as from recent in vitro studies (16) are consistent with the latter model. G\textsubscript{h} has also been reported to mediate activation of the large conductance Ca\textsuperscript{2+}-activated K\textsuperscript{+} channel in vascular smooth muscle cells (17) as well as inhibition of adenylyl cyclase in Balb/c 3T3 fibroblasts and bovine aortic endothelial cells (18).

The TGase and signaling activities of G\textsubscript{h} are reciprocally regulated by Ca\textsuperscript{2+} and GTP (5, 9). Receptor signaling by G\textsubscript{h} correlates with increased GTP binding (19). TGase activity is inhibited by guanine nucleotides (GTP\textsubscript{S} > GTP > GDP), whereas GTP photolabeling is inhibited by Ca\textsuperscript{2+} (5). GTP inhibition of TGase activity is inversely proportional to Ca\textsuperscript{2+} activation and is greatest under conditions where TGase activity is minimal. Extracellularly, where Ca\textsuperscript{2+} is present in millimolar concentrations and nucleotides are absent, TGase activity would be stimulated, thus allowing G\textsubscript{h} to function in tissue remodeling. In contrast, there is evidence that the low Ca\textsuperscript{2+} and high nucleotide concentrations present intracellularly, inhibit TGase activity (20), thus enabling G\textsubscript{h} to function as a G-protein in receptor signaling. Although there are additional factors modulating the TGase activity of G\textsubscript{h}, such as sphingosylphosphocholine (21) and G\textsubscript{h}-interacting proteins (22), Ca\textsuperscript{2+} and GTP appear to control the functional switch between the two activities of G\textsubscript{h}.

Residues involved in GTP binding by G\textsubscript{h} have not been elucidated. The only TGase structure available is that of the non-GTP-binding (5) human factor XIIIa (XIIIa) (23). XIIIa
monomers consist of four domains: a β-sandwich, an α/β core that contains the TGase active site catalytic triad (Cys-His-Asp), and two β-barrels. The high degree of homology among TGase proteins, particularly in their core domains, suggests conservation of tertiary structure. In contrast, there is no sequence homology between Gh and other GTP-binding proteins such as the monomeric and heterotrimeric G-proteins, elongation factors, or dynamin, each of which contains four consensus GTP-binding motifs (GXXXXGK/S/T), DXXG, NXXD, and (C/ S/A)X that fold into a structurally conserved GTP-binding site (24). Gh may thus have a novel GTP-binding site.

Our previous work localized GTP binding by Gh to a region involving amino acids 139–185 of the core domain (25). Models of Gh (25, 26) based on the crystal structure of IXIIIA predict that this region is distinct from, yet in close proximity to, the TGase active site. It is also in close proximity to residues 517–523 of barrel 1 that are involved in stabilization of GTP binding (26). Mutation of the active site Cys to Ser (C277S) abolished Gh activity without affecting signaling (10), indicating that although the TGase and GTP-binding sites are allosterically linked, signaling can function independently of TGase activity. In the present study, we have further localized GTP binding to a 15-amino acid segment between residues 159 and 173 and have generated mutants that are altered in their GTP binding and signaling properties but not in their TGase activity. These mutants, together with the TGase-deficient, signaling-unaffected mutant (C277S) should be valuable tools for determining the respective physiological or pathological roles of Gh, as a cross-linking enzyme and as a G-protein in vivo.

EXPERIMENTAL PROCEDURES

Constructs—Site-directed mutants were generated in rat Gh cDNA (25). Overlap polymerase chain reaction was used to construct a Gh/IXIIIA chimera in which Gh cDNA (positions 480–542) was substituted with human IXIIIA cDNA (positions 637–699). Using Gh cDNA, 520- and 1566-base pair fragments were amplified (Pfu DNA polymerase; Stratagene) with, respectively, primer pairs 1 (5′-CCGGAAATTCCTCCACCATGGAGAGGAGCTGGTC-3′) and 2 (5′-GACCTCTCCAACTTTTGTGCTTCTGCT-3′) and primer pairs 3 (5′-GGATGAGGAGCTGAAGGACCAATTCAGCCGCCGCGCTCCGCC-3′) and 4 (5′-GGATGAGGAGCTGAAGGACCAATTCAGCCGCCGCGCTCCGCC-3′) (fXIIIA sequence is italicized; overlapping sequences are underlined). Purified fragments (Brespin gel extraction kit; Geneworks, Australia) were boiled (5 min, Klenow buffer; Roche Molecular Biochemicals), chilled (ice, 2 min), extended (37 °C, 30 min, 1 mM dNTPs, Klenow fragment), purified (Brespin gel purification kit; Geneworks), and used in a polymerase chain reaction (Pfu DNA polymerase, primer pairs 1 and 4) to amplify a 2.1-kilobase pair fragment for cloning into the EcoRI and NotI sites of pGEX4T-2 (Amersham Pharmacia Biotech).

Purification of Recombinant Gh—Wild type (WT) and mutant Ghs were expressed as thrombin-cleavable GST-Gh fusion proteins (25). GST tags were cleaved from proteins bound to glutathione-Sepharose 4B (4 °C, 24 h; extraction buffer (25); protein/thrombin ratio, 14:1). Eluted Ghs were purified (Sephacryl S-200 column, 4 °C), and Gh content was approximated by Coomassie Blue R-250 staining after SDS-PAGE. WT Gh was further purified (Mono Q® column, 4 °C, final volume 600 mM NaCl gradient, 4 °C). A, OD (280-nm) profile of fractions 1–7 eluted from the Mono Q® column. B, Coomassie Blue-stained SDS-PAGE. C, Autoradiography (6-day exposure) of fractions 1–7. Molecular mass markers (kDa) are indicated.

GTP/βS Filter Binding Assays—GTP/βS binding (19) by WT or mutant Gh (6.7 nm) was assayed (20 mM Tris-HCl, pH 7.3, 150 mM NaCl, 1 mM EGTA, 10 mM dithiothreitol, 500 μM AppNHp, 25 mM GTP/βS) (1250 Ci/mmol; NEN Life Science Products), final volume 150 μl with or without 20 μM GTP/βS to determine nonspecific and total binding, respectively. Filtration buffer contained 20 mM Tris-HCl, pH 6.7, 150 mM NaCl, 1 mM EGTA.

GTPase Activity—The charcoal method was used to determine the GTPase activity of 200 nM WT or mutant Gh incubated with 1 μM [γ-32P]GTP (30 Ci/mmol; NEN Life Science Products) and 9 μM GTP as described (25).

Phosphatidylinositol Hydrolysis in Intact Cells—Intact, transiently transfected COS-1 cells were assayed for phosphatidylinositol hydrolysis as described (27).

Statistical Analyses—All comparisons were done using unpaired Student’s t test. p < 0.05 was considered significant.

RESULTS

Isolation and Sequence Analysis of GTP-binding Gh Peptides—To minimize nonspecific cross-linking, unbound GTP (fractions 2 and 3, Fig. 1) was separated from GTP-bound Gh by Mono Q® chromatography prior to photolabeling. Gh eluted as a single radiolabeled peak (fraction 5, Fig. 1), indicating a single radiolabeling site binding (19) by WT or mutant Gh (6.7 nm) was assayed (20 mM Tris-HCl, pH 7.3, 150 mM NaCl, 1 mM EGTA, 10 mM dithiothreitol, 500 μM AppNHp, 25 mM GTP/βS) (1250 Ci/mmol; NEN Life Science Products), final volume 150 μl with or without 20 μM GTP/βS to determine nonspecific and total binding, respectively. Filtration buffer contained 20 mM Tris-HCl, pH 6.7, 150 mM NaCl, 1 mM EGTA.

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ment (residues 159–173) at the start of the core domain (Fig. 2, A and C). This region is highly conserved in type II, but not other TGases (Fig. 2B). One peptide localized to barrel 1, indicating a possible additional GTP-interacting region within this domain.

**Mutagenesis of the Core GTP-binding Site and Identification of Specific Residues Required for GTP Binding**—Two mutagenesis strategies were adopted. First, a \( G_h/x IIIA \) chimeric cDNA was constructed, in which DNA encoding residues 162–179 of rat \( G_h \) was substituted with the equivalent region (encoding residues 207–224) of human \( x IIIA \). Second, a number of amino acids highly conserved in TGases II/\( G_h \), but not in other TGases, viz. Gln163, Gln164, Gln169, Ser171, and Lys173 (Fig. 2B), were mutated either to residues in \( x IIIA \) or to residues of equivalent volume that were either hydrophobic or maintained hydrogen bonding potential. Expression levels of Q163L and Q164L were lower than WT, and that of Q163L/Q164L was too low to purify.

Binding of GTP was assessed directly by \([\alpha-32P]\)GTP photo-labeling and by \([35S]\)GTP\(_S\) binding. Although photolabeling of all proteins (Fig. 3A, top panel) decreased in the presence of 5 mM MgCl\(_2\), only \( G_h/x IIIA \) and S171E photolabeled poorly. This was not due to a difference in protein loading, as indicated by Coomassie Blue staining of the gel (Fig. 3A, bottom panel). The small amount of photolabeling of \( G_h/x IIIA \) and S171E is probably due to nonspecific cross-linking of excess \([\alpha-32P]\)GTP, as was observed when photolabeled peptides were isolated without Mono Q\(_S\) purification prior to photolabeling. GTP\(_S\)

### Table I

| Protease | Fragment | Sequence | Fragment size | Determined | Theoretical |
|----------|----------|----------|---------------|------------|-------------|
|          |          |          | kDa           |            |             |
| V8       | 1        | 159YVLTQ...E186 | 3.5           | 3.3        |             |
| V8       | 2        | 159YVLTQGG...E266 | 10            | 12.3       |             |
| V8       | 3        | 159TSTGYQ...E266 | 14            | 16.7       |             |
| Lys-C    | 4        | 171ARFSLS...K173 | 14            | 11.1       |             |
| Lys-C    | 5        | 465LAEEK...K1589 | 14            | 14.1       |             |

* Amino acids determined by sequencing are italicized
* Estimated from SDS-PAGE.
* Based on protease recognition sites.

![Fig. 2. Identification of (\( \alpha-32P \))GTP-labeled \( G_h \) peptides. A, diagrammatic representation of (\( \alpha-32P \))GTP-labeled peptides (\#1–85) identified in Table I and their location relative to the four domains (\( \beta \)-sandwich, core, barrel 1, and barrel 2) of \( G_h \). Numbers denote boundary positions of peptides and domains; the 15-amino acid region that defines the core GTP-labeled segment is indicated by dashed vertical lines. B, alignment of the core GTP-labeled segment of TGases II/\( G_h \) with equivalent regions in other TGases is shown using single-letter amino acid code; shaded boxes indicate identical amino acids. Asterisks indicate amino acids targeted for site-directed mutagenesis. C, computer-generated model of \( G_h \) (25) based on crystal structure coordinates of XIIMA, showing the core GTP-labeled segment (orange). \( G_h \) active site residues (Cys277, His335, and Asp358), Ser171, and Lys173 discussed under “Results” are represented as ball-and-stick side chain groups; yellow represents sulfur, blue represents nitrogen, red represents oxygen, and white represents carbon atoms.*

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binding assays (Fig. 3B) indicated that, in addition to Gh/IXIIIA and S171E, K173L and K173N were impaired in GTP binding. This is informative, because in our hands, WT Gh has an affinity for GTP of ~25 nM, and affinities lower than this cannot be readily detected by filtration assays (28). Thus, the lack of GTP\&S binding by K173L and K173N, both of which photolabel, indicates that these two mutants have a lower affinity for GTP than WT. Despite impaired GTP binding, maximally activated TGase activities of Gh/IXIIIA, K173L, K173N, and S171E were equivalent to WT (Fig. 3C). S171C behaved like WT with respect to GTP photolabeling, GTP\&S binding, and maximal TGase activity. Mutants Q169L, Q164L, Q163L, and Q163D had both impaired TGase activity and GTP\&S binding.

Dose-response curves for Ca\&sup+\textsuperscript{2+} activation of TGase activities of Gh/IXIIIA, Ser\textsuperscript{171}, and Lys\textsuperscript{172} mutants were similar to WT (Fig. 4A, Table II), confirming that these mutations had intact TGase activity. As an indirect measure of GTP binding, GTP\&S inhibition of TGase activity was evaluated at both ~10% (Fig. 4B) and ~80% (Fig. 4C) of maximal Ca\&sup+\textsuperscript{2+}-stimulated TGase activity. TGase activity of both Gh/IXIIIA and S171E was resistant to GTP\&S inhibition (Table II). TGase activity of the other mutants was sensitive to GTP\&S inhibition but to varying degrees; K173L was significantly less sensitive to GTP\&S inhibition than WT (8-fold increase in IC\textsubscript{50} at 10% maximal activity, p = 0.002, n = 3; 6-fold increase in IC\textsubscript{50} at 80% maximal activity, p = 0.004, n = 3, Table II), whereas K173N was less affected and the IC\textsubscript{50} was only significantly different from WT at higher Ca\&sup+\textsuperscript{2+} concentrations (1.5-fold increase in IC\textsubscript{50} at 80% maximal TGase activity, p = 0.006, n = 3). TGase activity of S171C, on the other hand, was more sensitive to GTP\&S inhibition than WT (25% of WT IC\textsubscript{50} at 10% maximal TGase activity, p = 0.03, n = 3; 11% of WT IC\textsubscript{50} at 80% maximal TGase activity, p = 0.0002, n = 3).

GTase assays were performed to determine the effect of these mutations on GTP hydrolysis. GTase activity of both WT recombinant (Fig. 5) and purified guinea pig liver Gh is 0.01 pmol of GTP hydrolyzed/pmol protein/min and equivalent to that of c-Ha-Ras.\textsuperscript{2} GTP hydrolysis by S171C was equivalent to WT Gh, whereas K173N was impaired, K173L was barely detectable, and that of Gh/IXIIIA and S171E was undetectable.

Correlation of GTP Binding with Receptor Signaling—The ability of the Gh mutants to mediate agonist-stimulated phosphatidylinositol hydrolysis was compared with that of WT Gh and Gh\textsubscript{out}. COS-1 cells were transiently cotransfected with cDNAs encoding the \alpha\textsubscript{1B}-AR cDNA and either vector pMT4 (control), WT Gh, Gh mutants, or Gh\textsubscript{out} (Fig. 6). Basal accumulation of total \textsuperscript{[3H]}IP (IP\textsubscript{1}, IP\textsubscript{2}, and IP\textsubscript{3}) was not significantly different (data not shown). After stimulation with the AR agonist (\textsuperscript{[3H]}epinephrine (the presence of the \beta-AR antagonist, DL-propranolol (50 \textmu M)), significant enhancement of IP accumulation was observed in cells cotransfected with either K173N or S171C relative to \alpha\textsubscript{1B}-AR alone, indicating no impairment of signaling by these mutants. Indeed, maximal IP accumulation in S171C-cotransfected cells appeared to be significantly greater than in WT Gh-cotransfected cells and equivalent to that observed in Gh\textsubscript{out}-cotransfected cells. In cells cotransfected with K173L, (\textsuperscript{[3H]}epinephrine stimulation did not result in IP accumulation greater than that of \alpha\textsubscript{1B}-AR alone, indicating an inability of this mutant to signal. Interestingly, IP accumulation after (\textsuperscript{[3H]}epinephrine stimulation was less in cells cotransfected with Gh/IXIIIA or S171E than in cells transfected with \alpha\textsubscript{1B}-AR alone, suggesting a potential dominant negative effect of these mutants on signaling.

DISCUSSION

Unlike the heterotrimeric or monomeric GTP-binding proteins that contain consensus GTP-binding motifs and a structurally conserved GTP-binding site, the residues forming the Gh GTP-binding pocket have not been identified. Isolation of \textsuperscript{[32P]}GTP-labeled peptide fragments of Gh (Table I) localized GTP-labeling to a 15-amino acid segment (\textsuperscript{[109]VLTQQF-IYQGSVK\textsuperscript{173}}) at the start of the core domain (Fig. 2, A and C) and to an additional region (residues 465–589 in barrel 1). These data agree with previous work that localized GTP binding to residues 139–185 of the core (25) and implicated residues 517–523 of barrel 1 in stabilization of GTP binding (26). The conservation of residues 159–173 in the type II TGases of...
various species (but not in fXIIIA; TGases I, III, or IV; or band 4.2 (Fig. 2B)) is consistent with TGase II being the only group present in monomeric and heterotrimeric G-proteins. Available data would be required to superimpose the GTP-binding site found in monomeric and heterotrimeric G-proteins. Amino acids 159–173 of Gh are predicted to form a central six-stranded β-sheet, in which five strands are parallel and one is antiparallel (24). GTP is bound at the end of the β-strands mostly by hydrogen bond interactions to the main chain of either amino acids with small side chains (Gly and Ala) or amino acids in loops at the ends of the β-strands. Our current model of Gh (25) is based on crystal structure coordinates of the non-GTP-binding fXIIIA and predicts far fewer accessible main chain atoms than are seen in crystal structures of GTP-binding sites of other GTP-binding proteins. Amino acids 159–173 of Gh are predicted to form a β-strand, but although Ser171 and Lys173 are before a loop at the end of the β-strand, with another β-strand running antiparallel, significant structural rearrangement of the core and barrel 1 or 2 would be required to superimpose the GTP-binding site found in monomeric and heterotrimeric G-proteins. Available data from UV difference spectra (29), susceptibility to limited trypsin proteolysis (5, 30), and small angle neutron scattering (30) indicate that Gh undergoes conformational changes upon GTP binding (e.g. in the presence of GTP, the gyration radius of the scattering particle undergoes a small but significant decrease). Heterotrimeric and monomeric G-proteins share a structurally conserved GTP-binding site comprised of five α-helices and a central six-stranded β-sheet, in which five strands are parallel and one is antiparallel (24). 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for the lack of accessible main chain atoms.

Interestingly, our model of G\textsubscript{h} predicts that the \( \beta \)-strand formed from amino acids 159–173 interacts with part of the TGase-active site main chain scaffold that contains the catalytic residues Cys\textsuperscript{277} and His\textsuperscript{238}. Binding of GTP to this region would thus disrupt the TGase active site, thereby explaining the mechanism of GTP inhibition of TGase activity. The progressively greater impairment of both TGase activity and GTP binding upon site-directed mutagenesis of Gln\textsuperscript{169}, Gln\textsuperscript{164}, or Gln\textsuperscript{163}, respectively (Fig. 3) correlates with their progressively N-terminal \( \beta \)-strand location relative to Ser\textsuperscript{177} and supports the predicted interaction of this \( \beta \)-strand region with the TGase active site.

Ser\textsuperscript{171} may be involved in potential hydrogen bond interactions between negatively charged phosphate groups of GTP. The introduction of negatively charged Glu (S171E) abolished GTP binding and hydrolysis, whereas substitution of Cys for Ser (S171C), which maintains hydrogen bonding potential, had little effect on GTP binding or hydrolysis. The decreased affinity of S171C for Ca\textsuperscript{2+} (28 versus 11 \( \mu \)M for WT, Table II) would be the result of a concomitant conformational change that affects Ca\textsuperscript{2+}-binding. Alternatively, although Ser\textsuperscript{171} is not in a region that has homology to known Ca\textsuperscript{2+}-binding domains as discussed below, it is possible that Ser\textsuperscript{171} is a ligand for Ca\textsuperscript{2+} (or both Ca\textsuperscript{2+} and GTP) in a secondary Ca\textsuperscript{2+}-binding site. In this case, Ca\textsuperscript{2+} binding could inhibit GTP binding through either steric hindrance or local conformational changes. Substitution of Lys\textsuperscript{173} also impaired GTP binding and hydrolysis without affecting TGase activity. Lys\textsuperscript{173} may thus be the Lys residue that has been implicated in the GTP-binding site of erythrocyte TGase II based on covalent modification by dialdehyde-GTP (32). Although definitive evidence for involvement of this region in GTP binding will require the determination of the crystal structure of GTP-bound G\textsubscript{h}, the dialdehyde-GTP modification data (32), together with the localization of photolabeled peptides and characterization of the site-directed mutants presented here, are supportive evidence for direct involvement of Ser\textsuperscript{171} and Lys\textsuperscript{173} in binding GTP.

Recent work has unexpectedly demonstrated GTP inhibition of the TGase activity of TGase III, indicating that TGase III, like G\textsubscript{h}, can bind GTP. However, unlike G\textsubscript{h}, TGase III was reported to have no GTPase activity (33), although neither the mechanism nor sensitivity of detection was reported. TGase III has Ser (Ser\textsuperscript{166}) at the equivalent position of Ser\textsuperscript{171} in G\textsubscript{h} and Asn (Asn\textsuperscript{169}) at the equivalent position of Lys\textsuperscript{173} (Fig. 2B). Analogously to the K173N mutant of G\textsubscript{h}, then, it is likely that the presence of Asn (Asn\textsuperscript{169}) in TGase III allows GTP binding but does not support a readily detectable level of GTP hydrolysis.

Interestingly, the characteristics of GTP inhibition of TGase activity differ between G\textsubscript{h} and TGase III. In TGase III, GTP inhibition is not sensitive to Ca\textsuperscript{2+} concentration (33). The \( \xi \)III structure in the presence of Ca\textsuperscript{2+} indicates one main Ca\textsuperscript{2+}-binding site (34), which is conserved in G\textsubscript{h} (Asn\textsuperscript{398}, Asp\textsuperscript{400}, Glu\textsuperscript{447}, and Glu\textsuperscript{452}) and TGase III (Asp\textsuperscript{395}, Asp\textsuperscript{395}, Glu\textsuperscript{443}, and Glu\textsuperscript{448}). However, site-directed mutagenesis of Glu residues in this region of guinea pig G\textsubscript{a} did not completely inhibit enzymatic activity, indicating the presence of secondary Ca\textsuperscript{2+}-binding sites that influence enzyme function (35). This is consistent with enzyme kinetic studies (36) and equilibrium dialysis experiments (37) that have implicated binding of 2–6 Ca\textsuperscript{2+} ions to G\textsubscript{a}. Additional putative Ca\textsuperscript{2+}-binding sites in G\textsubscript{h} that are not conserved in TGase III may involve residues 242–254 and residues 409–421 (both of which satisfy all but the last ligand (Asp or Glu) of an EF-hand Ca\textsuperscript{2+}-binding motif in a relaxed PROSITE search (PROSCAN, Institute of Biology and Chemistry of Proteins, Lyon, France; data not shown)) and/or acidic regions comprising residues 146–162 (which overlap with the GTP-labeling site) and residues 229–234 (38). Ca\textsuperscript{2+} dependence of GTP inhibition of the TGase activity of G\textsubscript{h}, but not TGase III, may thus reflect conformational changes in G\textsubscript{h} that are associated with progressive binding of Ca\textsuperscript{2+} to more than one site, which in turn, progressively reduces optimal binding of GTP.

Importantly, receptor signaling by the site-directed mutants described in this study correlated primarily with GTP binding (Fig. 6), thereby demonstrating that GTP binding is required for signaling. Cotransfection into COS-1 cells of \( \alpha_{1B} \)-AR with S171C, which has slightly lower affinity for calcium and/or higher affinity for GTP\textsubscript{yS} (Fig. 4, Table II), resulted in higher maximal IP response after (-)-epinephrine stimulation than did WT G\textsubscript{h}. GTP hydrolysis by this mutant did not differ significantly from WT (Fig. 5). Cotransfection with K173N, which was slightly impaired in GTP binding (1.5-fold) and more so in GTP hydrolysis (10-fold) resulted in an IP response that was no different from WT, indicating that small differences in GTP binding affinity and hydrolysis are tolerated. In contrast, cotransfection with K173L, which showed significantly greater impairment of GTP binding (6-fold) and hydrolysis (50-fold), did not increase IP responses above control (\( \alpha_{1B} \)-AR alone) levels. Interestingly, cotransfection with either G\textsubscript{h}/\( \xi \)III or S171E, which did not bind or hydrolyze GTP, resulted in IP responses below those of \( \alpha_{1B} \)-AR alone, indicating a potentially dominant-negative effect. To establish the mechanism by which this might occur, we are currently determining whether these mutants bind other guanine nucleotides such as GDP and whether they interact with PLC\textsubscript{61} and \( \alpha_{1B} \)-AR.

The importance of the present study is 2-fold. First, we have localized residues required for GTP binding by G\textsubscript{h} and have shown that this binding, which probably involves a novel GTP-binding fold, is required for signaling by G\textsubscript{h}. Second, this is the first report of signaling-specific mutants of G\textsubscript{h} that are unaf-
fected in TGase activity. This work and our previous work describing TGase-deficient mutants unaffected in signaling (10) therefore demonstrate unambiguously that the signaling and TGase activities of G_h, although reciprocally regulated, are separable and can function independently. These specific signaling- and TGase-deficient mutants can now be used to dissect the respective physiological or pathological roles of G_h as a cross-linking enzyme and as a G-protein in cultured cells and in “knock-in” mouse models.

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REFERENCES

1. Folk, J. E. (1980) Annu. Rev. Biochem. 49, 517–531
2. Greenberg, C. S., Birckbichler, P. J., and Rice, R. H. (1991) FASEB J. 5, 3671–3077
3. Aeschlimann, D., and Paulsson, M. (1994) Thromb. Haemostasis 71, 402–415
4. Aeschlimann, D., Mosher, D., and Paulsson, M. (1996) Semin. Thromb. Hemostasis 22, 437–443
5. Achyuthan, K. E., and Greenberg, C. S. (1987) J. Biol. Chem. 262, 1901–1906
6. Lee, K. N., Birckbichler, P. J., and Patterson, M. K., Jr. (1989) Biochem. Biophys. Res. Commun. 162, 1370–1375
7. Gaudry, C. A., Verderio, E., Aeschlimann, D., Cox, A., Smith, C., and Griffin, M. (1999) J. Biol. Chem. 274, 30707–30714
8. Upchurch, H. F., Conway, E., Patterson, M. K., Jr., and Maxwell, M. P. (1991) J. Cell. Physiol. 149, 375–382
9. Nakazaki, H., Perez, B. M., Baek, K. J., Das, T., Hussain, A., Misono, K., Im, M.-J., and Graham, R. M. (1994) Science 264, 1593–1598
10. Chen, S., Lin, F., Iismaa, S., Lee, K. N., Birckbichler, P. J., and Graham, R. M. (1996) J. Biol. Chem. 271, 32385–32391
11. Vezza, R., Habib, A., and Fitzgerald, G. A. (1996) J. Biol. Chem. 274, 12774–12779
12. Park, E.-S., Won, J. H., Han, K. J., Suh, P.-G., Ryu, S. H., Lee, H. S., Yun, H.-Y., Kwon, N. S., and Baek, K. J. (1996) Biochem. J. 318, 263–269
13. Feng, J.-F., Rhe, S. G., and Im, M.-J. (1996) J. Biol. Chem. 271, 16451–16454
14. Murthy, S. N., Lomasney, J. W., Mak, E. C., and Lorand, L. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 11815–11819
15. Small, R., Feng, J.-F., Lorenz, J., Donnelly, E. T., Yu, A., Im, M.-J., Dern, G. W., II, and Liggett, S. B. (1999) J. Biol. Chem. 274, 21293–21296
16. Zhang, J., Tucholski, J., Lesort, M., Jope, R. S., and Johnson, G. V. W. (1999) Biochem. J. 345, 541–549
17. Lee, M.-Y., Chung, S., Bang, H.-W., Baek, K. J., and Uhm, D.-Y. (1997) Eur. J. Physiol. 433, 671–673
18. Gentile, V., Porta, R., Chiosti, E., Spina, A., Valente, F., Pezone, R., Davies, P. J. A., Alaadik, A., and Iliano, G. (1997) Biochim. Biophys. Acta 1357, 115–122
19. Im, M.-J., and Graham, R. M. (1990) J. Biol. Chem. 265, 18844–18851
20. Smethurst, P. A., and Griffin, M. (1996) Biochem. J. 313, 803–808
21. Lai, T.-S., Bielawska, A., Peoples, K. A., Hannuu, Y. A., and Greenberg, C. S. (1997) J. Biol. Chem. 272, 16295–16300
22. Baek, K. J., Das, T., Gray, C. D., Desai, S., Hwang, K.-C., Gachhui, R., Ludwig, M., and Im, M.-J. (1996) Biochemistry 35, 2651–2657
23. Yee, V. C., Pedersen, L. C., Le Trong, I., Bishop, P. D., Stenkamp, R. E., and Teller, D. C. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 7298–7303
24. Kjeldgaard, M., Nyborg, J., and Clark, B. F. C. (1996) FASEB J. 10, 1347–1368
25. Iismaa, S. E., Chung, L., Wu, M.-J., Teller, D. C., Yee, V. C., and Graham, R. M. (1997) Biochemistry 36, 11655–11664
26. Monsonego, A., Shani, Y., Friedmann, I., Paas, Y., Eizenberg, O., and Schwartz, M. (1997) J. Biol. Chem. 272, 3724–3732
27. Chen, S., Xu, M., Lin, F., Lee, D., Riek, P., and Graham, R. M. (1999) J. Biol. Chem. 274, 16320–16330
28. Bylund, D. B., and Toews, M. L. (1993) Am. J. Physiol. 265, L421–L429
29. Folk, J. E., Mulloy, J. P., and Cole, P. W. (1967) J. Biol. Chem. 242, 1838–1844
30. Casadio, R., Peverini, E., Mariani, P., Spinozzi, F., Carsuigi, F., Fontana, A., Pulverino di Laureto, P., Matteucci, G., and Bergamini, C. M. (1999) Eur. J. Biochem. 262, 672–679
31. Jones, D. T., Taylor, W. R., and Thornton, J. M. (1992) Nature 358, 86–89
32. Bergamini, C. M., and Signorini, M. (1993) Biochem. J. 291, 57–39
33. Hitomi, K., Kanehiro, S., Ikura, K., and Maki, M. (1999) J. Biochem. (Tokyo) 125, 1048–1054
34. Fox, B. A., Yee, V. C., Pedersen, L. C., Le Trong, I., Bishop, P. D., Stenkamp, R. E., and Teller, D. C. (1999) J. Biol. Chem. 274, 4917–4923
35. Ikura, K., Yu, C., Nagao, M., Saaki, R., Furuyoshi, S., and Kawabata, N. (1995) J. Biochem. 118, 11815–11819
36. Folk, J. E., Cole, P. W., and Mulloy, J. P. (1967) J. Biol. Chem. 242, 1651–2621
37. Bergamini, C. M. (1988) FEBS Lett. 239, 255–258
38. Nakashima, K., Nara, K., Hagiwara, H., Aoyama, Y., Ueno, H., and Hirose, S. (1991) Eur. J. Biochem. 202, 15–21
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