Involvement of G Protein-coupled Receptor Kinase 5 in Homologous Desensitization of the Thyrotropin Receptor*

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Homologous desensitization of G protein-coupled receptors involves agonist-dependent phosphorylation of receptors by G protein-coupled receptor kinases (GRKs). To identify GRK(s) that play a role in homologous desensitization of the thyrotropin (TSH) receptor, thyroid cDNA was amplified by polymerase chain reaction using degenerate oligonucleotide primers from highly conserved regions in GRK family. GRK5 is found in the predominant isoform expressed in the thyroid. Rat GRK5 cDNA was then isolated, which encodes a 590-amino acid protein with 95% homology to human and bovine homologs. Northern blot identified GRK5 mRNA of -3, 8, and 10 kilobases with highest expression levels in lung > heart, kidney, colon > thyroid. In functional studies using a normal rat thyroid FRTL5 cells, overexpression of GRK5 by transfecting the plasmid capable of expressing the sense GRK5 RNA suppressed basal cAMP levels and augmented the extent of TSH receptor desensitization, whereas suppression of endogenous GRK3 expression by transfecting the antisense GRK5 construct increased basal cAMP levels and attenuated the extent of receptor desensitization. Although exogenously overexpressed GRK6 also enhanced TSH receptor desensitization, we conclude that GRK5, the predominant GRK isoform in the thyroid, appears to be mainly involved in homologous desensitization of the TSH receptor.

Guanine nucleotide-binding regulatory (G) protein-coupled receptors transduce a wide variety of extracellular signals (hormones, neurotransmitters, odors, lights, chemoattractants, etc.) into intracellular signaling events, by activating or inhibiting specific effector enzymes (adenylyl cyclase, phospholipase C, phospholipase A2, ion channels, etc.) (1). A rapid loss of responsiveness also occurs following receptor activation by agonist ligand binding in this receptor superfamily. This process is called "homologous desensitization." The mechanisms of homologous desensitization have been extensively studied at the molecular level in rhodopsin and adrenergic receptors (2–4). Newly discovered G protein-coupled receptor kinases (GRKs) and arrestins are involved in this process. GRKs specifically recognize and phosphorylate the agonist-bound form of receptors in the active conformation. Subsequently, arrestin proteins bind exclusively to the phosphorylated and activated receptor, resulting in uncoupling of the receptor and G protein.

Six different types of GRKs have so far been cloned and characterized. cDNA for bovine β-adrenergic receptor kinase (β-ARK, now also called GRK2) was first isolated (5), followed by the cloning of cDNAs for bovine β-ARK2 (GRK3) (6), bovine rhodopsin kinase (GRK1) (7), human IT11 (GRK4) (8), human GRK5 (9), and human GRK6 (10, 11). Drosophila kinases GPRK-1 and -2 have also been identified (12). These GRKs can be divided into 3 different subgroups according to their homology, cellular localization, and distinct regulatory mechanisms (9, 10). The first subgroup includes GRK4 to 6 and GPRK-1, the second subgroup GRK1, GRK2, and GPRK-2, and the third subgroup GRK1. GRKs also seem to have different patterns of expression in tissues. GRK1 and GRK4, for instance, are primarily expressed in retinal cells and the testis, respectively, suggesting very specific functions. GRK1 (rhodopsin kinase) phosphorylates photolyzed rhodopsin (7). Although little is at present known about substrate specificity, the testis-specific expression of GRK4 implies that it might be a kinase for gonad-specific G protein-coupled receptors such as the lutropin and folliculin receptors (3). Whereas, other GRKs are expressed in many tissues, suggesting their broad roles in regulating many receptors.

The receptor for thyrotropin (thyroid stimulating hormone, TSH), that regulates thyroid growth and differentiated functions, is also a member of G protein-coupled receptors (13) and well known to undergo homologous, but not heterologous, desensitization (14–16). Although we have recently reported that only the hormone-occupied form of the TSH receptor, like the β-adrenergic receptor, is likely to be involved in homologous desensitization (17), the molecular mechanisms of TSH receptor desensitization remain largely unclear.

The aim of the present studies was, therefore, to characterize the GRK(s) that may play a role in TSH receptor homologous desensitization in the thyroid. Using reverse transcription and the polymerase chain reaction (RT-PCR), human and rat thyroid mRNA was studied to determine which types of GRK are present in these cells and whether these include any novel thyroid-specific GRK. We report herein that GRK5 appears to be the predominant GRK isoform in the thyroid cells. Furthermore, we have cloned and determined the nucleotide sequence of rat GRK5 cDNA of thyroid origin and provide evidence to support the functional role of GRK5 in homologous desensitization of the TSH receptor.

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‡The abbreviations used are: G-protein, guanine nucleotide-binding protein; GRK, G-protein coupled receptor kinase; ARK, adrenergic receptor kinase; TSH, thyrotropin; RT-PCR, reverse transcription-polymerase chain reaction; BS, Bluescript; bp, base pairs; wt wild type.
EXPERIMENTAL PROCEDURES

PCR—Oligonucleotide primers were synthesized corresponding to two highly conserved stretches of the catalytic domain of human GRK5: MNGGDL(K/F)F/F/H (5′-CAC AAT TCT GAA (CT/TTG)I IGA (CA/CT/TT IAA (AG/ITT) CCC/CA-3′, sense), where the italics are the restriction enzyme sites for EcoRI and SalI. Total RNA was extracted from a human thyroid tissue, obtained at the time of subtotal thyroidectomy from a patient with Graves’ disease, by the method of Han et al. (18) and from rat FRTL5 cells cultured in the presence of TSH by the acid guanidinium thiocyanate-phenol-chloroform method (19). FRTL5 cells, normal differentiated rat thyroid epithelial cell line (20), were cultured in Coon’s modified Ham’s F-12 medium supplemented with 5% fetal bovine serum and the addition of three hormones: 2 units/liter bovine TSH, 5 units/liter insulin, and 5 mg/liter transferrin (3H-medium). Sonatobin, hydrocortisone, and glycyrl-histidyl-lysine acetate were omitted. First strand cDNA was synthesized from 500 ng of total RNA using Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.) and random primers, a fraction of which was then amplified using 25 pmol of each primer, 200 mmol/liter of each dNTP, and 2 units of Taq polymerase in 10 mmol/liter Tris- HCl, pH 8.3, 50 mmol/liter KCl, 1.5 mmol/liter MgCl2, 0.01% gelatin, in a final volume of 50 μl. Thirty cycles of denaturation (94°C for 90 s), annealing (45°C for 90 s), and extension (72°C for 60 s) were conducted with an automated thermal cycler. All reactions were initiated with 5 min incubation for 95°C and terminated with 7 min incubation at 72°C. The approximately 300 base pair (bp) products were gel-purified, digested with SalI and EcoRI, and cloned into SalI and EcoRI digested pBS(+) vector (Stratagene). Clones were sequenced by the dye-terminator cycle chain termination methods (21) with a modified bacteriophage T7 DNA polymerase (Amerham) and T3 and T7 primers.

Construction and Screening of Rat FRTL5 DNA Library—Polyadenylated RNA was isolated from FRTL5 cells cultured in the presence of TSH by the acid guanidinium thiocyanate-phenol-chloroform method, followed by oligo(dT) cellulose chromatography. Double stranded cDNA was synthesized using the Amersham cDNA synthesis kit and was ligated into the EcoRI site of the λ ZAP II vector (Stratagene), with a yield of approximately 4 × 105 recombinant clones. The cDNA library was amplified in XL-1 Blue cells. The amplified library was screened by standard procedures (22), using, as a probe, a 300-bp PCR fragment obtained as described above. The cDNA probe was labeled with [α-32P]dCTP by the Multiprime DNA Labeling System (Amerham) to a specific activity of 1 × 108 cpmp μg DNA. The final washing was performed in 0.1 × SSC (1 × SSC is 0.15 mol/liter NaCl and 0.015 mol/liter sodium citrate, pH 7.0) and 0.1% SDS at 55°C, followed by autoradiography. The isolated clones were then excised with R048 helper phage from the λ ZAP II phage to yield the inserts in pBS. The sizes of the inserts were estimated by the Excel digestion assay (23). After purifying the inserts, using a gel electrophoresis. Nucleotide sequences of selected clones were determined by the deoxyxynucleotide chain termination methods following the ExoMung procedure (Takara Shuzo, Japan). All the sequences in Fig. 1 were verified from both DNA strands. Nucleotide homology searches were performed against sequences in GenBank.

Tissue Distribution Study by Northern Blot Analysis—Total RNA was extracted from thyroid, lung, heart, kidney, colon, and liver in a 12-week-old Wistar rat by the acid guanidinium thiocyanate-phenol-chloroform method. 20 μg of total RNA was subjected to electrophoresis in 1% agarose, 2.2 m formaldehyde gels and blotted onto Hybond nylon membrane paper (Amerham) by standard techniques (22) using 10 × SSC and 0.1% SDS at 42°C for 4 h in 0.5× SSC, 0.5% SDS and nitrocellulose at 42°C for 4 h in 0.2× SSC, 0.1% SDS. The blots were probed with the 5′-32P-labeled probe and washed extensively with 10× SSC, 0.5% SDS and nitrocellulose at 42°C for 4 h in 0.2× SSC, 0.1% SDS. The blots were probed with the 5′-32P-labeled probe and washed extensively with 10× SSC, 0.5% SDS and nitrocellulose at 42°C for 4 h in 0.2× SSC, 0.1% SDS. The blots were probed with the 5′-32P-labeled probe and washed extensively with 10× SSC, 0.5% SDS. The blots were probed with the 5′-32P-labeled probe and washed extensively with 10× SSC, 0.5% SDS.

RESULTS

Using degenerate oligonucleotide primers corresponding to highly conserved sequences in the catalytic domain of members of the GRK family, the DNA fragments of the expected size (approximately 300 bp) were successfully amplified by PCR from human and rat thyroid cDNA. The PCR products were then subcloned into pBS and their nucleotide sequences were determined. As summarized in Table 1, of 30 cDNA clones obtained from a human thyroid, 27 were identical to human GRK5, 2 were GRK4, and 1 was GRK6. In the rat thyroid, of 20 cDNA clones sequenced, 19 and 1 showed approximately 90% homology to human GRK5 and -4, respectively, suggesting these may be rat GRK5 and 4. Rat GRK4 and -5 cDNAs have not previously been cloned. Neither GRK1, -2, nor -3 were observed in human and rat thyroids. No novel GRK mRNA species was identified. Since these data indicate that the predominant GRK isoform

G Protein-coupled Receptor Kinase in Thyroid

10144
expressed in the thyroid is likely to be GRK5 and because a well characterized, normal rat thyroid epithelial cell line, FRTL5 cells, is a very useful model for further functional studies, we attempted to clone rat GRK5 cDNA. 

4

In the first series of experiments, these transfectants as well as the wt-FRTL5 cells, cultured in TSH-free medium for 7 days, were incubated with 10 units/liter TSH for up to 24 h. As shown in Fig. 4A, maximum intracellular cAMP concentrations were observed 30 min after TSH stimulation in each cell type. The extent of desensitization was the wt-FRTL5 cells, cultured in TSH-free medium for 7 days, were incubated with 10 units/liter TSH for up to 24 h. As shown in Fig. 4A, maximum intracellular cAMP concentrations were observed 30 min after TSH stimulation in each cell type. The extent of desensitization was

**TABLE I**

| Isomers of the GRKs | Thyroid | Human | Rat |
|---------------------|---------|-------|-----|
| GRK1 (rhodopsin kinase) | 0 | 0 | - |
| GRK2 (β-ARK) | 0 | 0 | - |
| GRK3 (β-ARK) | 0 | 0 | - |
| GRK4 | 2 | 1a | - |
| GRK5 | 27 | 19b | - |
| GRK6 | 1 | 0 | - |
| Number of clones sequenced | 30 | 20 | - |

a Homologous to human GRK4.
b Homologous to human GRK5.

**Fig. 1.** Deduced amino acid sequence of rat GRK5. Deduced amino acid sequence of the full-length rat GRK5 is shown. Predicted amino acid sequence is numbered on the right.

**Fig. 2.** Distribution of GRK5 messenger RNA. 20 μg of total RNA from various tissues of rat were separated on 1% agarose-formaldehyde gel, transferred to nylon membrane, and probed with 32P-labeled PCR product (A) and cyclophilin cDNA (B). The blot was washed at high stringency condition, followed by autoradiography at −80°C. Samples were hybridized with sense GRK5 cDNA (lane 1), liver (lane 2), heart (lane 3), kidney (lane 4), colon (lane 5), and lung (lane 6), kb, kilobases.

(20 μg of total RNA) demonstrated little detectable bands for GRK4 and GRK6 even by longer exposure (data not shown).

To clarify the functional properties of GRK5 in thyroid cells, especially in the TSH receptor signal transductive system, the cDNA in the sense of antisense orientation (GRK5S or GRK5AS) was stably transfected into rat thyroid FRTL5 cells. After selection with G418, multiple independent cell lines were selected for each transfectant. The data obtained with two clones for each transfectants are shown in Figs. 3 and 4. The FRTL5-GRK5S-1 and -2 expressed approximately 6- and 4-fold higher levels of GRK5 mRNA and 5- and 3-fold higher levels of the protein, respectively, than did the wild type (wt) FRTL5 cells (Fig. 3). In FRTL5-GRK5AS, the expression of the antisense message was detected by RT-PCR with the sense GRK5-specific oligonucleotide primer in the RT reaction (data not shown). FRTL5-GRK5AS-1 and -2 expressed approximately one-forth and one-half of sense GRK5 mRNA and one-third and one-half of the protein, respectively, compared to those in the wt-FRTL5 cells.

In the first series of experiments, these transfectants as well as the wt-FRTL5 cells, cultured in TSH-free medium for 7 days, were incubated with 10 units/liter TSH for up to 24 h. As shown in Fig. 4A, maximum intracellular cAMP concentrations were observed 30 min after TSH stimulation in each cell type. The magnitude of cAMP increase was very similar among these clones (data not shown). In the wt-FRTL5 cells, cAMP levels then gradually declined to 49.3 ± 2.3, 41.0 ± 2.6, and 56.7 ± 3.1% (% maximum, mean ± S.D.) after 2, 4, and 24 h TSH stimulation, respectively. The extent of desensitization was

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**Homologous to human GRK5.**

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**TABLE I**

| Analysis of cloned G protein-coupled receptor kinase cDNAs amplified from human and rat thyroids by RT-PCR |
|-------------------------------------------------|
| cDNAs for GRK were amplified from human and rat thyroid RNA by RT-PCR, subcloned, and sequenced (see “Experimental Procedures” for detail). |

| Isomers of the GRKs | Thyroid | Human | Rat |
|---------------------|---------|-------|-----|
| GRK1 (rhodopsin kinase) | 0 | 0 | - |
| GRK2 (β-ARK) | 0 | 0 | - |
| GRK3 (β-ARK) | 0 | 0 | - |
| GRK4 | 2 | 1a | - |
| GRK5 | 27 | 19b | - |
| GRK6 | 1 | 0 | - |
| Number of clones sequenced | 30 | 20 | - |

a Homologous to human GRK4.
b Homologous to human GRK5.
more pronounced in the cells stably expressing the sense GRK5 RNA (FRTL5-GRK5S). For example, cAMP levels 2, 4, and 24 h after TSH stimulation were 26 ± 1.5, 13.0 ± 1.0, and 25.3 ± 1.2%, respectively, in FRTL5-GRK5S-1 (p < 0.01 versus the wt-FRTL5 in each point). On the contrary, in cells expressing the antisense GRK5 RNA (FRTL5-GRK5AS), the extent of desensitization after 2, 4, and 24 h incubation with TSH was less evident (for example, 63.3 ± 2.5, 59.7 ± 7.5, and 76.0 ± 6.6% in FRTL5-GRK5AS-1, p < 0.05 versus the wt-FRTL5 in each point).

In the second series of experiments, the wt-FRTL5, FRTL5-GRK5S-1, and FRTL5-GRK5AS-1 were pretreated with 10 units/liter TSH for 4 h, washed extensively, and were then stimulated with 10 units/liter TSH for 30 min (Fig. 4B). The reasons for the 4-h pretreatment of the cells with TSH in these experiments are (i) the data obtained in the foregoing studies suggested that 4 h incubation of the cells with TSH was sufficient for the maximal desensitization (Fig. 4A), and (ii) previous studies have shown that the TSH receptor down-regulation, another mechanism of the diminished receptor responsiveness, is not manifest after 4 h treatment of FRTL5 cells with TSH (28). As shown in Fig. 4B, cAMP response to the second TSH stimulation was lower in FRTL5-GRK5S than in the wt-FRTL5 cells (10.3 ± 0.6% of the maximum response in FRTL5-GRK5S versus 28.0 ± 4.6% in the wt-FRTL5, p < 0.01), but higher in FRTL5-GRK5AS (44.7 ± 7.1%, p < 0.05). Furthermore, the basal cAMP level prior to TSH pretreatment was significantly lower in FRTL5-GRK5S cells than in the wt-FRTL5 (1.0 ± 0.1 pmol/well in FRTL5-GRK5S versus 1.3 ± 0.1 pmol/well in the wt-FRTL5, p < 0.05), and that in FRTL5-GRK5AS was slightly, but not significantly, higher than that in the wt-FRTL5 (1.6 ± 0.3 pmol/well, p > 0.05). Differences in the basal levels of cAMP became more evident after 4 h pretreatment with TSH. Thus, those of the wt-FRTL5, FRTL5-GRK5S, and FRTL5-GRK5AS were 8.3 ± 1.5, 2.3 ± 0.6, and 16.3 ± 1.5 pmol/well (p < 0.01), respectively. These data clearly demonstrate that the expression levels of GRK5 was positively correlated with the extent of TSH receptor desensitization.

To evaluate kinase-receptor specificity, another isotype of GRK, GRK6 (10), was also stably expressed in FRTL5 cells (FRTL5-GRK6S). Two clones, FRTL5-GRK6S-1 and -2, which expressed exogenous GRK6 mRNA (Fig. 5), were selected. In these cells, as shown in Fig. 6, cAMP levels 2 and 4 h after TSH stimulation were significantly lower in the FRTL5-GRK6S cells (for example, 37.2 ± 11.0 and 17.7 ± 7.9% in FRTL5-GRK6S-1 versus 74.2 ± 9.8 and 43.5 ± 10.2% in the wt-FRTL5, p < 0.05 in each point), suggesting that the TSH receptor desensitization can also be influenced by other isotypes of GRKs.

**DISCUSSION**

In the present studies, we evaluated the molecular mechanisms of homologous desensitization of the TSH receptor in the thyroid. From the extensive studies on desensitization of adrenergic receptors and rhodopsin (2–4), we speculated that, like these receptors, phosphorylation of the agonist-bound TSH receptor by GRK(s) and subsequent binding of arrestin(s) to phosphorylated TSH receptor might be involved in receptor desensitization. In the present studies we focused on one of these proteins, GRK, and first attempted to clarify subtypes of GRK expressed in the thyroid by RT-PCR. We also wished to determine if there was a novel thyroid-specific GRK. Although no novel GRK was detected in the thyroid, our data demon-
strate that, of 6 different types of GRKs, GRK5 appears to be the predominant isoform expressed in both human and rat thyroids. The fact that little message was detected for GRK4 and GRK6 in Northern blot analysis with rat thyroid RNA (20 µg of total RNA) supports this finding. The expression level of GRK5 mRNA in the thyroid is, however, not higher than that in other tissues such as lung, colon, heart, and kidney in Northern blot analysis.

We next studied the functional properties of GRK5 in rat FRTL5 thyroid cells by the cloning and transfection of rat GRK5 cDNA. Enhancement and suppression of GRK5 expression was achieved by stable transfection of the cloned sense and antisense cDNAs, respectively. The present studies clearly demonstrate that overexpression or underexpression of GRK5 in vivo increased or attenuated, respectively, homologous desensitization of the TSH receptor. These data, together with the fact that the GRK5 may be the predominant GRK isoform in the thyroid, implicates GRK5 in TSH receptor homologous desensitization. Similar approaches have recently been employed by others and demonstrate in vivo functional properties of GRKs. Thus, overexpression of β-ARK is reported to enhance β2-adrenergic receptor desensitization (29), and suppression of β-ARK expression by antisense oligonucleotides, overexpression of a β-ARK dominant negative mutant, or neutralizing antibodies against β-ARK2 successfully attenuates desensitization of β2-adrenergic, m2 muscarinic receptors, or odorant receptor (30–33). Furthermore, our data also suggest that GRK5 limits the extent of TSH receptor homologous desensitization in FRTL5 cells, a finding consistent with the previous studies with β2-adrenergic receptor (29).

The incomplete attenuation of TSH receptor desensitization in the cells expressing the antisense GRK5 mRNA can be explained largely by the incomplete suppression of the endogenous GRK5 mRNA expression (Fig. 4A), although another possibility cannot be excluded that other kinases such as GRK4 and 6, which are expressed in the thyroid only in a very small quantity, together may also participate desensitization of the TSH receptor because exogenously overexpressed GRK6 enhanced desensitization of the TSH receptor (Fig. 6). Although it is reported that some GRKs prefer certain types of receptor, the substrate specificity of GRKs is in general not strict (2, 3). Indeed GRK5 phosphorylates rhodopsin, β2- and α2-adrenergic and m2-muscarinic receptors in vitro (4, 9, 34). Which GRK(s) are involved in desensitization of the TSH receptor depends not only on the intrinsic ability of individual GRKs to desensitize the receptor but also on the concentration of individual GRKs in the thyroid cells.

From these data, we speculate that GRK5 may be capable of phosphorylating the TSH receptor, although there is at present no direct evidence supporting this thesis. It has been demonstrated that phosphorylation of rhodopsin and the β2-adrenergic receptor by GRKs occurs in the cytoplasmic C-terminal tails (35), whereas the phosphorylation site by GRK in the α2-adrenergic receptor appears to be in the third cytoplasmic loop (36). We and others have shown that the truncated TSH receptor in which the serinedithreonine-rich cytoplasmic C-terminal tail of the receptor is deleted undergoes homologous desensitization, like the full-length receptor (17, 37, 38), suggesting other sites, such as the third cytoplasmic loop of the TSH receptor, may be targeted by GRKs. Further studies regarding agonist-dependent phosphorylation of the TSH receptor by GRK5 will be required. This kind of study could be employed in reconstituted systems with purified proteins. However, purification of the TSH receptor has not yet been attained (39). Furthermore, although it has recently been reported that crude membranes from insect cells overexpressing GRKs, rather than purified receptors, can be used for phosphorylation studies (40), the functional full-length TSH receptor cannot be expressed in insect cells. This is probably due to the incomplete or improper glycosylation of the receptor protein in insect cells (41, 42). In summary, the present studies demonstrate that (i) GRK5 appears to be the predominant isoform of GRK in the thyroid, and (ii) the expression levels of GRK5 are positively correlated with the extent of TSH receptor desensitization. From these data we conclude that GRK5 appears to be involved in homologous desensitization of the TSH receptor in the thyroid.

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