IDENTIFICATION OF FOUR SPLICE VARIANTS*

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A novel human G protein-coupled receptor kinase was recently identified by positional cloning in the search for the Huntington’s disease locus (Ambrose, C. J. Ames, M., Barnes, G., Lin, C., Bates, G., Altherr, M., Duyao, M., Groot, N., Church, D., Wasmuth, J. J., Lehrah, H., Houseman, D., Buckler, A., Gusella, J. F., and MacDonald, M. E. (1993) Hum. Mol. Genet. 1, 697-703). Comparison of the deduced amino acid sequence of GRK4 with those of the closely related GRK5 and GRK6 suggested the apparent loss of 32 codons in the amino-terminal domain and 46 codons in the carboxyl-terminal domain of GRK4. These two regions undergo alternative splicing in the GRK4 mRNA, resulting from the presence or absence of exons filling one or both of these apparent gaps. Each inserted sequence maintains the open reading frame, and the deduced amino acid sequences are similar to corresponding regions of GRK5 and GRK6. Thus, the GRK4 mRNA and the GRK4 protein can exist as four distinct variant forms.

The human GRK4 gene is composed of 16 exons extending over 75 kilobase pairs of DNA. The two alternatively spliced exons correspond to exons II and XV. The genomic organization of the GRK4 gene is completely distinct from that of the human GRK2 gene, highlighting the evolutionary distance since the divergence of these two genes. Human GRK4 mRNA is expressed highly only in testis, and both alternative exons are abundant in testis mRNA.

The four GRK4 proteins have been expressed, and all incorporate [3H]palmitate. GRK4 is capable of augmenting the desensitization of the rat luteinizing hormone/chorionic gonadotropin receptor upon coexpression in HEK293 cells and of phosphorylating the agonist-occupied, purified β-adrenergic receptor, indicating that GRK4 is a functional protein kinase.

G protein-coupled receptor kinases (GRKs)† are a family of serine/threonine protein kinases that phosphorylate G protein-coupled receptor proteins (1, 2). Receptors are phosphorylated by GRKs in vitro on multiple serine and threonine residues on intracellular loops and/or the carboxyl-terminal tail. GRK-phosphorylated receptors are bound by arrestin proteins, leading to the functional removal of the receptor from the signaling pathway by preventing further receptor coupling to G proteins (3). Agonist-occupied, activated G protein-coupled receptors are phosphorylated by GRKs, but inactive receptor proteins are not recognized by these kinases as substrates (1, 2). The GRKs, along with arrestin proteins that bind to phosphorylated receptors and prevent coupling to G proteins, mediate homologous desensitization of hormonal responses (1).

Six GRKs have been identified by purification and by cloning. Rodopsin kinase (GRK1) in the visual system phosphorylates light-bleached rhodopsin on its carboxyl-terminal tail (1, 3). Arrestin bound to phosphorylated rhodopsin prevents further activation of transducin, dampening activation of retinal cGMP phosphodiesterase (3). The “β-adrenergic receptor kinases,” GRK2 and GRK3, were identified by their functional ability to phosphorylate β-adrenergic receptors and are widely distributed throughout the body (2). GRK2 and GRK3 have been shown to phosphorylate a variety of G protein-coupled hormone and neurotransmitter receptors (2). Together with the somatic β-arrestin proteins, GRK2 and GRK3 have been implicated in the uncoupling of receptors from their respective G proteins, a mechanism leading to homologous desensitization (1, 3).

A putative member of the family of G protein-coupled receptor kinases was recently identified by a positional cloning strategy, due to the proximity of its gene to the Huntington’s disease locus (4). Originally named IT11, this sequence has been renamed GRK4 in an effort to systematize the nomenclature of these enzymes (1). GRK4 is most similar in sequence to mammalian GRK5 (5) and GRK6 (6) and to Drosophila GPRK2 (7). Together, these sequences define a subfamily of GRK enzymes that is distinct from the more extensively characterized rodopsin kinase and β-adrenergic receptor kinase subfamilies. Both GRK5 and GRK6 have been functionally expressed and shown to phosphorylate several G protein-coupled receptors (5, 6, 8, 10). Functional activity of the GRK4 kinase has not yet been reported, and GRK4 is the least well understood member of the GRK family. Saliese et al. (11) have recently described an alternatively spliced exon encoding 32 amino acids in the amino-terminal region of human GRK4. In this paper, we define

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† The abbreviations used are: GRKs, G protein-coupled receptor kinases; LH, luteinizing hormone; CG, chorionic gonadotropin; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; PIP2, phosphatidylinositol 4,5-bisphosphate; kb, kilobase pair(s).
the structure of the human GRK4 gene and show that the GRK4 gene transcript undergoes extensive alternative splicing to generate four distinct forms of GRK4 mRNA that encode four forms of the GRK4 protein. We demonstrate the functional ability of GRK4 to augment desensitization of the LH/CG receptor in transfected cells and to phosphorylate the agonist-occupied, purified β2-adrenergic receptor.

MATERIALS AND METHODS

Amplification of cDNAs—DNA amplification was performed by the polymerase chain reaction using standard conditions (12). Each 100-μl reaction contained 1×-Thermus aquaticus DNA polymerase buffer, 200 μM each dNTP, 500 nM each oligonucleotide primer, and 10 ng of first-strand cDNA template. Reactions were assembled except for polymerase and heated for 5 min at 95°C, and 2.5 units of Taq DNA polymerase (Promega) were added. Reactions were cycled 35 times for 1 min at 95°C, 1 min at 60°C, and 3 min at 72°C, followed by a final 10-min extension at 72°C.

The aminoterminal coding region of the GRK4 cDNA was amplified from human testis first-strand cDNA using the primers 5′-ctctggtcgcggcatag (in the 5′-untranslated region) and 5′-aagcgatgtctgggaaattctcag (antisense to RVHAVNY), while the carboxyterminal coding region was amplified using the primers 5′-aaagcgattctgcggaggtggttggtcggcatag (antisense to EFPKQC). Product DNA bands were cut out from agarose gels, subcloned into the pCR-TOPO vector (Invitrogen) or digested with BamHI and EcoRI (underlined sites) and subcloned into pBSII (Stratagene).

Amplification of Genomic DNA (Introns)—Human genomic DNA or individual cosmid DNAs were amplified by a long-accurate PCR technique (13). Taq and Taq DNA polymerases (Promega) were mixed 20:1 by volume and carried out using standard buffer and nucleotide conditions as described above. Reactions were cycled 35 times for 1 min at 95°C, 1 min at 55–65°C, and 10 min at 72°C, followed by a final 10-min extension at 72°C. Reactions were performed with a series of primers spanning individual introns and their flanking exons as determined by DNA sequencing. The GRK4 gene was contained on the overlapping cosmids BC10A12 (14), L142C5 (15), BJ56w4-3 (16), and 8C10A12 (14), L142C5 (15), BJ56w4-3 (16), and 8C10A12 (14).

DNA Sequencing—Double-stranded sequencing of cDNAs was carried out by chain termination using Sequenase 2 (Amersham Corp./U. S. Biochemical Corp.) and specific primers. For direct sequencing from cosmid DNAs, cycle sequencing was performed using radiolabeled oligonucleotide primers and the fmolTM sequencing kit (Promega). Primers located randomly throughout the cDNA sequence were used to identify intron locations, and specific primers were prepared to sequence through each exon and into the exon/intron junction.

Northern Blotting—Nylon filters containing 2 μg of human tissue poly(A) RNAs transferred from agarose gels were obtained from CLON-TECH (human MT1 and MTN 11). Blots were hybridized (12) overnight in a preincubation solution containing 5×SSC buffer, 50% formamide, 100 μg/ml each of alternative exons 1 and XV. The blot was washed in 0.2 × SSC buffer at 65°C and exposed to x-ray film for 5 days at ~70°C. Blots were then stripped of probe and rehybridized at 55°C with end-labeled antisense 48-mer oligonucleotides specific for the amino-terminal (bases 306–353) and carboxy-terminal (bases 1839–1886) alternative exons. Oligonucleotide probes were washed in 2 × SSC buffer at 55°C and exposed to x-ray film for 3 days at ~70°C.

Antiserum and Immunoblotting—A fusion protein of glutathione S-transferase and the carboxy-terminal 115 amino acids of GRK4a (the long form including exon XV) was constructed by inserting the amplified GRK4 DNA fragment (see above) into the BamHI and EcoRI sites of the pGEX system (Pharmacia Biotech Inc.). The protein was expressed by induction of Escherichia coli strain NM522 bearing the pGEX-GRK4-CT plasmid with 500 μM isopropyl-1-thio-β-D-galactopyranoside and growth for 2 h at 30°C, and the fusion protein was purified using glutathione-Sepharose as described (5). Antiserum was raised in rabbits using this purified protein as immunogen (5).

Affinity-purified GRK4 antipeptide antibody was prepared against a peptide sequence from GRK4a that was obtained from Santa Cruz Biochemicals. The antigen peptide (IPWQEDCLTMVPSEKEV) is interrupted in the GRK4a and GRK4b sequences by the addition of exon XV, and this antibody was found to recognize only GRK4a and GRK4b (data not shown).

Immunoblotting was performed as described (5), using the crude GRK4-CT antiserum at 1:2500 dilution or affinity-purified antibody at 1 μg/ml. Briefly, protein samples were separated by SDS-PAGE on 10% acrylamide gels, transferred to nitrocellulose membranes, and blocked by incubation with 3% bovine serum albumin in phosphate-buffered saline. Primary antisera in 3% bovine serum albumin was allowed to bind for 1 h, and washed, and the membrane was incubated for 1 h with goat anti-rabbit IgG-alkaline phosphatase conjugate (Pierce) for 1 h. Avidin-alkaline phosphatase conjugate was included to detect biotinylated molecular weight standards (Bio-Rad). After washing, the membrane was developed using enhanced blue reagents (Promega).

Expression of GRK4 Forms—The full-length GRK4 variants were recombinantly expressed in SF9 cells and in Escherichia coli using individual cDNA GRK4 inserts and BaculoGold baculovirus DNA (Pharmingen). Expression of GRK4 forms in SF9 cells was induced by infection of 1.5 × 10^7 cells/ml with the appropriate recombinant baculovirus at a multiplicity of infection of 5. Cells were grown for 48 h after infection; harvested by centrifugation; and stored frozen in 20 mM HEPES, pH 7.2, 5 mM EDTA, and 20 mM NaCl supplemented with a mixture of protease inhibitors (2 μg/ml each of aprotinin, leupeptin, pepstatin, and 100 μM phenylmethylsulfonyl fluoride) (buffer A).

Palmitate Labeling and Immunoprecipitation—CHO-COS7 cells were transfected with 10 μg of GRK4 cDNAs in the pRK5 vector using lipofectamine (Life Technologies, Inc.). The cells were grown in Dulbecco’s modified Eagle’s medium containing 10% bovine serum. Cells were transfected by the calcium phosphate precipitation method (12) with 5 μg of rat LH/CG receptor cDNA and 10 μg of pRK5 vector (buffer A) in the presence of 12.5 μg of pRK5 vector or pRK5 containing individual GRK4 cDNAs. Cells were split into 12-well plates (2 × 10^5 cells/well) 1 day after transfection and labeled by incubation with 2 μCi/ml [3H]labeled palmitic acid, 200 μM [3H]myristic acid, or 100 μM [3H]sodium palmitate labeling mixture (DuPont NEN) for 24 h, beginning 24 h after transfection. Cells were harvested 48 h after transfection in radioimmunoprecipitation assay buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS), which was stored at –80°C. After immunoprecipitation with antiserum, and analyzed by SDS-PAGE as described previously for GRK6 (19). Dried gels, pretreated with Amplify (Amersham Corp.), were exposed to x-ray film for 2 months at ~80°C.

Whole Cell Desensitization Assays—HEK-293 cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% bovine serum. Cells were transfected by the calcium phosphate precipitation method (12) with 5 μg of rat LH/CG receptor cDNA (20) in the pRK5 vector, frozen in 10 ml of buffer A plus 0.1% Lubrol. Fractions containing the C-terminal 100-1000 mM NaCl in buffer A with 100 mM NaCl to reduce Lubrol to 0.1%, filtered through a 0.2-μm Nalgene filter, and passed over a Mono-Q HR 5/5 column (Pharmacia Biotech Inc.). GRK4 protein was eluted with a 100-ml gradient of 100-1000 mM NaCl in buffer A plus 0.1% Lubrol. Fractions containing immunoreactive GRK4 eluted at 400–500 mM NaCl and were pooled for use in functional assays.

Partial Purification of GRK4a—SF9 cells were collected with 100 ml of culture infected with GRK4a baculovirus, frozen in 10 ml of buffer A, thawed and lysed by 10 strokes of a Dounce homogenizer. Crude lysates were spun at 1000 × g to separate unlysed cells and nuclei. The lysate was supplemented with deionized Lubrol PX to 1% (w/v) and NaCl to 100 mM, and loaded for 1 h at 4°C. The lysate was spun at 100,000 × g for 1 h, and the supernatant was resolved by gel filtration on a 10-ml column of Sephacryl S-200 in buffer A with 100 mM NaCl to reduce Lubrol to 0.1%. Fractions were pooled and measured using the radioimmunoprecipitation assay described above for GRK6 (19).

Receptor Phosphorylation Assay—The ability of partially purified GRK4 to phosphorylate purified β2-adrenergic receptor in phospholipid vesicles was assessed as described previously for GRK5 (5). Briefly, tests were performed in a 25-μl total volume containing 100 μM [γ-32P]ATP (500–2000 dpm/μmol), 1 pmol of β2-adrenergic receptor in phospholipid vesicles, 20 mM Tris, pH 7.5, 2 mM EDTA, 100 mM MgCl2, 1 mM dihydrothreitol, and 10 μg/ml protein kinase A inhibitor (14–21-amide peptide). Assays were incubated for 10 min at 30°C after addition of freshly prepared (+)-isoproterenol or (-)-propranolol (each at 100 μM). Assays

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were stopped by addition of Laemmli sample buffer, and products were separated on 10% SDS-polyacrylamide gels (12). Gels were fixed, stained, and dried for autoradiography using x-ray film.

Human β₂-adrenergic receptors were purified from baculovirus-infected S19 cells by alphenol affinity chromatography and reconstituted into phospholipid vesicles as described (23). Receptors were reconstituted in vesicles of 100% phosphatidylcholine or in vesicles of 5% phosphatidylinositol 4,5-bisphosphate (PIP₂) and 95% phosphatidycholine. Lipids were all 99% pure grade from Sigma.

RESULTS

Splice Variants of GRK4—Comparison of the deduced amino acid sequence of human GRK4 (4) with that of bovine GRK5 (5) and other G protein-coupled receptor kinases indicated the absence of two stretches of residues in GRK4 that are conserved in other members of this family of kinases. One apparent gap of 32 amino acid residues occurred after Gln17 in the amino-terminal domain of GRK4, while the second gap of 46 amino acids occurred after Glu515 in the carboxyl-terminal domain (numbering based on the longest (GRK4α) sequence reported here). To determine whether these gaps were present in the mRNA encoding GRK4, PCR amplification across both of these regions was performed using cDNA prepared from human testis poly(A) RNA from two patient samples. For both cDNA samples, each of the amino- and carboxyl-terminal reactions yielded product bands of sizes consistent with the reported sequence (containing the apparent gaps), as well as larger bands consistent with a sequence having these gaps filled (data not shown). Surprisingly, the longer products were more abundant in all reactions.

All product bands were subcloned and sequenced. Comparison of the DNA sequences of the short and long PCR-amplified clones indicated that the longer clones were analogous to the shorter clones and to the previously reported GRK4 sequence, with additional nucleotide sequences occurring precisely at the predicted gaps, for each of the amino- and carboxyl-terminal products. These insertions each maintained the open reading frame, and deduced amino acid sequences of these insertions corresponded well to sequences found in GRK5 and GRK6 (Fig. 1). The amino-terminal insertion is identical to that reported by Sallese et al. (11).

GRK4 Genomic Structure—The presence of alternative internal sequences in the amino- and carboxyl-terminal regions of GRK4 suggested the existence of four distinct forms of GRK4 mRNA and thus of GRK4 protein. These variants could arise from alternative splicing of a single GRK4 gene transcript at two distinct sites. To determine whether these inserted sequences lie on distinct exons within the GRK4 gene, the exon/intron organization of the human GRK4 gene was determined. Gene fragments corresponding to all exons and all exon/intron junctions were sequenced directly from individual cosmid DNAs using specific radiolabeled oligonucleotide primers. The size of each intron was determined by polymerase chain reaction using pairs of primers from the flanking exons and the corresponding cosmid DNA as template. A schematic diagram of the GRK4 gene structure is shown in Fig. 2, and the precise exon/intron junction sequences are summarized in Table I. The GRK4 gene, exclusive of promoter regions, spans ~75 kb and is composed of 16 exons. The trapped exons used to identify the GRK4 gene (4) correspond to exons I and X. The amino-terminal insertion corresponds to exon II, of 96 bases (32 codons), flanked by 18- and 4.3-kb introns. The carboxyl-terminal insertion corresponds to exon XV, of 138 bases (46 codons), flanked by 825- and 2050-base pair introns. Since these observed alternative exons are appropriately located within the GRK4 gene, four forms of GRK4 mRNA result from alternative splicing of a single gene.

Four GRK4 Forms—We have called the longest form of GRK4, with both the amino- and carboxyl-terminal alternative exon sequences, GRK4α. The deduced GRK4α protein sequence, which is essentially colinear with GRK5 and GRK6, has 578 amino acids and a predicted molecular mass of 66.5 kDa. The next shorter form, lacking only the amino-terminal alternative exon (32 codons), is GRK4β (546 amino acids, 62.9 kDa), while the variant lacking only the carboxyl-terminal alternative exon (46 codons) is GRK4γ (532 amino acids, 61.2 kDa). GRK4γ is identical to the splice variant described by Sallese et al. (11), who called it GRK4A. The shortest variant, missing both alternative exons, is GRK4δ (500 amino acids, 57.6 kDa). This is the originally described GRK4 sequence and has been called IT11 (4) and GRK4β (11).

Several differences were noted between the sequences obtained here and the originally reported sequence (4), and several polymorphisms were noted within the samples analyzed here. All numbering refers to the longest (GRK4α) form reported here. Gly562 was observed in all cDNA clones from two patient cDNA samples, in amplified genomic fragments from

![Fig. 1. Human GRK4 alternative exons.](http://www.jbc.org/)

![Fig. 2. Organization of the human GRK4 gene.](http://www.jbc.org/)
an independent patient, and in cosmid BJ56, rather than Asp as described previously. The original IT11A clone (4) was resequenced in this region and found to encode Asp562 as reported. In one patient cDNA sample, all clones contained Leu61, Val142 instead of Arg61, Ala142, which was found in all other cDNA and genomic DNA samples. Cosmid L142C5 encodes Ile247 instead of Val. Cosmid BJ56 carries a silent C\(^3\)T polymorphism within Ser519. Both cDNA samples, one genomic DNA sample, and cosmid BJ56 all encode Ala486, while cosmid BJ56w4-3 encodes Val486, the one previously reported polymorphism in the human GRK4 sequence (4).

**GRK4 mRNA Distribution**—Based on PCR/Northern analysis of human cDNA libraries, GRK4 mRNA was reported to have a widespread, but low level, distribution in human tissues (4, 11). The original IT11A clone was obtained from a human brain cDNA library (4). However, Northern analysis of six baboon tissue mRNAs indicated a significant level of GRK4 mRNA only in intestine (4). To more fully characterize the pattern of GRK4 mRNA expression in man, Northern analysis was performed using the IT11A clone (4) as a probe, a 2.5-kb band was observed only in human testis poly(A) RNA (Fig. 3A). An additional weak band was evident at 4.5 kb in brain and skeletal muscle mRNAs. This 4.5-kb band is distinct from those of all known GRK4-related GRKs and may represent an additional GRK4-like family member or alternatively processed GRK4 mRNA. Thus, GRK4 mRNA is highly abundant in testis, in agreement with the distribution and message size in baboon (4).

**Expression of Four Forms of GRK4**—The four variants of the GRK4 cDNA were each reconstructed for expression studies. An antisera prepared to the last 115 amino acids of GRK4 was used to assess the expression and subcellular localization of the four GRK4 proteins. It has been reported that GRK5 and GRK6 proteins are associated with cell membranes (5, 19), in contrast to the cytosolic localization of GRK1, GRK2, and GRK3 (1, 2). Lysates from GRK4 baculovirus-infected Sf9 cells were separated into cytosolic and particulate fractions, and equivalent volumes were separated by SDS-PAGE. Anti-GRK4 antisera detected each GRK4 protein form, while no immunoreactivity was apparent in uninfected Sf9 cells (Fig. 4). For all forms, substantially more immunoreactivity was seen in the membrane fraction than in the soluble fraction. The sizes of the four recombinant GRK4 proteins were in accord with their predicted molecular masses, from 58 to 66 kDa. Similar sizes and membrane association were seen in transfected COS cells (data not shown). Thus, GRK4, like GRK5 and GRK6, appears to have significant basal membrane association.

**Palmitoylation of GRK4 Proteins**—It has recently been demonstrated that GRK6 is palmitoylated on carboxy-terminal cysteine residue(s) and that this lipid modification is important for the membrane localization of the GRK6 protein (19). GRK5, GRK2, and GRK1 are not palmitoylated (19). The ability of GRK4 proteins to incorporate \[^{3}H\]palmitate was determined, as GRK4 shares one carboxy-terminal cysteine residue with GRK6 (but is not found in GRK5) and ends with a unique carboxy-terminal cysteine residue. COS cells expressing the individual GRK4 forms were labeled with \[^{3}H\]palmitate or with a mixture of \[^{35}S\]cysteine and \[^{35}S\]methionine. GRK4 proteins from labeled cells were immunoprecipitated using anti-GRK4 antiserum and separated by SDS-PAGE. A fluorograph is shown in Fig. 5. Like GRK6, all four GRK4 proteins incorporated \[^{3}H\]palmitate. GRK4 palmitoylation is likely to play a role in the membrane association of the kinase, as has

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**Table I**

| Exon/intron functions | exon size | Intronic Name Size(bp) |
|-----------------------|-----------|-----------------------|
| 5'UTR...3'UTR         |           | A 18000               |
| 5'UTR...3'UTR         |           | B 4300                |
| 5'UTR...3'UTR         |           | C 3500                |
| 5'UTR...3'UTR         |           | D 12000               |
| 5'UTR...3'UTR         |           | E 3600                |
| 5'UTR...3'UTR         |           | F 1800                |
been shown for GRK6 (19). 2

Functional Receptor Desensitization by GRK4 Forms—To assess the functional ability of the GRK4 variants to desensitize G protein-coupled receptors, we chose to examine the effects of GRK4 on potentially colocalized receptors. Using immunoblotting to identify the native GRK4 protein forms in testis and mature sperm has been unsuccessful due to a prominent nonspecific band of 65 kDa in all rabbit preimmune and immune sera tested (data not shown). Mature sperm did not appear to contain large amounts of GRK4 protein, although substantial GRK3 was detected, in agreement with a previous report (24). In the absence of finer localization of GRK4 expression within testis, it seemed reasonable to expect expression in Leydig and/or Sertoli cells, where the enzyme would have access to either the LH/CG or follicle-stimulating hormone receptors, respectively, as potential substrates.

We therefore chose to examine the ability of GRK4 proteins to modulate signaling by the rat LH/CG receptor transiently expressed in HEK293 cells. Individual GRKs were cotransfected with LH/CG receptor cDNA, and cAMP accumulation was assessed after stimulation of [3H]adenine-labeled cells with human CG (Fig. 6). In the absence of added GRK cDNAs, human CG produces a dose-dependent cAMP accumulation (data not shown). In the presence of GRK2 cDNA, a GRK that is known to be active on many G protein-coupled receptors, human CG-induced cAMP accumulation is reduced by 38% at 200 ng/ml human CG. This loss of stimulation reflects GRK-mediated processes occurring in the continued presence of the hormone, which occur naturally but are augmented in the presence of exogenous GRK2 (9, 25). Transfected GRK4a produces a degree of desensitization (36% decrease at 200 ng/ml human CG) that is comparable to that achieved by transfected GRK2. Similar results were observed at 50 ng/ml human CG (data not shown).

Individual GRK4 variant cDNAs were each cotransfected with LH/CG receptor cDNA, and cAMP accumulation was assessed after stimulation of [3H]adenine-labeled cells with human CG (Fig. 6). In the absence of added GRK cDNAs, human CG produces a dose-dependent cAMP accumulation (data not shown). In the presence of GRK2 cDNA, a GRK that is known to be active on many G protein-coupled receptors, human CG-induced cAMP accumulation is reduced by 38% at 200 ng/ml human CG. This loss of stimulation reflects GRK-mediated processes occurring in the continued presence of the hormone, which occur naturally but are augmented in the presence of exogenous GRK2 (9, 25). Transfected GRK4a induces a degree of desensitization (36% decrease at 200 ng/ml human CG) that is comparable to that achieved by transfected GRK2. Similar results were observed at 50 ng/ml human CG (data not shown).

2 R. H. Stoffel and R. J. Lefkowitz, unpublished observations.
GRK4a, GRK4b, and GRK4g, although GRK4g did not appear significantly different from the control at this concentration (data not shown). Thus, all four GRK4 variants appear capable of decreasing signaling through the G protein-coupled LH/CG receptor.

GRK4 Kinase Activity in Vitro—Extracts from cells expressing GRK4 proteins showed no activity above background for the agonist-dependent phosphorylation of the G protein-coupled receptor rhodopsin or β2-adrenergic receptor (data not shown and Ref. 11). This is similar to the weak activity of GRK6, but is in contrast to the readily detectable activity of GRK1, GRK2, GRK3, and GRK5. To circumvent this difficulty, the activity of recombinant GRK4a was assessed after partial purification from baculovirus-infected Sf9 cells. The high association of expressed GRK4 with the particulate fraction prompted the use of detergent to remove enzyme from the membrane. Crude cell lysates were extracted with Lubrol, and the extract was passed over a Mono-Q column to purify and concentrate the GRK4 protein. The activity of the eluted GRK4a protein was assessed using purified β2-adrenergic receptor as substrate (Fig. 7). As the lipid composition of vesicles has recently been shown to have a great effect on the activity of several GRKs (23, 26, 27), GRK4a was tested using the ω2-adrenergic receptor reconstituted into vesicles containing 100% phosphatidylcholine as well as 95% phosphatidylinositol and 5% PIP2. Using purified receptor reconstituted into phosphatidylcholine vesicles, GRK4a was unable to phosphorylate the β2-adrenergic receptor in the presence of isoproterenol or propranolol. Given receptor vesicles containing PIP2, GRK4a phosphorylated the β2-adrenergic receptor in the presence of isoproterenol, but not in the presence of propranolol. This agonist-dependent receptor phosphorylation is the hallmark of the GRKs and indicates that GRK4 is indeed an active GRK enzyme. Like other GRKs (23), GRK4 exhibits a requirement for PIP2 in membranes containing substrate receptors. It is also apparent that GRK4 does not undergo autophosphorylation, despite the presence of a serine residue cognate to the autophosphorylation site mapped in GRK1 and GRK5 (5, 28).

DISCUSSION

Characterization of the genomic organization of the human GRK4 gene verified the existence of two alternatively spliced exons identified by polymerase chain reaction amplification of the GRK4 cDNA. The GRK4 gene is composed of 16 exons separated by 15 introns and spans over 75 kb of chromosomal DNA. Except for the first intron, which is 16–20 kb long, the GRK4 introns average 4 kb in length. In the human GRK2 gene, 21 exons are found over only 23 kb since all introns except the first average only 500 base pairs (29). Partial characterization of the mouse GRK3 gene reveals an organization identical

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3 J. A. Pitcher and R. J. Lefkowitz, unpublished observations.
demonstrates that the precursors of the cloning of GRK cDNAs highly similar to GRK2 (dGPRK1) and GRK4 (dGPRK2) from Drosophila melanogaster clearly demonstrates that the precursors of the GRK2 and GRK4 genes diverged before insects diverged from the lineage leading to chordates. GRK2 and GRK4 may be seen as models for their two subfamilies of GRKs, and it seems probable that the organization of the genes for the GRK4-related GRK5 and GRK6 enzymes will be more similar to that of the GRK4 gene. GRK1 (rhodopsin kinase) is intermediate in similarity between GRK2 and GRK4, so it will be interesting to compare the structure of the GRK1 gene with those of GRK2 and GRK4.

The GRK4 gene transcript undergoes alternative splicing to yield four distinct forms of GRK4 mRNA. This occurrence of alternative splicing is unique among the known GRK enzymes. The four GRK4 mRNAs arise from the presence or absence of exon 11 and/or exon XV. These alternative exons maintain the open reading frame and encode four GRK4 proteins that differ in the presence or absence of 32 amino acids in the amino-terminal region (11) or 46 amino acids in the carboxy-terminal region. The locations of these alternative exons within the GRK4 protein suggest that they may play important functional roles in regulation of the enzyme, while the catalytic domains of all GRK4 forms are identical.

In this work, we have demonstrated that GRK4 is an active protein kinase with the activated receptor substrate recognition expected of a GRK enzyme. GRK4α phosphorylated purified β2-adrenergic receptor in the presence of isoproterenol, but only using vesicles that contained PIP2. GRK4 appeared essentially inactive at phosphorylating β2-adrenergic receptors reconstituted into pure phosphatidylincholine vesicles. GRK5 autophosphorylation (26) and activity, GRK6 activity, and GRK2 activity (23, 27) all exhibit similar lipid cofactor requirements. PIP2-dependent activity appears to be an under-appreciated common feature of GRKs, although its physiological significance remains unexplored. The previous inability to measure the activity of GRK4-α (11) may be due to use of a soluble cell fraction rather than detergent-extracted proteins, use of rhodopsin as a substrate, or lipid composition of the rod outer segment membranes.

The carboxy-terminal domain of GRKs is involved in their subcellular localization. For GRK1 (rhodopsin kinase), the primary amino acid sequence encodes a CAAX box that directs post-translational farnesylation, proteolysis, and carboxy-methylation (30). GRK1 is a cytosolic enzyme in the rod outer segment, but translocates to the membrane upon light activation of rhodopsin. GRK1 mutant that lacks a CAAX motif fails to be prenylated and exhibits a deficit in translocation (31). GRK2 and GRK3 also appear cytosolic in unstimulated cells, but translocate to the cell membrane upon agonist activation of G protein-coupled receptors (31). This translocation is due to association of GRK2 with free G protein βγ-subunits and PIP2 through a carboxy-terminal region that includes a pleckstrin homology domain (23, 32, 33). Removal of this carboxy-terminal region renders GRK2 unable to translocate to activated receptors (33). GRK4 contains neither a CAAX motif for protein prenylation nor a G protein βγ-subunit-binding domain. GRK5 and GRK6 also lack prenylation or βγ-subunit binding, but exhibit a significant degree of association with cellular membranes. GRK5 has a highly basic carboxy-terminal sequence, which has been proposed to serve as anchor for GRK5 to phospholipids in the membrane (5). GRK4 also has a basic carboxy-terminal sequence, which is present only in the two variants containing exon XV (GRK4αx and GRK4γx). The absence of this basic domain in GRK4β and GRK4δ did not lead to striking losses in the apparent membrane association of these proteins. The carboxy-terminal region of GRK6 has no basic region, but contains cysteine residues that have been identified as sites for the post-translational palmitoylation (19). Palmitoylated GRK6 is found only associated with cellular membranes (19). All four GRK4 proteins incorporate palmitate, presumably via their carboxy-terminal cysteine residue or on an internal cysteine residue conserved with GRK6. While the functional role of GRK4 and GRK6 palmitoylation remains to be explored, this lipid clearly contributes to membrane localization of the proteins. In addition, palmitoylation and de-palmitoylation are dynamic processes within the cell, so it is possible that palmitoylation of GRK4 and GRK6 may be regulated in a signal-dependent manner. Regulated palmitoylation and de-palmitoylation have been observed for other signal transduction components (34, 35).

Although the amino-terminal domain of the GRKs is thought to be involved in recognition by the kinase of the activated G protein-coupled receptor, much less is known about the role of this region of the kinases (1, 2). An antipeptide antibody raised to the GRK1 amino terminus has been shown to block recognition of activated rhodopsin (36). It remains possible that the amino-terminal splice variants of GRK4 differ in their receptor substrate preferences or activation-dependent recognition requirements. Testing these possibilities will require comparing the activity of GRK4 variants to recognize and phosphorylate several distinct receptor substrates.

Comparison of the ability of the four GRK splice variant proteins to augment desensitization of the LH/CG receptor in transfected cells revealed that all GRK4 forms are functional. The GRK4β, GRK4δ, and GRK4γ variants appeared as active as GRK2, while the GRK4γ variant appeared weaker. This assay may be insensitive to subtle differences among the kinases, as the expression level of the various enzymes was not directly quantified. However, all of the GRK4 forms appear to have some activity, so the alternative splicing does not render any forms completely inactive. More quantitative comparisons among the four variants will require purification of all four enzymes to equivalent extents for assay against various substrate receptors.

The relative abundance of the four native GRK4 proteins is unknown. In the amplification of the GRK4 amino- and carboxy-terminal regions, the longer variant cDNA bands were more abundant, suggesting a predominance of exon 11- and exon XV-containing mRNAs. This is in contrast to the report of Sallesse et al. (11), who observed that the short amino-terminal form of GRK4 was most common in brain cDNA. Direct immunohistochemical detection of GRK4 proteins in testis has proven unsuccessful, although specific affinity-purified antibodies should allow this question to be addressed. Affinity-purified antibodies raised against a GRK4β carboxy-terminal peptide obtained commercially also failed to recognize native GRK4y and GRK4δ proteins in testis membranes or cytosol (data not shown), supporting the hypothesis that the longest (GRK4α) form may be the most abundant form in testis.

It is noteworthy that GRK4 mRNA is essentially limited to testis. Very low levels of GRK4 mRNA are present in several other tissues, including brain (4, 11). Of the known GRKs, only
the retinal GRK1 has such a limited tissue distribution (37) and thus a defined native substrate receptor, rhodopsin. The widespread and generally overlapping distributions of GRK2, GRK3, GRK5, and GRK6 hamper efforts to understand their individual functions. Little is yet known about the receptor substrate preferences of GRKs, although certain receptors can be phosphorylated equivalently by many GRKs (8, 9, 38). The limited distribution of GRK4 may facilitate definition of the G protein-coupled receptors it regulates. Although it is not yet known which of the three main testis cell types express GRK4, several receptors are known to be present in each cell type (39). Leydig cells express LH/CG receptor and gonadotropin-releasing hormone receptor. Sertoli cells contain follicle-stimulating hormone receptor and glucagon receptor. The various germ cell stages express bombesin BB₃ receptors and a variety of olfactory-like receptors. In addition, several G protein-coupled receptor mRNAs are expressed at high levels in testis, although their cellular localizations have not been defined. These include the adenosine A₂ receptor, cannabinoid CB₁ receptor, and vasoressin V₁a receptor, as well as several “orphan” receptors. All of these receptors are potential substrates for GRK4. Further studies characterizing the function of GRK4 will focus on regulation of those receptors expressed in the testis cell type(s) determined to contain GRK4. Differential regulatory properties of the individual GRK4 variants may be more observable on those receptors to which the enzymes normally have access.

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Characterization of the G Protein-coupled Receptor Kinase GRK4: IDENTIFICATION OF FOUR SPLICE VARIANTS
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