Identification, Purification, and Characterization of GRK5, a Member of the Family of G Protein-coupled Receptor Kinases*

(Received for publication, May 18, 1993, and in revised form, October 6, 1993)

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A novel member of the family of G protein-coupled receptor kinases (GRKs), named GRK5, has been cloned from bovine taste epithelium. The cDNA sequence predicts a 590-amino acid protein with high overall similarity to rhodopsin kinase. GRK5 mRNA is found most abundantly in lung, heart, retina, and lingual epithelium, but is expressed very little in brain, liver, kidney, or testis. GRK5 expressed in Sf9 cells was purified to apparent homogeneity. GRK5 major autoprophosphorylation sites were mapped to Ser** and Thr**. Purified GRK5 phosphorylates rhodopsin in a light-dependent manner and β-adrenergic receptor in an agonist-dependent manner and phosphorylates the C-terminal tail regions of both receptor proteins. GRK5 possesses neither a CAAX motif specifying protein prenylation like rhodopsin kinase nor similarity to the G protein βγ-subunit binding domain of β-adrenergic receptor kinases. GRK5 phosphorylation of rhodopsin or β-adrenergic receptor is not stimulated by G protein βγ-subunits. The GRK5 protein does not undergo agonist-dependent translocation from cytosol to membranes as do β-adrenergic receptor kinase and rhodopsin kinase, but rather appears to associate with membranes constitutively. GRK5 thus appears functionally similar to other characterized GRKs, but has distinct regulatory properties which may be important for its cellular function.

Activation of G protein-coupled receptors such as the β-adrenergic receptor (βAR)1 and rhodopsin has been shown to lead to the specific phosphorylation of the receptor by a serine/threonine protein kinase activity which recognizes only the agonist-occupied or activated conformation of the receptor as substrate (1–3). This phosphorylated receptor then interacts with an arrestin protein, which prevents the further coupling of the receptor to G proteins, and thus results in a desensitized state (4, 5).

The G protein-coupled Receptor Kinases (GRKs) which mediate this activation-dependent phosphorylation of receptors have only recently been characterized at the molecular level (6). Two widely distributed β-adrenergic receptor kinase isoforms, βARK1 and βARK2, have been cloned and shown to phosphorylate βAR (7, 8). Rhodopsin kinase has recently been cloned from the retina and shown to phosphorylate rhodopsin (9). However, neither the βARKs nor rhodopsin kinase exhibits specificity limited to the receptors for which they were named, but can phosphorylate diverse G protein-coupled receptor types (10–13), although the extent of this diversity is not yet clear. Quite recently, three additional rhodopsin kinase-related sequences have been reported. One, named IT11 (or GRK4), was cloned positionally due to proximity to the Huntington's disease locus (14). The GRK4 message is abundant mainly in testis, but the functional properties and receptor specificity of this putative kinase are not yet known (14). The GRK5 and GRK6 kinase cDNAs were recently cloned from human heart, and the expressed enzymes were shown to phosphorylate rhodopsin in a light-dependent manner (15, 16).

GRKs mediate homologous desensitization of receptors in many tissues, although particular GRKs appear to predominate in specific tissues. Rhodopsin kinase plays a role in inactivation of visual stimulation (17). Similarly, βARK2 has recently been implicated in receptor desensitization in the olfactory system (18, 19). As part of an effort to characterize G protein-coupled receptor kinases which may play a role in adaptation of taste responses, we screened taste tissue to determine which receptor kinases are present and identified an unknown rhodopsin kinase-like sequence (which has recently been cloned from human heart (15)). We report here on the cDNA sequence, tissue distribution, purification, and functional properties of this G protein-coupled receptor kinase, GRK5.

EXPERIMENTAL PROCEDURES

Polymerase Chain Reaction—Oligonucleotide primers were synthesized corresponding to two absolutely conserved stretches of G protein-coupled receptor kinase catalytic domains (7–9) which contain residues not found in most other protein kinases: G(EVK)GGFGE (5′-CACCGGCTGAGGIGGIGGGGTCG-3′) and G(YIFMAFAPV (5′-GCCTCTCGAGGACYTCIGGIGCCATRWAICC). First strand cDNA was synthesized from 1 µg of poly(A) RNA using oligo(dT) primer and SuperScript Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.) (20). cDNA from 10 ng of bovine circumvallate papillae poly(A) RNA was amplified using 500 µM primers, 200 µM concentration of each dNTP, 1.5 mM MgCl2, and 2.5 units of Taq DNA polymerase, and 1 x buffer (Promega) for 55 cycles at 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 3 min (20). The 500-base pair product was gel-purified and subcloned into pBluescript II (Stratagene) using Xhol sites in

1 The abbreviations used are: βAR, β-adrenergic receptor; βARK, β-adrenergic receptor kinase (subtypes 1 and 2); GRK, G protein-coupled receptor kinase; GRK5, rhodopsin, rhodopsin treated with endo-
protease Asp-N to remove the C-terminal 19 amino acids; ROS, rod outer segments; bp, base pair(s); kb, kilobase(s); PCR, polymerase chain reaction; HPLC, high performance liquid chromatography; PAGE, poly-
crylamide gel electrophoresis.

2 Nucleotide codes are: I = inosine, M = A or C, Y = A or T, R = A or G, and W = A or T.

* This work was supported in part by the National Institutes of Health Grant HL 16037. The costs of publication of this article were defrayed
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Vol. 269, No. 9, Issue of March 4, pp. 6832–6841, 1994

Printed in U.S.A.

The Journal of Biological Chemistry
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the primers. Clones were classified using unique restriction sites and DNA sequencing.

cDNA Cloning—Oligo(dT)- and random-primed cDNA libraries were prepared in pgt10 using the SuperScript cDNA kit (Life Technologies, Inc.), and screened by hybridization to the 500-bp PCR fragment using standard techniques (20). Individual virus inserts were subcloned as EcoRI/SphI or BglII/SphI fragments into pCAGGS (CLONTECH) or as BglII/SphI fragments into pMAD2 using the cohesive ends of pMAD2. PCR products were inserted into the pGEM-3Z vector (Promega) using the cohesive ends of the primer. After restriction digestion with SspI, the final construct was inserted into the BamHI and EcoRI sites of pGEX-2T vector (Pharmacia) using restriction sites built into the S' ends of the primers. The resulting pGEX-GRK5C7 plasmid was grown in NMS22 strain E. coli. Bacteria were treated with isopropyl-β-D-thiogalactopyranoside to induce expression of the glutathione S-transferase/GRK5 fusion protein, which was purified using glutathione-Sepharose (Pharmacia) as described for glutathione S-transferase/bARK C-terminal fusion proteins (23). The purified glutathione S-transferase/GRK5 fusion protein was used to immunize rabbits as previously described for bARK iso-

ymes (24).

Preparation of GST/GRK5 C-terminal Fusion Protein and Antiserum—The C-terminal fragment of GRK5 was expressed in Escherichia coli as a fusion protein with glutathione S-transferase. Briefly, oligonucleotide primers corresponding to bases 1561–1578 (encoding PPFPVF) and the inverse complement of bases 1930–1947 (encoding STGSS(stop)) were used to amplify the C-terminal fragment from the pGEX-GRK5C7 plasmid as described, but the reaction was terminated by passage through a Bio-Spin-30 column (Bio-Rad) equilibrated with 20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 10 mM MgC12, 1 mM dithiothreitol, 10 μg/ml protein kinase A inhibitor (1-24-amide peptide) for 15 min at 24 °C. Incubations also contained ATP (10 μM) and were separated by addition of Laminin sample buffer and separated on SDS-polyacrylamide gels. Gels were fixed, stained with Coomassie Blue, and dried for autoradiography with x-ray film. Incorporated disintegration/min was quantified by Cerenkov counting of protein bands cut from the dried gel.

Receptor Phosphorylation Assays—For receptor phosphorylation, enzyme was incubated as in autophosphorylation assays, except that incubations also contained urea-washed rod outer segment membranes containing rhodopsin or phospholipid vesicles reconstituted with β2-adrenergic receptor. For rhodopsin, assays were incubated at 24 °C in incubation buffer containing 100 μM MgC12, 1 mM dithiothreitol, 10 μg/ml protein kinase A inhibitor-(1-24)amide peptide, and the mixture of protease inhibitors, but not containing ATP. Incubations were performed at 24 °C for 5 min in the dark or light, and reactions were terminated at 100,000 × g for 20 min. Supernatant fractions were removed and supplemented with 25 μl of rhodopsin, while pelleted membranes were resuspended in the original volume of incubation buffer. The reaction was then continued for 10 min prior to addition of [γ-32P]ATP, and the incubations continued for 10 min in the light. Reactions were stopped with SDS-PAGE sample buffer and separated on 12% gels to determine rhodopsin phosphorylation.

Peptide Phosphorylation Assay—The BARK and rhodopsin kinase substrates were used in conjunction with a phosphopeptide REPPRPEKREF (PEP) which was purified and immunoprecipitated with the same rabbit serum used for the above procedures. Peptides were phosphorylated by in vitro incubation with the appropriate kinase. The reaction was stopped by boiling samples for 5 min, and samples were separated on 12% SDS-PAGE gels. Phosphorylated peptides were visualized by autoradiography. In addition, purified rhodopsin kinase was used to phosphorylate a synthetic peptide PPFWD-RHS (described above) and a synthetic peptide STGSS in the dark or light as indicated. For β-adrenergic receptor, assays were performed at 24 °C in incubation buffer containing 100 μM MgC12, 1 mM dithiothreitol, 10 μg/ml protein kinase A inhibitor-(1-24)amide peptide, and the mixture of protease inhibitors, but not containing ATP. Incubations were performed at 24 °C for 5 min in the dark or light, and reactions were terminated at 100,000 × g for 20 min. Supernatant fractions were removed and supplemented with 25 μl of rhodopsin, while pelleted membranes were resuspended in the original volume of incubation buffer. The reaction was then continued for 10 min prior to addition of [γ-32P]ATP, and the incubations continued for 10 min in the light. Reactions were stopped with SDS-PAGE sample buffer and separated on 12% gels to determine rhodopsin phosphorylation.

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Fig. 1. Nucleotide and deduced amino acid sequence of the GRK5 cDNA. A, schematic map of clones obtained. Positions of relevant restriction sites are shown. B, sequence of the GRK5 cDNA was determined by completely sequencing both strands of clones 7.1, 19.1, and 39.1 and portions of clones 19.2 and 35.1, as well as the original PCR fragment. No significant differences among clones were observed. Sites matching underlined.

**Results**

Degenerate primers corresponding to kinase catalytic domain sequences conserved only among known members of the G protein-coupled receptor kinase family were used to amplify a 500-bp product from bovine circumvallate papillae cDNA by PCR. Of 24 clones analyzed, 15 were PARK1 (GRK2) and 1 was PARK2 (GRK3). No rhodopsin kinase (GRK1) clones were obtained, but 8 clones encoding a novel rhodopsin kinase-like sequence were obtained. A screen of 2 x 10^6 recombinant viruses of an unamplified circumvallate cDNA library using this 500-bp fragment as a probe led to the identification of 5 independent clones (Fig. 1B). The assignment of the first ATG codon as the translation start was determined by completely sequencing both strands of clones 7.1, 19.1, and 39.1 and portions of clones 19.2 and 35.1, as well as the original PCR fragment. No significant differences among clones were observed. Sites matching underlined.
initiator methionine is based on the high degree of similarity of the deduced amino acid sequence of the N-terminal domain to those of other receptor kinases and the presence of an upstream, in-frame stop codon. The open reading frame encodes a 590-amino acid protein with a predicted molecular mass of 68 kDa. The central protein kinase catalytic domain contains several features characteristic of the G protein-coupled receptor kinase subfamily, including the sequence DLG in the kinase subdomain VII (9, 31), and is highly similar to the catalytic domains of rhodopsin kinase (57% identity, 78% similarity) and the recently identified GRK4 or IT11 kinase (79% identity, 91% similarity) (9, 14). The 138-amino acid C-terminal domain is short like those of rhodopsin kinase and GRK4 kinase, but contains neither a CAAX motif specifying protein prenylation like rhodopsin kinase (32) nor any similarity to the G protein βγ-subunit binding region identified in βARK1 and βARK2 (23).

Comparison of the GRK5 deduced protein sequence with those of the previously identified mammalian G protein-coupled receptor kinases indicates that this sequence is the bovine homolog of the recently identified human GRK5 receptor kinase (99% similarity) (15). GRK5 is closely related to the GRK4 kinase (80.7% similarity) (14) and to rhodopsin kinase (68.4% similarity) (9), but shares only 58.6% similarity with βARK1 and βARK2 (7, 8). An additional receptor kinase recently identified in human neutrophils and human heart, termed GRK6 (16, 33), is also highly similar to the GRK5 sequence (83.6% similarity). These sequence differences can thus be grouped into three distinct subfamilies: the GRK5 receptor kinase, together with the GRK4 and GRK6 kinases, form one subfamily, while βARK1 and βARK2 form a second subfamily. Rhodopsin kinase is more closely related to the GRK4/GRK5/GRK6 subfamily than to the two βARKs, but in light of its limited tissue distribution as well as its distinct mechanism of regulation and intracellular localization involving C-terminal farnesylation and carboxymethylation, rhodopsin kinase is best considered as a distinct subfamily. Of the known nonmammalian receptor kinases, the Drosophila GPRK2 sequence is a member of the GRK4/GRK5/GRK6 subfamily and shares 79.6% similarity with the GRK5 sequence, while the Drosophila GPRK1 sequence (57.2% similarity) encodes a member of the βARK subfamily (34).

The GRK5 receptor kinase PCR fragment was obtained as part of an effort to identify taste-specific G protein-coupled receptor kinases. To ascertain whether GRK5 might represent such an enzyme, its mRNA distribution was determined by Northern blotting (Fig. 2). Equivalently high mRNA levels were observed in both taste (circumvallate and fungiform papillae) and adjacent non-taste lingual epithelium, indicating that expression of the GRK5 receptor kinase is not unique to taste tissues nor apparently enriched there. Highest expression was evident in lung, heart, and retina, while little or no expression was detected in brain cortex, kidney, liver, and testis. Two messages, a major band of 2.8 kb and a minor species of 7 kb, were observed in all cases, perhaps due to the use of alternative polyadenylation signals. This distribution is in agreement with the human GRK5 mRNA distribution, which was also found to be high in skeletal muscle and placenta (15).

To examine the functional properties of the GRK5 kinase, the cDNA was expressed in Sf9 cells from the strong baculovirus polyhedrin promoter. Cell supernatants from infected cells exhibited a markedly increased ability to phosphorylate rhodopsin in rod outer segment membranes in the presence but not absence of light (data not shown; 15). Therefore, the expressed GRK5 enzyme was subjected to protein purification according to protocols devised for the purification of βARK enzymes. Sf9 cell cytosolic extracts were sequentially chromatographed on S-Sepharose and on heparin-Sepharose to yield a 400-fold enrichment of the GRK5 kinase. This preparation is substantially (>80%) pure as assessed by Coomassie Blue staining of SDS gels. One μg each of purified preparations of recombinant bovine GRK5, rhodopsin kinase, and βARK1 purified from baculovirus-infected Sf9 cells, separated by SDS-PAGE and stained with Coomassie Blue, are shown in Fig. 3A.

To verify that this purified protein represents the GRK5 kinase, a gel containing 100 ng of each of these three GRK proteins was immunoblotted using an anti-GRK5 antibody (Fig. 3B). This polyclonal serum was raised against a glutathione S-transferase fusion protein containing the C-terminal 128 residues of GRK5. Based on sequence similarities, this serum is predicted to also recognize GRK4 and GRK6 proteins, but not rhodopsin kinase or the two βARKs. The antibodies readily
detect the purified recombinant GRK5 protein, but fail to recognize equivalent amounts of purified βARK1 or rhodopsin kinase proteins.

The ability of the purified GRK5 enzyme to phosphorylate rhodopsin in rod outer segment membranes was characterized. Phosphorylation reactions were incubated in the light for various times to determine the linearity of the enzymatic activity. Rhodopsin phosphorylation by GRK5 appears linear over 20 min (Fig. 4A), while kinase autophosphorylation is maximal within 5 min (not shown). No phosphorylation of rhodopsin occurs when incubations are performed in the dark, where rhodopsin is in the inactive state. This receptor activation dependence of kinase activity toward receptor substrates is a hallmark of the G protein-coupled receptor kinases. Incubation of the rod outer segment preparation without added GRK5 leads to virtually no rhodopsin phosphorylation, indicating the absence of endogenous rhodopsin kinase or other GRKs in the membrane preparations (not shown).

The ability of the GRK5 kinase to interact with light-activated rhodopsin was measured by determining the $K_{m}$ of GRK5 for rhodopsin phosphorylation. Various concentrations of rod outer segment membranes were incubated with purified GRK5 in the light, and rhodopsin phosphorylation was quantified (Fig. 4B). GRK5 was determined to have a $K_{m}$ value of 2.2 ± 0.7 μM for rhodopsin. This is close to the values estimated for rhodopsin kinase (0.6-3.0 μM) (38, 37) and for βARK1 (4.0-6.0 μM) (38, 39).

Both βARK1 and rhodopsin kinase have been shown to be inhibited by polyamines such as heparin (40, 41). The ability of heparin to inhibit GRK5 kinase phosphorylation of rhodopsin was assessed (Fig. 4C). Heparin inhibited the light-dependent phosphorylation of rhodopsin by GRK5 with an $IC_{50}$ of 46 ± 10 μg/ml. This is intermediate between the heparin $IC_{50}$ for βARK1 (770 ng/ml) and for rhodopsin kinase (1 mg/ml) (40, 41).

The βARK1 and βARK2 enzymes have been shown to be activated by heterotrimeric G protein βγ-subunits, due to βγ-binding to a domain in the C-terminal tail of the kinase and concomitant localization of the kinase to the membrane (28, 42). Rhodopsin kinase, which lacks this βγ-binding domain, is not activated by βγ-subunits (26). Purified GRK5 and βARK1 were assayed for their ability to phosphorylate rhodopsin in the absence or presence of βγ-subunits purified from bovine brain or from bovine retina (Fig. 5). Rhodopsin phosphorylation by

**Fig. 3. Identification of purified recombinant GRK5.** A, 1 μg each of purified SF9 cell-expressed bovine GRK5 (lane 1), rhodopsin kinase (lane 2), and βARK1 (lane 3) were separated on a 10% SDS-PAGE gel and stained with Coomassie Blue. Bio-Rad prestained molecular weight standards shown are phosphorylase b (106,000), bovine serum albumin (80,000), ovalbumin (49,500), carbonic anhydrase (32,500), soybean trypsin inhibitor (27,500), and lysozyme (18,500). B, 100 ng of bovine GRK5 (lane 4), rhodopsin kinase (lane 5), and βARK1 (lane 6) were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose. The blot was incubated with anti-GRK5 C-terminal antibody at a 1:2500 dilution in 3% bovine serum albumin and Western Blue substrate (Promega).

**Fig. 4. Rhodopsin phosphorylation by GRK5.** A, time course of rhodopsin phosphorylation by GRK5. Purified GRK5 (1 pmol) was incubated with 25 pmol of rhodopsin for the indicated times at 24 °C in the light (open squares) or for 30 min in the dark (closed square). Reactions were separated by SDS-PAGE on a 10% gel and dried for autoradiography. Radioactivity in excised rhodopsin bands is plotted versus incubation time for the autoradiogram shown. B, rhodopsin concentration dependence of GRK5 phosphorylation. Purified GRK5 (1 pmol) was incubated with the indicated concentration of rhodopsin for 15 min at 24 °C in the light. Reactions were separated by SDS-PAGE on a 10% gel and dried for autoradiography. Rhodopsin bands were excised from the dried gel, and radioactivity was quantified by Cerenkov counting. $K_{m}$ values were derived from double-reciprocal plots of data. C, heparin inhibition of rhodopsin phosphorylation by GRK5. Purified GRK5 (1 pmol) was incubated with 25 pmol of rhodopsin for 15 min at 24 °C in the light in the presence of the indicated concentration of heparin. Reactions were separated by SDS-PAGE on a 10% gel and
**Fig. 5. Effects of G protein βγ-subunits on the rhodopsin phosphorylating activity of GRK5 and βARK1.** Purified GRK5 or βARK1 (3 pmol) were incubated with 25 pmol of rhodopsin for 10 min at 24°C in the light or dark in the absence (-) or presence (+) of 100 nm brain G protein (G$_{βγ}$) or retinal (T$_{βγ}$) βγ-subunits as indicated. Reactions were separated by SDS-PAGE on a 10% gel and dried for autoradiography. Phosphorylated rhodopsin bands are shown.

βARK1 is markedly enhanced by added brain βγ-subunits and less stimulated by transducin βγ-subunits as previously demonstrated (28), but rhodopsin phosphorylation by GRK5 is not significantly altered by either form of βγ-subunits. In our studies, GRK5 and βARK1 have nearly an equivalent ability to phosphorylate rhodopsin in the absence of added βγ-subunits (60–70 nmol of phosphate/min/mg of protein), but βARK1 plus brain βγ-subunits has approximately 10-fold higher activity than GRK5.

The ability of the GRK5 kinase to phosphorylate the G$_{βγ}$-coupled β$_2$-adrenergic receptor was also assessed, using recombinant human β$_2$AR affinity-purified from Sf9 cell membranes and reconstituted into phospholipid vesicles (Fig. 6). GRK5 phosphorylates the β$_2$AR in an isoproterenol-dependent manner, indicating that GRK5 recognizes the agonist-activated form of the β$_2$AR as substrate. Further, addition of G protein βγ-subunits has no stimulatory effect on GRK5 phosphorylation of the β$_2$AR. Interestingly, GRK5 has an approximately 7-fold higher ability to phosphorylate β$_2$AR than does βARK1 in the absence of added βγ-subunits, while βARK1 plus brain βγ-subunits is approximately 2-fold better than GRK5.

Incubation of rhodopsin kinase with Mg-ATP leads to the rapid and extensive autophosphorylation of the enzyme at Ser$^{366}$ and Thr$^{489}$ (35), while the βARK enzymes are autophosphorylated only to low stoichiometry (38) and do not contain phosphorylatable serine or threonine residues in these cognate positions (7, 8). The GRK5 sequence does share Ser$^{484}$/Thr$^{485}$ in the cognate positions with rhodopsin kinase, and GRK5 extensively autophosphorylates (see Figs. 6 or 8A). To define the sites of GRK5 autophosphorylation, 3 nmol of purified enzyme was incubated with [γ$^{32}$P]ATP for 15 min, and the autophosphorylated protein was digested with endoproteinase Asp-N. Peptide fragments were separated by HPLC, yielding one major radioactive peak (67% of counts) and two minor peaks (20% and 12% of counts) (Fig. 7A). The most radioactive fraction (No. 50) was subjected to a second HPLC step with a more shallow gradient which resolved a radioactive peptide peak containing 45% of the applied counts and a broad peak of radioactivity apparently containing several peptides (Fig. 7B). This major peak (fraction 77) was subjected to amino acid sequencing and was determined to correspond to the predicted proteolytic fragment of residues 479 to 491, DIEQPSTVKGVNL. Phosphoamino acid analysis of this peptide fraction indicated that under these hydrolysis conditions, most of the $^{32}$P label was associated with phosphothreonine (70%) and only 30% with phosphoserine. The less radioactive peak (fraction 80) had a mixed sequence which included the serine-rich C-terminal 55-residue fragment. Therefore, the major autophosphorylation sites on GRK5, Ser$^{484}$/Thr$^{485}$, are conserved with those of rhodopsin kinase. Additional minor sites remain to be defined.

Phosphorylation of rhodopsin by rhodopsin kinase occurs on serine and threonine residues in the C-terminal tail of the protein (43), and enzymatic removal of the last 19 amino acids of the tail using endoproteinase Asp-N eliminates these phosphorylation sites from the remaining truncated rhodopsin fragment (27). Rod outer segment membranes were digested over-
night with endoprotease Asp-N to produce truncated \textsuperscript{G\textsubscript{329}}, rhodopsin by removal of the last 19 amino acids (27). This results in the increased mobility of the truncated rhodopsin in SDS gels. Incubation of this truncated rhodopsin with GRK5 kinase in the light does not result in any phosphorylation of the \textsuperscript{G\textsubscript{329}},rhodopsin protein (Fig. 8A). The ability of GRK5 to interact with the truncated \textsuperscript{G\textsubscript{329}},rhodopsin appears unimpaired as assessed by augmentation of peptide phosphorylation activity (see below). Alternatively, if rhodopsin previously phosphorylated by GRK5 (or rhodopsin kinase or \textsuperscript{\beta}ARK1) is then digested with endoproteinase Asp-N, the radioactivity is lost from the rhodopsin band (but not entirely lost, due to incomplete digestion) but fails to appear in the \textsuperscript{G\textsubscript{329}},rhodopsin fragment (Fig. 8B). Therefore, phosphorylation of rhodopsin by GRK5, \textsuperscript{\beta}ARK1, or rhodopsin kinase occurs predominantly if not entirely within the C-terminal 19 amino acids of the opsin protein.

Phosphorylation of the \textsuperscript{\beta}2AR by \textsuperscript{\beta}ARK1 occurs on the C-terminal tail of the receptor, since enzymatic removal of the tail (up to membrane span 7) using carboxypeptidase Y eliminates phosphorylation (44), while mutagenesis of the tail region to remove potential phosphorylation sites reduces the ability of the receptor to undergo homologous desensitization (45). If \textsuperscript{\beta}2AR previously phosphorylated by GRK5 (or rhodopsin kinase or \textsuperscript{\beta}ARK1) is then digested with carboxypeptidase Y, the radioactivity is lost from the remaining receptor fragment (Fig. 8C), indicating that phosphorylation by GRK5, \textsuperscript{\beta}ARK1, or rhodopsin kinase occurs predominantly or entirely on the C-terminal tail of the \textsuperscript{\beta}2-adrenergic receptor. Thus, for two distinct receptor proteins, each of these three kinases appears to phosphorylate the same or overlapping sets of serine and threonine residues.

The ability of both \textsuperscript{\beta}ARK1 and rhodopsin kinase to phosphorylate a series of synthetic peptide substrates has been characterized (27, 30, 46). The peptide RRREEEEEEAAA has been shown to be substrate for both \textsuperscript{\beta}ARK1 and rhodopsin kinase (30, 46). Phosphorylation of this peptide by GRK5 was linear only over 20–25 min (not shown), in contrast to PARK1 and rhodopsin kinase which give linear activity over at least 2 h (30). The GRK5 enzyme was incubated for 15 min with various concentrations of the RRREEEEEEAAA peptide to characterize its phosphorylation (Fig. 9A). GRK5 was determined to have a \( K_m \) of 1.4 ± 0.5 mM for this peptide with a calculated \( V_{max} \) of 3 nmol of phosphate/min/mg of protein for this enzyme preparation. These values are intermediate between those previously determined for \textsuperscript{\beta}ARK1 and rhodopsin kinase, with \( K_m \) values of 1 mM and 2 mM, respectively (30).

Peptide substrates for G protein-coupled receptor kinases are generally quite poor (having about 1000-fold lower \( K_m \) than activated receptor). However, the peptide phosphorylation activity of \textsuperscript{\beta}ARK1 and rhodopsin kinase can be augmented by the presence of activated (but not inactive) receptor in the assay (27, 46), since binding of the kinase to an activated receptor appears to stimulate the kinase catalytic activity. Assays containing 1 mM RRREEEEEEAAA peptide were incubated in the presence of rhodopsin in the light or in the dark to determine whether binding to rhodopsin stimulates the peptide phosphoryltransferase activity of the GRK5 enzyme (Fig. 9B). Rod outer segment membranes containing native rhodopsin as well as ROS membranes containing endoproteinase Asp-N-treated rhodopsin were tested. Compared to the absence of rhodopsin, addition of rhodopsin or truncated \textsuperscript{G\textsubscript{329}},rhodopsin stimulated the phosphorylation of the peptide 2–3-fold when incubations were performed in the light. In the dark, addition of either rhodopsin preparation had no effect. Thus, like rhodopsin kinase and \textsuperscript{\beta}ARK, the GRK5 enzyme appears to undergo an activation step upon binding to an activated receptor, which is
varying concentrations of the peptide RRREEEEESAAA, from 0.125 to 4 mM. Assays were terminated by spotting triplicate 25-μl aliquots onto Whatman P81 filters and immediately washing in phosphoric acid.

GRK5. Purified GRK5 (1 pmol) was incubated with 1 pmol of rhodopsin or G<sup>329</sup>-rhodopsin for 15 min at 30°C in the light or dark, as indicated. This experiment was repeated twice.

| Light | S | M |
|-------|---|---|
| ++    |   |   |
| +     |   |   |
| -     |   |   |
| --    |   |   |
| +++   |   |   |

**Fig. 10. Association of GRK5 with ROS membranes.** Purified GRK5 (5 pmol) was incubated with 25 pmol of rhodopsin for 5 min in assay buffer not containing ATP in the dark or light and pelleted at 100,000 × g. Supernatant fractions (S) and pelleted ROS membranes (M) were assayed for rhodopsin phosphorylation to determine GRK5 activity.

**DISCUSSION**

**Similarities to Other GRKs**—The GRK5 kinase, like other characterized G protein-coupled receptor kinases, is capable of phosphorylating rhodopsin and β<sub>2</sub>AR, but only when the receptors are in the (light or hormone) activated state. Activated rhodopsin (or a non-substrate truncated rhodopsin) is also capable of stimulating the catalytic activity of GRK5 toward peptide substrate, presumably by binding to noncatalytic sites of the enzyme, as has been demonstrated for both rhodopsin kinase (27) and βARK1 (46). Removal of the C-terminal of rhodopsin with endoproteinase Asp-N or of the β<sub>2</sub>AR with carboxypeptidase Y eliminates GRK5 phosphorylation sites on these receptors. Whether the GRK5, rhodopsin kinase, and βARK1 sites on the C termini of these two receptor proteins are distinct, overlapping, or identical is not yet known. The similarity of the substrate sites on these two proteins, however, indicates that these three GRK enzymes may recognize the same activated conformational determinants in the receptors and may all serve equivalent cellular roles in marking the receptors for arrestin proteins.

Like rhodopsin kinase, GRK5 is extensively autophosphorylated. This autophosphorylation is quite rapid and appears unaffected by the presence of light-activated rhodopsin (Fig. 8A) or by agonist activation of the β<sub>2</sub>AR (Fig. 6, upper band). Ser<sup>484</sup> and Thr<sup>485</sup> in GRK5 are the cognate residues of the major autophosphorylation sites identified on rhodopsin kinase (27) and also appear to be the major autophosphorylation sites in GRK5. The minor rhodopsin kinase autophosphorylation site at Ser<sup>21</sup> is not conserved in the GRK5 sequence. Additional radioactive peptide fragments of GRK5, which have not yet been further characterized, may represent minor autophosphorylation sites. Autophosphorylation of rhodopsin kinase has been reported to play a functional role in regulating kinase interaction with receptors (47); whether GRK5 autophosphorylation plays a similar role is not yet known.

The relative activity of GRK5 and βARK1 against rhodopsin and β<sub>2</sub>AR have been compared. If βARK1 alone phosphorylates independent of the ability of the receptor to be a productive kinase substrate.

Previously characterized GRK enzymes undergo activated receptor-dependent "translocation" from cytosolic fractions to membranes. That is, in the presence of inactive receptors, rhodopsin kinase and βARK1 appear predominantly soluble, but, upon addition of agonist to β<sub>2</sub>AR or light to rhodopsin, the kinases appear associated with the membrane (1, 6). Membrane association of rhodopsin kinase requires the post-translational farnesylation of the kinase (26), while membrane association of the βARKs requires the additional presence of G protein βγ-subunits in the membrane (26, 28). Since GRK5 appears to neither bind βγ-subunits nor to have sequences directing post-translational protein prenylation, it was of interest to determine whether GRK5 also undergoes an activated receptor-dependent translocation from cytosol to membranes. GRK5 enzyme was added to ROS membranes in the dark or light for 5 min, and reactions were centrifuged to separate soluble from membrane fractions. The partitioning of GRK5 between these two fractions was assessed by subsequent rhodopsin phosphorylation assays (Fig. 10). In marked contrast to rhodopsin kinase and βARK1, the majority of GRK5 activity appears membrane-associated even in the absence of rhodopsin activation, and light activation fails to influence the amount of kinase activity associated with the ROS membranes. Interestingly, COS cells contain a native GRK5-like immunoreactive band which appears in the membranes but not the cytosol, but overexpression of GRK5 leads to significant accumulation of the enzyme in the cytosolic fraction (data not shown). Thus, the native GRK5 protein may be constitutively associated with the membrane and perhaps with receptors.

**Fig. 9. Peptide phosphorylation by GRK5.** A, kinetics of peptide phosphorylation by GRK5. Purified GRK5 (1 pmol) was incubated with varying concentrations of the peptide RRREEEEESAAA, from 0.125 to 4 mM. Assays were terminated by spotting triplicate 25-μl aliquots onto Whatman P81 filters and immediately washing in phosphoric acid. Specific phosphorylation was calculated as the difference with a reaction containing no peptide. Kinetic parameters were derived from double-reciprocal plots using the Sigma-plot program. B, effect of rhodopsin and G<sup>329</sup>-rhodopsin on peptide phosphorylation activity of GRK5. Purified GRK5 (1 pmol) was incubated with 1 mM RRREEEEESAAA peptide in the absence or presence of 25 pmol of rhodopsin or G<sup>329</sup>-rhodopsin for 15 min at 30°C in the light or dark, as indicated. This experiment was repeated twice.
rhodopsin with a relative activity of 1, addition of G protein βγ-subunits increases the activity to 10–20; GRK5 phosphorylates rhodopsin with a relative activity of 1. Similarly, if βARK1 alone phosphorylates β2AR with a relative activity of 1, addition of G protein βγ-subunits increases this activity to 10–20; GRK5 phosphorylates β2AR with a relative activity of 7–8. Thus, relative to βARK1 plus βγ-subunits, GRK5 is better able to phosphorylate the β2AR than rhodopsin. The lower apparent activity of GRK5 toward either substrate compared to fully (βγ) activated βARK1 may be intrinsic or may be due to the lack of some ancillary factor for GRK5 (such as βγ-subunits for βARK enzymes).

The GRK5 mRNA appears to have an extensive somatic distribution. The mRNA encoding the related GRK6 kinase is even more widely distributed (16, 33), while the GRK4 mRNA is found primarily in testes (14). Thus, various G protein-coupled receptors are expected to be natural substrates for the GRK5 enzyme and other members of this GRK subfamily. The range of potential receptor substrates has not been determined for any of the receptor kinases, but the availability of diverse cloned receptors and kinases should facilitate this mapping process. Conversely, the involvement of βARK enzymes in hormonal desensitization has been assessed using heparin as a “specific” βARK inhibitor in permeabilized cells (48), but these concentrations of heparin would also be expected to inhibit GRK5 activity. More recent work using βARK subtype-specific antibodies to inhibit the enzymes has shed light on the role of βARK2 in olfactory desensitization (18, 19). A similar approach may be useful to elucidate the role of GRK5 in hormonal desensitization in various tissues, such as heart.

Two possibilities exist for GRK specificity: either there are distinct differences in receptor substrate specificity among GRKs or GRK subfamilies or all GRKs are essentially functionally equivalent. Clearly, GRK5 can phosphorylate the β2AR, as can GRK6 (16). Both enzymes then qualify to be called “βARKs.” βARK1 activated by βγ-subunits (presumably the native circumstance) is better able to phosphorylate the β2AR than is GRK5, but not by a very great extent. Given the divergence between these two kinases, it is perhaps surprising that differences in substrate recognition domains do not produce a larger difference in phosphorylating activity toward a given receptor substrate. However, only a miniscule fraction of the known G protein-coupled receptors have been tested as GRK substrates, and distinct differences may well exist. Nevertheless, from the initial receptor phosphorylation site mapping experiments described here, GRK5 appears to phosphorylate essentially the same sites on rhodopsin and the β2AR as do βARK1 and rhodopsin kinase and would thus be expected to lead to the same desensitizing effect on receptor coupling to G proteins.

**Differences from Other GRKs**—The GRK5 enzyme, however, appears to have some aspects to its regulation which are distinctly different from those of previously characterized GRKs. Rhodopsin kinase and both βARK isozymes have been shown to be cytosolic enzymes which translocate to the membrane in response to receptor activation (by light or by hormone) (1, 20). This translocation is associated with stimulation of kinase activity, presumably by making the activated receptor substrate available to the enzyme without altering the catalytic activity of the kinase (28). Translocation of rhodopsin kinase requires the C15 farnesylation of the enzyme at its C-terminal CAAX motif (26), while translocation of both βARK isozymes is dependent on interaction of a C-terminal domain with prenylated G protein βγ-subunits released upon receptor activation of coupled G proteins (23, 42). The GRK5 sequence (and the GRK4 and GRK6 sequences) has no CAAX motif for direct prenylation, nor does it have sequences similar to the βγ binding region defined on βARK1 and βARK2 (23). The activity of GRK5 to phosphorylate rhodopsin or the β2AR is not appreciably affected by the βγ-subunits of G proteins from brain or retina, nor does the glutathione S-transferase fusion protein containing the C-terminal portion of GRK5 appear to bind to βγ-subunits.

Assays for the activated receptor-dependent translocation of GRK5 from solution to ROS membranes surprisingly demonstrate that the kinase appears to associate with the membranes constitutively, even in the absence of receptor activation. Native GRK5-like immunoreactive proteins are present in several cell lines, including COS, and these proteins are associated primarily with the membrane fractions. This pattern of agonist-independent enzyme localization to the membrane is similar to that seen with a mutant rhodopsin kinase modified with the C20 geranylerfanyl lipid rather than the native C15 farnesyl lipid (26). GRK5 also appears to have a higher basal (agonist-occupied receptor) activity than βARK1 alone (Fig. 6), but is similar to the activity of βARK1 plus βγ in the presence of propranolol. One possible explanation for this higher basal activity is that the GRK5 enzyme is already close to its substrates on the membrane, as is the “activated” βARK1-βγ complex. This implies that no other factors are likely to play a role in localizing the GRK5 enzyme to the membrane, as do βγ-subunits for the βARK enzymes. It is interesting to note that the last 46 residues of the GRK5 kinase are predominantly positively charged, hydroxyl- or amine-containing amino acids. Whether this strongly basic domain (pI = 11.3) plays a role in kinase localization is not yet known. However, the mechanisms responsible for regulation of GRK5 (and related kinases) access to membrane receptor substrates are quite distinct from the recently defined βARK and rhodopsin kinase paradigms.

**Acknowledgments**—We thank Dr. Susan Schiffman for discussions and advice on taste bud dissection, Humphrey Kendall and Carl Stone for DNA sequencing, Carl Stone and Alan Payne for generation of anti-GRK5 polyclonal sera, Grace Irons for virus and cell culture, Darrell Peptide HPLC, Judy Phelps for amino acid sequencing, and Drs. Jeff Benovic and Bodduluri Haribabu, respectively, for sharing Refs. 2. Benovic, J. L., Strasser, R. H., Caron, M. H.; 3. Kwatra, M. M., Schwinn, D. A, Schreurs, J., Blank, J. R., and 4. Wilden, W., Hall, S. W., and Kuhn, H. (1986) *Proc. Natl. Acad. Sci. U. S. A.* 83, 2797–2801; 5. 1174–1178;

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