Development of a Yeast Bioassay to Characterize G Protein-coupled Receptor Kinases

IDENTIFICATION OF AN NH$_2$-TERMINAL REGION ESSENTIAL FOR RECEPTOR PHOSPHORYLATION*

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G protein-coupled receptor kinases (GRKs) specifically bind and phosphorylate the agonist-occupied form of G protein-coupled receptors. To further characterize the mechanism of GRK/receptor interaction, we developed a yeast-based bioassay using strains engineered to functionally express the somatostatin receptor subtype 2 and exhibit agonist-dependent growth. Here, we demonstrate that agonist-promoted growth was effectively inhibited by co-expression with either wild type GRK2 or GRK5, whereas catalytically inactive forms of these kinases were without effect. In an effort to identify residues involved in receptor interaction, we generated a pool of GRK5 mutants and then utilized the bioassay to identify mutants selectively deficient in inhibiting agonist-promoted growth. This resulted in the identification of a large number of mutants, several of which were expressed, purified, and characterized in more detail. Two of the mutants, GRK5-L3Q/K113R and GRK5-T10P, were defective in receptor phosphorylation and also exhibited a partial defect in phospholipid binding and phospholipid-stimulated autophosphorylation of the kinase. In contrast, these mutants had wild type activity in phosphorylating the non-receptor substrate tubulin.

To further characterize the function of the NH$_2$-terminal region of GRK5, we generated a deletion mutant lacking residues 2–14 and found that this mutant was also severely impaired in receptor phosphorylation and phospholipid-promoted autophosphorylation. In addition, an NH$_2$-terminal 14-amino acid peptide from GRK5 selectively inhibited receptor phosphorylation by GRK5 but had minimal effect on GRK2 activity. Based on these findings, we propose a model whereby the extreme NH$_2$-terminus of GRK5 mediates phospholipid binding and is required for optimal receptor phosphorylation.

A diverse array of extracellular stimuli transduce their signals through interaction with G protein-coupled receptors (GPCRs). A critical process that occurs in most cells is the regulation of hormonal responsiveness, a phenomenon often termed desensitization. Whereas multiple mechanisms contribute to the regulation of GPCR function, G protein-coupled receptor kinases (GRKs) and arrestins play an important role in many cells (1–3). GRKs comprise a family of serine/threonine kinases that are uniquely able to associate with the agonist-occupied form of receptors (3–5). Signaling is terminated upon receptor phosphorylation and the subsequent binding of arrestins, effectively uncoupling receptor from G protein. Mammalian GRKs are classified into three subgroups according to their sequence homology: GRK1 and -7; GRK2 and -3; and GRK4, -5, and -6 (4–6). GRKs share a common structural organization that includes a moderately conserved (~20% identity) NH$_2$-terminal domain of ~185 residues, a conserved (~50% identity) central catalytic domain of ~330 residues, and a poorly conserved COOH-terminal domain of ~80–180 residues (4, 5).

An important feature of GRKs is their ability to specifically interact with activated receptors. Although the specific determinants required for this recognition are not well defined, a few studies have suggested a role for the GRK NH$_2$-terminal region in receptor interaction. For example, site-specific antibodies directed against regions of the NH$_2$ terminus of GRK1 blocked phosphorylation of light-activated rhodopsin but had no effect on phosphorylation of a peptide substrate (7). In addition, NH$_2$-terminal truncation mutants of GRK1 and GRK2 as well as point mutants targeting a conserved acidic residue in the NH$_2$ terminus resulted in a greatly decreased ability of these GRKs to phosphorylate rhodopsin, whereas activity toward a peptide substrate was unaffected (8). In addition to interaction with the receptor, previous studies implicating negatively charged phospholipids in GRK activation also have suggested a role for the NH$_2$ terminus in phospholipid association (9).

In the present study we attempted to further elucidate the role of the NH$_2$ terminus of GRKs with respect to receptor interaction. We make use of an expression system whereby analysis of receptor-GRK association was facilitated through functional co-expression of these proteins in yeast cells. *Saccharomyces cerevisiae* utilizes GPCRs as well as heterotrimeric G proteins to regulate mating between two haploid yeast cells (10–12). Mating pheromones bind to pheromone-specific GPCRs encoded by the STE2 and STE3 genes. Upon activation, these pheromone receptors promote the activation of the G protein, GPA1, which then activates a downstream signal transduction pathway. As a result, transcriptional activation of additional components of the mitogen-activated protein kinase cascade occurs, leading to cell cycle arrest. Previously, it has signaling; SST-14, somatostatin-14; SSTR2, somatostatin receptor subtype 2; β$_2$AR, β$_2$-adrenergic receptor.
been demonstrated that mutations of certain components of the yeast pheromone response pathway eliminates the characteristic growth arrest response (13). This in turn led to the development of a yeast-based bioassay using strains that were constructed to functionally express the rat somatostatin receptor subtype 2 (SSTR2) and consequently induce growth upon activation by somatostatin peptide. In our investigation, we coexpressed mammalian GRKs with the SSTR2 in yeast to determine any possible phenotypic effects of these kinases. Interestingly, our results revealed that coexpression of GRK2 or GRK5 resulted in loss of the agonist-dependent growth response observed with receptor alone. This bioassay was then used to select NH2-terminal mutants of GRK5 that are selectively deficient in inhibiting agonist-promoted growth. We demonstrate that several residues within the NH2-terminal region of GRK5 are important for phosphorylation of receptor substrates. In addition, we also demonstrate that this region plays a role in mediating the association of GRK5 with phoshopilids. Thus, our studies have identified an NH2-terminal domain of GRK5 important for receptor phosphorylation and phospholipid interaction.

**EXPERIMENTAL PROCEDURES**

**Materials**—Restriction endonucleases were from New England Biolabs and Roche Applied Science. Somatostatin-14 and α-factor peptide were from Bachem. SP-Sepharose was from Amersham Biosciences, whereas phosphatidylcholine (soybean type II-5) was from Sigma. Mouse α-inter-K (anti-GRK-4) and 5-bromo-4-chloro-3-indolyl phosphate, NBT/BCIP (Bluelight) and anti-α-factor antibodies (gift from J. Jones) were from Clontech. PCR COOH terminus were from Upstate Biotechnology. Horseradish peroxidase-conjugated anti-rabbit antibody was from Sigma. Plasmid mini-prep, PCR purification, and gel extraction kits were from Qiagen, whereas ECL reagents were from Pierce. FluGENE transfection reagent was from Roche Applied Science and [γ-32P]ATP was from Invitrogen. Escherichia coli 101-10000 DNA ligase was from Stratagene. AMOY11 (MATα ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 Ole-17) was crossed, the resulting zygotes were identified microscopically and cultured on YPD plates. The diploid cells were induced to sporulate on appropriate media and the tetrads were dissected on YPD plates. Four spore tetrads were assessed for the presence of required nutrient markers. MPY566 (MATα ura3 leu2 his3 lys2-801 ade2 trp1 can1-100) was used for further strain construction.

**Plasmid Construction**—The plasmid pMP222 was constructed by amplifying a fragment encoding the rat SSTR2 by PCR using pHJ2 as template and synthetic oligonucleotides (MP0249, TCTCAAGCTTTAAAATGGAGATGAGCTCTGAG; MP0250, TCTCAAGCTTCTGACTGGTCGGAG) that added a 5′ HindIII site followed by a yeast translation initiation site and a 3′ BglII site. The fragment was cut with HindIII and BglII and cloned into corresponding sites in pPGK (14). The translation initiation site and a 3′ terminal SST2 coding sequence were replaced with those of GRK5 (MELENIVANTVLLK) as well as a scrambled peptide (TILLKVAVNNELEM) were synthesized by the solid state Merrifield method on an Applied Biosystems autoclaved synthesizer. Synthetic oligonucleotides corresponding to residues 1–14 of GRK5 were annealed and ligated directly to the plasmid at XhoI and 3′-KpnI sites. The coding sequence of the GRK5 gene was amplified using the primers pFUS2-CAN1 was cloned into pCRII (Invitrogen) and sequenced. The fragments were excised and assembled into pbKs (Stratagene) forming pSst2-G418r. The fragment was cloned into pCR (Invitrogen) and sequenced. The fragments were excised and assembled into pRS306 (16) forming pFUS2-CAN1. pFUS2-CAN1 was linearized with XbaI prior to transformation.

**PCR Mutagenesis**—Full-length human GRK5 in pcDNA3 was used as template for PCR amplification using (sense) and 5′-CCACGGCGCTTACAGGCA-3′ (antisense, encoding residues 213–218 of GRK5). To increase the frequency of random mutations, a mutagenic mixture of dNTPs containing 5 mM dCTP was used in conjunction with a final concentration of 5 mM MnCl2 in PCR. Amplification involved denaturing template DNA at 94 °C for 1 min, annealing for 45 s at 55 °C for 1 min and extension for 1.5 min at 72 °C for 30 s, with a final extension for 5 min at 72 °C. The resulting PCR products from 10 separate reactions were then pooled and purified.

**Construction of GRK5 Yeast Expression Vectors**—Wild-type human GRK5 in pcDNA3 was digested with BamHI and XbaI and subcloned into the yeast expression vector Yplac111, which includes a GALI/10 promoter. Yplac111-GRK5 was digested at 2 internal restriction sites, BamHI and Bsu36I, to excise a 462-base pair open reading frame (encoding residues 1–154 of GRK5). Randomly mutated GRK5 PCR products were digested with BamHI and Bsu36I and ligated into previously digested Yplac111-GRK5 to reconstruct full-length GRK5 cDNAs.

**Construction of MPY576fc**—Haploid S. cerevisiae strains YPH499 (MATα ura3-52 leu2Δ his3Δ200 lys2-801 ade2-101 trpl-Δ63, Stratagene) and MOYO11 (MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 Ole-17) were crossed, the resulting zygotes were identified microscopically and cultured on YPD plates. The diploid cells were induced to sporulate on appropriate media and the tetrads were dissected on YPD plates. Four spore tetrads were assessed for the presence of required nutrient markers. MPY566 (MATα ura3 leu2 his3 lys2-801 ade2 trp1 can1-100) was used for further strain construction.

**Standard yeast media and culture conditions were employed (18).**

**MPY566 was modified by sequential deletion of several genes in the mating signal transduction pathway leading to yeast strains that produce a sensitive growth-based readout of GPCR activation. DNA mediated transformation of S. cerevisiae was carried out using the lithium acetate method (19). The far1 gene was deleted using the far1LYS2 construct in pLP80 (14). The SSTR2 gene was inactivated by replacement with the Sac1-Hxol fragment in pSST2-G418r, a construct that permits expression of a Sac2-G418r fusion protein, by selecting for G418 resistance on plates containing α-mating factor. The FUS1 gene was replaced with the FUS1-HIS3 allele in S. cerevisiae pS11497 (20). Finally, the FUS2 gene was modified by pFUS2-CAN1 using the pop-in/pop-out replacement procedure (21). FUS2 coding sequences were replaced with those of CAN1, thus placing CAN1 expression under control of the pheromone inducible FUS2 promoter. As the modified loci in MPY576fc were confirmed by PCR analysis. As described above, the MPY576fc is capable of agonist-induced vegetative growth on selective media lacking histidine, G418 resistance, and sensitivity to the toxic arginine analog, canavanine.

**Bioassay**—Cultures of the yeast strain containing the SST2 alone (MPY576fc(pMP222), or SST2 plus GRK5 (MPY576fc(pMP222, Yplac111-GRK5)) were grown overnight in synthetic complete media containing glucose (2%) and lacking uracil (to select for receptor alone), or containing galactose (2%) and lacking uracil and leucine (to select for receptor and GRK5), then centrifuged for 10 min at 1000 × g. Pelleted cells were re suspended in 1 mL of sterile water and subsequently diluted 100-fold. Diluted cells (0.5 mL) were then spread on agar plates lacking uracil, histidine or lacking histidine, leucine, and uracil. Plates were also contained 4 mg 3-aminol,1,2,4-triazole to inhibit background growth. Sixty pmol of somatostatin-14 peptide (SST-14) in a total volume of 5 μL was then spotted in the center of the plate and allowed to dry. Plates were incubated for 48–56 h until growth appeared on around the point of agonist application. Analogous experiments using α-factor peptide (5 mg/mL) were performed similarly. S. cerevisiae strains containing 20 different mutations of GRK5 were used to select for sensitivity to the toxic arginine analog, canavanine. S. cerevisiae strains containing 20 different mutations of GRK5 were used to select for sensitivity to the toxic arginine analog, canavanine.
at 30 °C for 1 h by heat shock treatment for 5 min at 42 °C in the presence of MgSO4. Cell suspensions were then plated on agar containing galactose and lacking uracil, leucine, and histidine (+4 mM 3-amino-1,2,4-triazole). 180 nmol of SST-14 peptide were added to the plates and incubated for 7 days. Colonies were then patched onto plates containing galactose and lacking uracil, leucine, and histidine and incubated in the presence or absence of SST-14 for 3–5 days.

Expression of GRK5 in Yeast—GRK5 expression was confirmed by extraction of protein from yeast cells followed by Western blotting. Briefly, cultures of yeast strain MPY576 (pMP225, Ycp1lac11-GRK5) containing SSTR2 and GRK5 expression vectors were grown to stationary phase in selective raffinose (1%) media, then washed and diluted 1:10 into selective galactose media. Cultures (10 ml) were grown to log phase in the presence of Tris-HCl, pH 8.0, 10 mM MgCl2, 1 mM EDTA, 100 mM NaCl, 1 mM ω-μ leupeptin, 1 μg/ml pepstatin, 40 μg/ml phenylmethylsulfonyl fluoride). Pelleted cells were resuspended in 100 μl of buffer A (20 mM Tris-HCl, pH 8.0, 10 mM MgCl2, 1 mM EDTA, 100 mM NaCl, 1 μl/ml leupeptin, 1 μg/ml pepstatin, 40 μg/ml phenylmethylsulfonyl fluoride). Pelleted cells were resuspended in 100 μl of buffer A and 50 μl of this suspension was then diluted with an equal volume of SDS sample buffer for 10 min. Equal amounts of total protein were loaded on a 10% SDS-polyacrylamide gel, electrophoresed, and subsequently electroblotted onto nitrocellulose membranes. Membranes were blocked for 30 min in 5% nonfat dry milk in 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20 (TBST), then incubated for 1 h with GRK5 monoclonal antibody diluted 1:5000 in TBST + 3% dry milk. Membranes were washed 3 times in TBST, incubated for 1 h with peroxidase-labeled goat anti-mouse antibody (1:2000) in TBST + 5% dry milk, washed, and developed using ECL chemiluminescence reagent.

Recovery of GRK5 Plasmids and Retransformation—Total DNA from individual isolates was extracted from yeast cells. Briefly, cells were grown overnight in selection media containing 1% yeast extract, 2% peptone, and 2% glucose. Cells were then centrifuged for 5 min at 1000 × g and resuspended in a final volume of 1 ml of 1x sorbitol, pH 7.5, 0.1 x EDTA, 0.5 mg/ml zymolyase 60, 0.1 M EDTA. Genomic DNA was transformed into the competent bacterial strain MC1066 that specifically selects for the Leu marker of the GRK5-expressing plasmid. DNA was prepared from individual colonies and transformed into the competent bacterial strain MPY576 (pMP225, Ycp1lac11-GRK5) containing GRK5 expression vectors were grown to stationary phase in selective raffinose (1%) media, then washed and diluted 1:10 into selective galactose media. Cultures (10 ml) were grown to log phase in the presence of Tris-HCl, pH 8.0, 10 mM MgCl2, 1 mM EDTA, 100 mM NaCl, 1 μl/ml leupeptin, 1 μg/ml pepstatin, 40 μg/ml phenylmethylsulfonyl fluoride). Pelleted cells were resuspended in 100 μl of buffer A and 50 μl of this suspension was then diluted with an equal volume of SDS sample buffer for 10 min. Equal amounts of total protein were loaded on a 10% SDS-polyacrylamide gel, electrophoresed, and subsequently electroblotted onto nitrocellulose membranes. Membranes were blocked for 30 min in 5% nonfat dry milk in 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20 (TBST), then incubated for 1 h with GRK5 monoclonal antibody diluted 1:5000 in TBST + 3% dry milk. Membranes were washed 3 times in TBST, incubated for 1 h with peroxidase-labeled goat anti-mouse antibody (1:2000) in TBST + 5% dry milk, washed, and developed using ECL chemiluminescence reagent.

Development of a Yeast Bioassay to Identify GRK Mutants

Purification of GRK5—Autophosphorylation reactions contained 4 pmol of purified wild type or mutant GRK5 in 20 μl of buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 0.1 μl EDTA). Reactions were incubated for 1–30 min at 30 °C and quenched with SDS sample buffer. Samples were electrophoresed and the 32P-labeled proteins were excised and counted. Autophosphorylation reactions performed in the presence of phospholipids contained 0.85 mg/ml soybean phosphatidylinositol vesicles. GRK5 Binding to Phospholipides—Phospholipid vesicles were prepared by sonication 85 mg of crude soybean phosphatidylinositol in 5 ml of buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 0.1 μl EDTA) on ice for 4 times for 20 s. Phospholipid association with GRK5 was analyzed by incubating 80 ng (20 nm) of purified or partially purified GRK5 in the presence or absence of the indicated amount of phospholipid in 60 μl of buffer (20 mM Tris-HCl, pH 8.0, 2 mM MgCl2, 100 mM NaCl, 0.015% Triton X-100) for 5 min at 30 °C. Reactions were pelleted at 200,000 × g for 15 min and pellet and supernatant fractions were solubilized in SDS sample buffer. Equal aliquots of each fraction were electrophoresed, transferred to nitrocellulose, and visualized by immunoblotting using mouse monoclonal anti-GRK5 antibodies. Optical density of developed bands was assessed by densitometry and the amount of GRK5 bound to lipid was expressed as a percentage of the total.

RESULTS AND DISCUSSION

Development of a Yeast Bioassay to Analyze GRK Interaction with GPCRs—The mating pheromone pathway in the budding yeast S. cerevisiae utilizes a GPCR-heterotrimeric G protein complex to regulate mating between haploid cells. During the mating process, pheromone peptide binds to GPCRs encoded by the STE2 and STE3 genes that correspond to the MATa and MATα mating types, respectively. Subsequently, a signal transduction cascade is initiated that activates mitogen-activated protein kinase homologues encoded by the FUS1 and KSS1 genes, as well as regulatory protein kinases encoded by the STE7 and STE11 genes. Under normal conditions, the activation of this pathway results in cell cycle arrest mediated by the protein product of the FAR1 gene (10–12). However, by introducing specific genetic mutations into a given strain, this response can be eliminated. When FAR1 is deleted, cell growth is permitted to continue in the presence of the activated pheromone pathway. Based on this concept, a novel expression system was developed that allows yeast cells expressing the mammalian somatostatin receptor subtype 2 to couple to the pheromone pathway in the presence of the peptide agonist SST-14 (13). Activation of this pathway can then be used in a growth selection by placing a HIS3 reporter into transcriptional elements of the FUS1 gene. Expression of the His3 protein is thus placed under control of the pheromone response cascade such that binding of SST-14 and subsequent activation of the pathway induces the Fus1 promoter. His3 protein expression then permits auxotrophic growth of yeast cells on media lacking histidine. In accordance with this observation, an appropriate strain was constructed (MPY576fc) that functionally expresses the SSTR2 and exhibits agonist-dependent growth upon exposure to somatostatin (13).

Although several GPCRs have been demonstrated to couple with the yeast pheromone response pathway (27), the choice of SSTR2 as a model was based on its ability to more effectively couple to the pheromone pathway compared with several other mammalian receptors that were tested. Moreover, previous studies suggested that SSTR2 is likely phosphorylated and...
regulated by GRKs in mammalian cells (28–30), although this has not been directly demonstrated. Thus, we hypothesized that co-expression of a GRK with the SSTR2 would result in agonist-promoted receptor phosphorylation and potentially result in reduced G protein coupling and inhibition of agonist-dependent growth. To test this hypothesis, SSTR2 was expressed in MPY576fc under control of the \textit{PGK1} promoter that produces high level expression in both glucose and galactose-containing medium. GRK5 was coexpressed using a galactose-inducible, glucose-repressible promoter. In the presence of glucose, conditions under which little or no GRK5 expression is expected, growth was observed in the presence of SST-14 (Fig. 1A, upper left panel). However, SST-14 promoted growth was abolished in the presence of galactose demonstrating that GRK5 effectively inhibits SSTR2-promoted growth (Fig. 1A, lower left panel). To test whether the observed non-growth phenotype was dependent on the catalytic activity of GRK5, we co-expressed a catalytically inactive form of GRK5 (GRK5-K215R) (24) with the receptor. Expression of GRK5-K215R had no effect on agonist-promoted growth suggesting that disruption of the growth phenotype requires the kinase activity of GRK5 (Fig. 1A, lower right panel). We also tested GRK2 and GRK2-K220R in the same manner and found that wild type GRK2 effectively inhibited agonist-promoted growth, whereas the catalytically inactive kinase had no effect on agonist-promoted growth (data not shown). Thus, this bioassay provides an effective readout of GRK/GPCR interaction and should be useful for structure-function analysis as well as screening for

| GRK5 mutant<sup>a</sup> | Mutations | Agonist-dependent growth<sup>b</sup> |
|------------------------|-----------|---------------------------------|
| WT                     |           | –                               |
| 70                     | D52N, V87G| ++                              |
| 72                     | L3Q, K113R| +++                             |
| 78                     | Deletion 29–153| + |
| 86                     | I56F, P75S| +++                             |
| 87                     | T10P      | +++                             |
| 101                    | N55, V116A| +++                             |
| 113                    | N91, P37L, S40C, E72V, P75S | + |
| 121                    | K34Q, K103R, C138S, E151V | + |
| 128                    | I6N, T109S, I62N, P75L, P83S, K103R, L141R | +++ |
| 129                    | I6N, V87G, S143T, Y152H | ++ |
| 136                    | K26N      | +                               |
| 140                    | E72V      | +                               |
| 154                    | P75L, V116A| + |

<sup>a</sup> Clones were sequentially numbered based on the order in which they were identified.

<sup>b</sup> Relative amounts of growth in the qualitative patch-test assays are represented by plus signs, with growth similar to strains containing SSTR2 alone represented by +++. Mutant GRK5 expression was assessed by Western blotting and determined to be similar to wild type GRK5 levels that completely inhibit growth (shown as – on the growth scale).
compounds that modulate GRK/GPCR interaction. Such screening could conceivably be targeted toward a particular GRK or GRK/GPCR combination.

To test the receptor specificity of the GRK5-dependent phenotype, we also assessed whether GRK5 disrupted the pheromone response pathway. Cells co-expressing SSTR2 and either GRK5 or GRK5-K215R were examined for growth in the presence of α-factor peptide, the natural agonist for the Ste2 receptor (Fig. 1B). These assays showed comparable levels of agonist-dependent growth in either the presence or absence of GRK5 expression, suggesting that neither wild type GRK5 nor GRK5-K215R have a significant effect on α-factor-promoted growth. Taken together, these results suggest that the GRK-induced phenotype is mediated by GRK-promoted phosphorylation of SSTR2 resulting in reduced receptor/G protein coupling and reduced growth. Based on these findings, we next used this bioassay to identify GRK5 mutants that are selectively deficient in receptor interaction.

Identification of Amino-terminal GRK5 Mutants—Based on our observations of a GRK5-specific growth phenotype, we developed a selection assay in which a library of GRK5 mutants could be analyzed for an altered ability to suppress growth. To specifically target the GRK5 NH2 terminus, we randomly mutated the first 218 amino acids of GRK5 by PCR. We then cut this fragment at two internal restriction sites that excised the first 154 residues of the NH2 terminus. A GRK5 mutant library was then generated by replacing the same fragment of wild type GRK5 in the galactose-inducible yeast expression vector.
with the mutant cDNAs. The GRK5 mutant library was then co-transformed into strain MPY576/p(MP222) containing the SST2R and the cells were grown on the appropriate selection media. Transformants that exhibited growth in the presence of SST-14 were predicted to express GRK5 mutants that had been disrupted in receptor phosphorylation. Initially, we isolated ~200 transformants from the mutant library that exhibited agonist-dependent growth.

To account for the possibility that some of the isolates obtained through the selective transformation could grow because of sporadic adaptive mutations on the media plates, each transformant was subjected to a round of screening whereby individual colonies were patched onto selective media in the presence of agonist. A second round of screening in either the presence or absence of agonist confirmed candidate GRK5 mutants exhibiting growth exclusively in the presence of agonist. Upon re-testing each of these clones in patch assays, we found that ~80% of the original 200 grew in an agonist-dependent manner. To test the possibility that the growth phenotype was because of reduced GRK5 expression, we tested the mutants that demonstrated agonist-dependent growth by extracting total protein from yeast cells followed by Western blotting. Analysis of these clones revealed that ~35% of those tested exhibited expression levels comparable with wild type GRK5. Those mutants that did not express at all or expressed very poorly were eliminated as candidates for further analysis. For the transformants that demonstrated expression similar to wild type GRK5, plasmids containing mutant cDNAs were then recovered and re-transformed into strain MPY576/p(MP222) under the appropriate selection conditions. For each transformation, several isolates were re-tested using patch assays in the presence or absence of SST-14 to confirm the reproducibility of the mutant phenotype. Mutant GRK5 cDNAs were then sequenced to determine changes in amino acid residues. Based on the selection criteria, we generated a pool of 13 mutants to consider for further study (Table 1).

Fig. 2 depicts the location of the various mutations identified in our study. Although the mutated residues were spread over the entire NH2-terminal region, there are particular high concentrations of mutations localized to the first 10 residues and to the central portion of the NH2-terminal domain (residues 72 to 87) suggesting the functional importance of these regions. Although GRK5 interaction with Go subunits has not been reported, this central region falls within the regulator of G protein signaling (RGS) homology domain in GRKs (encompassing residues 40–180 in GRK5) that mediates GRK2 interaction with Goq (31–34). Glu-72, Pro-75, and Val-87 may be particularly important because multiple clones were identified with these residues mutated. Our studies also identified one mutation (I62N) that falls within the previously identified caveolin-binding domain of GRKs (35). Interestingly, several mutants identified in our screen (K24Q, K26N, I33F, and P37L) reside within a calmodulin-binding domain of GRKs (35). Interestingly, several mutants identified in our screen (K24Q, K26N, I33F, and P37L) reside within a calmodulin-binding domain in GRK5 that is localized between residues 20 and 39 (36–39). This particular region of GRK5 has also been implicated in phophatidylinositol bisphosphate binding suggesting that mutation of these residues might result in decreased activity toward the receptor because of disruption of lipid interaction (9). In fact, two of the residues identified in our screen (Lys-24 and Lys-26) appear to be directly involved in phosphatidylinositol bisphosphate-dependent GRK5 activity (9).

We can also speculate on the potential effect of these mutations by comparing GRK5 with the recently solved crystal structure of GRK2 (40). Interestingly, the GRK2 structure reveals that the RGS, kinase, and pleckstrin homology domains form an equilateral triangle that is ~80 Å on a side. The RGS domain has extensive contacts with both the kinase (~1700 Å2 of surface area) and pleckstrin homology (~1400 Å2 of surface area) domains. Moreover, the RGS domain consists of two discontinuous regions with the characteristic nine-helix bundle in the NH2-terminal region and two additional helices (a10–
11) following the kinase domain. Although GRK5 lacks the COOH-terminal pleckstrin homology domain found in GRK2, it seems likely that GRK5 will share many of the structural features of GRK2. Because the RGS domain of GRK5 will likely contact both the kinase and COOH-terminal regions, proper folding of this region will play an important role in maintaining a functional kinase. In this regard, several of the mutations (I62N, F83S, V87G, L141R, and possibly Y152N) are within the

### Table II

Kinetic parameters for wild type and mutant GRK5

| GRK5 Substrate | Km \( \mu M \) | \( V_{\text{max}} \) nmol/min/mg | \( V_{\text{max}}/K_m \) |
|----------------|-------------|------------------|-----------------|
| WT Rhodopsin   | 8.0 ± 1.7   | 500 ± 127        | 63              |
| L3Q/K113R Rhodopsin | 50.0 ± 6.6   | 400 ± 79         | 8               |
| T10P Rhodopsin  | 7.1 ± 0.9   | 100 ± 35         | 14              |
| WT Tubulin     | 0.029 ± 0.001 | 5.98 ± 0.23   | 206             |
| L3Q/K113R Tubulin | 0.030 ± 0.002 | 5.88 ± 0.10 | 196             |
| T10P Tubulin   | 0.026 ± 0.002 | 6.02 ± 0.13   | 232             |

**Fig. 5.** Effect of NH₂-terminal mutations on GRK5 interaction with phospholipids. A, autophosphorylation of GRK5. 4 pmol of GRK5 was autophosphorylated in buffer containing 20 mM Tris-Cl, pH 8.0, 2 mM EDTA, 4 mM MgCl₂, and 0.1 mM \(^{32}\)P-ATP (1000 cpm/pmol) in a total reaction volume of 20 \( \mu l \) in the presence (closed symbols) or absence (open symbols) of 1 \( \mu g \) of soybean phosphatidylcholine liposomes. The reactions were incubated for the indicated times and stopped by the addition of 5 \( \mu l \) of SDS sample buffer, followed by electrophoresis on a 10% SDS-polyacrylamide gel and autoradiography. \(^{32}\)P Incorporation in GRK5 was determined by excising and counting the GRK5 bands.

B, GRK5 purified from SF9 cells was incubated in the presence of liposomes (1 mg/ml) made from crude soybean phosphatidylcholine (PL) as described under “Experimental Procedures.” Incubations were pelleted at 200,000 \( \times g \), supernatant fractions were removed, and supernatant and pellet fractions were solubilized in SDS sample buffer. Equal aliquots of both fractions were electrophoresed, transferred to nitrocellulose membranes, immunoblotted with monoclonal anti-GRK5 antibodies, and visualized using ECL. Optical density of developed bands was determined by densitometry and the amount of pelleted GRK5 was expressed as a percentage of the total. The results shown represent the mean ± S.E. of four to eight separate experiments. WT, wild type.
hydrophobic core of the GRK5 RGS domain and might be expected to disrupt folding.

Effect of NH₂-terminal Mutations on GRK5 Activity—From the initial pool of 13 mutants, we chose clones 72 (L3Q/K113R), 86 (P75S/I33F), and 87 (T10P) for further analysis because these particular mutants demonstrated strong growth phenotypes (Table I) as well as a small number of residues mutated. Leu-3 is conserved in all GRKs, Pro-75 is conserved in GRK4–6, Ile-33 and Lys-113 are conserved in GRK4 and -5, and Thr-10 is conserved in GRK5 and -6. We used the baculovirus expression system to express and purify wild type GRK5 and the three mutant kinases (Fig. 3). Fig. 3 demonstrates that all of the kinases used in our analysis were highly purified.

Our initial studies compared the ability of wild type GRK5, GRK5-L3Q/K113R, GRK5-T10P, and GRK5-P75S/I33F to phosphorylate the receptor substrate rhodopsin. GRK5-P75S/I33F phosphorylation of rhodopsin was comparable with wild type GRK5, whereas the L3Q/K113R and T10P mutants had significantly reduced activity (Fig. 4A). To verify that the L3Q/K113R and T10P mutants had a general defect in GPCR phosphorylation we also compared their ability to phosphorylate purged H92522AR, a receptor that serves as an in vivo substrate for GRK5 (41). These studies demonstrated that the L3Q/K113R and T10P mutants were also effectively disrupted in H92522AR phosphorylation, whereas P75S/I33F was similar in activity to wild type GRK5 (data not shown). Because we were primarily interested in mutants with a deficiency in receptor phosphorylation, we did not pursue the characterization of P75S/I33F further.

To address which residue contributes to the observed defect in receptor phosphorylation, we constructed and expressed the individual point mutants GRK5-L3Q and GRK5-K113R in Sf9 cells. We then compared the ability of lysates from wild type, L3Q, K113R, and L3Q/K113R baculovirus-infected cells to phosphorylate rhodopsin. Our results revealed that K113R exhibited activity comparable with wild type GRK5, whereas L3Q was largely defective in rhodopsin phosphorylation (Fig. 4C). Thus, Leu-3 appears to play an important role in promoting receptor phosphorylation.

To better characterize the activity of the L3Q/K113R and T10P mutants, we performed a kinetic analysis by varying
either the rhodopsin or tubulin concentration (Table II). These experiments revealed a $K_m$ of 8 H9262 M and $V_{max}$ of 500 nmol/min/mg for wild type GRK5-mediated phosphorylation of rhodopsin, values similar to those previously reported (26). For GRK5-L3Q/K113R, the $K_m$ for rhodopsin was increased 6-fold (50 H9262 M), whereas there was only a minimal decrease in $V_{max}$ (400 nmol/min/mg). By comparison, GRK5-T10P had a 5-fold reduction in $V_{max}$ (100 nmol/min/mg) with essentially no change in $K_m$ (7.1 H9262 M). These data suggest that the two mutants may have different mechanistic defects in phosphorylating rhodopsin. Kinetic analysis comparing mutant and wild type GRK5 phosphorylation of tubulin revealed no significant changes in $K_m$ or $V_{max}$. Thus, these mutants appear to retain full catalytic activity and are selectively defective in receptor phosphorylation.

Role of the NH$_2$ Terminus in Phospholipid Binding—Previous studies have demonstrated an important role of the COOH-terminal domain in targeting GRKs to the phospholipid bilayer (4). GRK5 interacts with phospholipids primarily through a COOH-terminal polybasic domain localized between residues 552 and 562 (23). GRK5 interaction with phospholipid stimulates autophosphorylation that, in turn, enhances the ability of the kinase to phosphorylate receptor substrates (43). To address whether GRK5-L3Q/K113R and GRK5-T10P have altered autophosphorylation, we measured the rate of autophosphorylation in the presence or absence of phospholipid. Whereas autophosphorylation in the absence of phospholipid was comparable for the wild type and mutant GRKs, the rate of GRK5-L3Q/K113R and GRK5-T10P autophosphorylation in the presence of phospholipid was significantly attenuated compared with wild type GRK5 (Fig. 5A). Wild type GRK5 showed an ~4-fold increase in autophosphorylation in the presence of phospholipid, whereas L3Q/K113R and T10P were only enhanced ~2-fold.

To address whether the defect in autophosphorylation was contributing to the reduced receptor phosphorylation, we made use of a GRK5 autophosphorylation site mutant that functionally mimics the autophosphorylated state of the kinase (GRK5-S484D/T485D) (43). Because autophosphorylation enhances GRK5 activity, if the deficiency in receptor phosphorylation results from decreased autophosphorylation, then incorporation of the S484D/T485D mutation into the L3Q/K113R and T10P mutants should overcome this deficiency. Phosphorylation of rhodopsin by purified wild type, L3Q/K113R, and T10P versions of GRK5-S484D/T485D revealed that the S484D/T485D mutation did not overcome the reduced ability of L3Q/K113R and T10P mutants to phosphorylate rhodopsin (data not shown). Thus, the observed defect in receptor phosphorylation does not appear to be because of a defect in phospholipid-stimulated autophosphorylation.

The reduced phospholipid-stimulated autophosphorylation of L3Q/K113R and T10P suggests that these mutants are either directly disrupted in phospholipid binding or are unable to undergo phospholipid-mediated conformational changes that enhance autophosphorylation. To address these possibilities, we tested whether L3Q/K113R and T10P exhibited altered
phospholipid binding by measuring the ability of wild type and mutant GRK5 to co-sediment with phospholipid vesicles. At a fixed lipid concentration, we found that L3Q/K113R and T10P show an ~50 and ~85% reduction, respectively, in phospholipid binding compared with wild type GRK5 (Fig. 5B). These results are somewhat surprising because the major phospholipid-binding domain in GRK5 is localized to the COOH terminus (23). Nevertheless, to further establish that the mutants are disrupted in phospholipid binding, we also generated COOH-terminal truncations that remove the major phospholipid-binding domain in GRK5 (residues 552–590). Phospholipid binding analysis of the truncated wild type and mutant GRK5 revealed that the mutants retained their reduced ability to bind phospholipids (Fig. 5C). Taken together, these results suggest that the NH$_2$-terminal region of GRK5 is directly involved in phospholipid binding.

Residues 1–14 Constitute a Receptor/Phospholipid Binding Domain—Analysis of the NH$_2$-terminal domain of GRK5 using protein secondary structure software predicts that the first 14 amino acids of GRK5 form an amphipathic a-helix (Fig. 6A). To further establish the importance of this domain, we made a truncation mutant (GRK5A2–14) that lacks residues 2–14. Partially purified GRK5A2–14 was completely defective in phosphorylating rhodopsin (Fig. 6B) but had similar activity to wild type GRK5 in phosphorylating tubulin (Fig. 6C). The mutant was also defective in phospholipid-stimulated autophosphorylation (Fig. 6D). Thus, GRK5A2–14 has properties very similar to those observed for the L3Q/K113R and T10P mutants. These data provide further support that the NH$_2$-terminus of GRK5 plays an important role in phospholipid interaction and receptor phosphorylation.

Interestingly, one previous study that analyzed NH$_2$-terminal truncation mutants of GRK1 and GRK2 also concluded that the NH$_2$-terminal region is important for receptor phosphorylation and revealed an important role of a conserved glutamate (Glu-7 in GRK1, Glu-5 in GRK2) in this process (8). This glutamate is also conserved in GRK4 family members (Glu-4 in GRK5) and we would predict that it has an important role in GPCR phosphorylation. Interestingly, point mutants of this glutamate in GRK1 and -2 retained their ability to be activated by COOH-terminal truncated rhodopsin and to bind to rod outer segments in a light-dependent manner. These results suggest that this conserved glutamate is essential for effective receptor phosphorylation but might not play a direct role in receptor binding.

The role of the GRK5 NH$_2$ terminus in receptor phosphorylation was further analyzed using synthetic peptides. A peptide corresponding to residues 1–14 of GRK5 effectively inhibited GRK5 phosphorylation of rhodopsin with an IC$_{50}$ of ~20 $\mu$M, whereas a scrambled peptide containing the same amino acids had no effect (Fig. 7A). Interestingly, the GRK5 peptide had no significant effect on the ability of GRK2 to phosphorylate rhodopsin (Fig. 7B). Thus, even though the NH$_2$-terminal 15 residues of GRK2 (8) and GRK5 (Figs. 6 and 7) appear essential for receptor phosphorylation, our results suggest mechanistic differences in how this region of GRK2 and GRK5 functions. Although the NH$_2$-terminal 28 amino acids are missing in the GRK2 crystal structure, the structure predicts that the extreme NH$_2$ terminus is on the GPCR/lipid binding face of the protein and thus might contribute to these interactions (40).

The 14-amino acid peptide also inhibited phospholipid-stimulated autophosphorylation of GRK5 (Fig. 7C) but had no effect on tubulin phosphorylation (data not shown). Interestingly, the addition of phospholipid to the rhodopsin phosphorylation assay was able to effectively attenuate the inhibitory effects of the peptide on GRK5 activity, suggesting that the peptide might directly bind to phospholipid or might inhibit GRK5 binding to phospholipid (Fig. 7D). Taken together, these results suggest that the 14-amino acid NH$_2$-terminal region of GRK5 plays an important role in mediating kinase association with phospholipids and the subsequent phospholipid-mediated autophosphorylation and receptor phosphorylation.

Proposed Role of the GRK5 NH$_2$ Terminus in Receptor Phosphorylation—Previous studies that identified the COOH-terminal phospholipid-binding domain of GRK5 suggested that other regions within the kinase could interact with lipid in a regulatory manner (23). Here, we have identified an NH$_2$-terminal domain of GRK5 that appears important for both phospholipid association and receptor phosphorylation. We propose a mechanism whereby the extreme NH$_2$ terminus of GRK5 mediates GRK5 interaction with phospholipid through a putative amphipathic $\alpha$-helical domain. This interaction promotes autophosphorylation of the kinase and is essential for optimal receptor phosphorylation. Although this mechanism may be somewhat unique to GRK5 given the importance of autophosphorylation in its function, it is evident that the NH$_2$-terminal domain of multiple GRKs plays a critical role in mediating receptor phosphorylation (Ref. 8 and this study).

Summary—In this study we developed a yeast-based bioassay that provides an effective readout of GRK/GPCR interaction. This enabled us to screen for mutations in GRK5 that disrupt GPCR interaction and identify an important role for the NH$_2$ terminus in regulating GRK activity. Our results suggest that the extreme NH$_2$-terminal domain of GRK5 mediates a variety of important aspects of GRK function, including phospholipid binding and autophosphorylation, that culminate in effective GPCR phosphorylation. In addition, our findings are the first to suggest mechanistic differences in how this NH$_2$-terminal domain functions in GRK2 and GRK5 family members. Future studies will attempt to identify the mechanistic basis for these findings and may lead to strategies for selectively regulating GRK function by targeting the extreme NH$_2$ terminus. Identification of specific regulators of GRK/GPCR interaction may have important therapeutic implications given the proposed role of GRKs in such diseases as heart failure and hypertension (6, 44).

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