Many G protein-coupled receptors are phosphorylated and regulated by a distinct family of G protein-coupled receptor kinases (GRKs) that specifically target the activated form of the receptor. Recent studies have revealed that the GRKs are also subject to post-translational regulation. For example, GRK5 activity is strongly inhibited by protein kinase C phosphorylation and by Ca\(^{2+}\)-calmodulin binding. Ca\(^{2+}\)-calmodulin binding also promotes GRK5 autophosphorylation, which further contributes to kinase inhibition. In this study we identify two important structural domains in GRK5, a phospholipid binding domain (residues 552–562) and an autoinhibitory domain (residues 563–590), that significantly contribute to GRK5 localization and function. We demonstrate that the C-terminal region of GRK5 (residues 563–590) contains residues autophosphorylated in the presence of calmodulin as well as the residues phosphorylated by protein kinase C. Deletion of this domain increases the apparent affinity of GRK5 for receptor substrates 2–3-fold but has no effect on non-receptor substrates. These findings define residues 563–590 of GRK5 as an autoinhibitory domain with efficacy that is regulated by phosphorylation. Another C-terminal domain in GRK5 that appears to be functionally important is found between residues 552 and 562. Deletion of this region significantly inhibits kinase phosphorylation of membrane-bound receptor substrates but has no effect on soluble substrates. Additional studies reveal that this domain is critical for GRK5 interaction with phospholipids and for the intracellular localization of the kinase. Interestingly, similar regions in GRK4 and GRK6 appear to be palmitoylated (and involved in membrane interaction), suggesting evolutionary conservation of the function of this domain.

A large variety of extracellular stimuli transmit their signals via interaction with cell surface G protein-coupled receptors (GPCRs)\(^{1}\) (1). A fundamental process of regulating the responsiveness of a system, termed desensitization, uses numerous mechanisms initiated by phosphorylation of the receptor. Receptor phosphorylation results in uncoupling of receptor and G protein and may also control processes such as GPCR internalization and down-regulation. The phosphorylation of GPCRs can be mediated either by second messenger-dependent kinases, such as protein kinase A and protein kinase C (PKC), or by G protein-coupled receptor kinases (GRKs). The six mammalian GRKs identified to date can be divided into three subfamilies based on their sequence homology (2, 3): GRK1 (rhodopsin kinase) forms one group; GRK2 (\(\beta\)-adrenergic receptor kinase) and GRK3 constitute a second; and GRK4, GRK5, and GRK6 constitute the third subfamily.

One feature of GRKs that plays a critical role in regulating the interaction of the soluble kinases with integral membrane GPCRs is their ability to associate with the plasma membrane. Interestingly, the various GRKs use distinct mechanisms for membrane interaction, including modification of the C-terminal domain by lipids (GRK1, GRK4 and GRK6) and association with G\(\alpha\) subunits and acidic phospholipids (GRK2 and GRK3) (4). The specific determinants of membrane interaction for GRK5 are poorly understood. Although GRK5 does not appear to be modified by lipids, it displays a high level of membrane association, possibly via direct binding of a C-terminal polybasic domain with phospholipids (5). However, it has been also suggested that GRK5 contains an N-terminal phosphatidylinositol-4,5-bisphosphate (PIP\(_2\)) binding site that can promote membrane association (6).

Additional mechanisms of GRK regulation have been identified. Of particular interest is the calcium-dependent regulation of GRK activity. Recent studies have demonstrated that GRK2 can be phosphorylated by PKC, resulting in an 2–3-fold activation of the kinase, possibly via an increased association of GRK2 with membranes (7, 8). Conversely, GRK5 is significantly inhibited after phosphorylation by PKC because of a decreased activity and decreased affinity for receptor (9). In the visual system, GRK1 has been shown to be inhibited by the Ca\(^{2+}\)-binding protein recoverin (10). Although regulation by recoverin may be specific for GRK1, a universal mediator of calcium signaling, calmodulin, appears to inhibit all other GRK subtypes, with GRK5 being the most sensitive (11–13).

We have demonstrated recently that calmodulin stimulates autophosphorylation of GRK5 and that this phosphorylation inhibits GRK5 activity, in a manner similar to the PKC-mediated phosphorylation (13). Here we show that the residues autophosphorylated in the presence of calmodulin (as well as the residues phosphorylated by PKC) lie within a C-terminal 28-amino acid region of GRK5 (residues 563–590). This region of GRK5 appears to function as an autoinhibitory domain, because its deletion increases GRK5 affinity for both phospholipids and receptor. We also demonstrate that the sequence immediately upstream (amino acids 552–562) of this region is critical for GRK5 association with phospholipids and for the
intracellular localization of the kinase. Thus, our studies reveal two novel structural domains that play an important role in GRK5 function and regulation.

EXPERIMENTAL PROCEDURES

Materials—Restriction endonucleases, the Expand high fidelity polymerase chain reaction (PCR) system, and other molecular biology reagents were purchased from New England Biolabs or Boehringer Mannheim. SP-Sepharose was obtained from Amersham Pharmacia Biotech. Calmodulin (bovine brain, >98% pure), phosphatidylcholine (PC) (soybean type II-S, contains ~17% PC), and 1-α-phosphatidydinsitol-4,5-diphosphate (bovine brain, >98% pure) were from Sigma. Phosphatidylerine (bovine brain, 99% pure) was from Avanti Polar Lipids, Inc. Affinity-purified polyclonal antibodies against GRK5 (residues 571–590) and GRK6 (residues 525–544) were from Santa Cruz Biotechnology, Inc. Fluorescein isothiocyanate-conjugated anti-rabbit antibody and SlowFade mounting medium were from Molecular Probes, Inc. Peptides corresponding to GRK5 residues 547–565 (KGGLQRR-LFKFRQHQNNSKS) and 556–571 (KRQHQNNSKSSPSKST) were synthesized by the solid-state Merrifield method on an Applied Biosystems synthesizer and purified by reverse phase high performance liquid chromatography. All other materials were from sources previously described (9).

Transfection of COS-1 Cells and Assessment of GRK Autophosphorylation—Expression plasmids for GRKs were constructed by cloning the coding sequence of human GRK5 (14) in the vector pcDNA3. Mutant GRK5 sequences were generated using PCR, and the PCR-derived portions of the constructs were then sequenced in their entirety using the dideoxy chain termination method. COS-1 cells were grown to ~80–90% confluency in 60-mm dishes and transfected with 4 μg of DNA per dish using LipofectAMINE per the manufacturer’s instructions (Life Technologies, Inc.). Forty-eight hours after transfection cells were harvested and GRKs were partially purified by chromatography on SP-Sepharose as described (13). Typically, partially purified GRK samples contained 7–20 ng of GRK5/μl.

Three-μl aliquots of the partially purified GRK5 were then assayed by incubation without 1 μl of calmodulin in 25 μl of 20 μM Tris·HCl, pH 8.0, 0.4 mM MgCl₂, 0.1 mM CaCl₂, 0.1 mM [γ-32P]ATP (1000 cpm/μl) for 15 min at 30 °C. Reactions were stopped by placing on ice and adding 120 μl of buffer A (20 mM Tris·HCl, pH 8.0, 300 mM NaCl, 40 mM NaF, 1 mM EDTA, 0.05% Tween 20) and 5 μl of rabbit polyclonal antiserum generated against GST-GRK5(489–590) (9). Samples were incubated on ice for 15 min, after which 20 μl of a 50% protein A-agarose suspension were added, and the reactions were incubated and additional 30 min. The resin was washed twice with 1 ml of buffer A, and labeled proteins were eluted with two 35-μl portions of the constructs were then sequenced in their entirety using the dideoxy chain termination method. The GST-GRK5 fusion proteins were expressed in Escherichia coli and purified over glutathione-Sepharose using standard procedures. The purity of the proteins was >95% as determined by Coomassie Blue staining. Protein concentrations were determined by dye binding assay (Bio-Rad) using bovine serum albumin as a standard.

To assess the ability of GST-GRK5 fusion proteins to bind phospholipids, 2 μg of the indicated fusion protein were incubated in the presence or absence of 1.7 mg/ml soybean phosphatidylcholine vesicles in 60 μl of 20 μM Tris·HCl, pH 8.0, 2 mM MgCl₂, 100 mM NaCl on ice for 10 min. Reactions were then pelletted and processed by gel electrophoresis as described above. Proteins were visualized by Coomassie Blue staining.

Immunofluorescence Microscopy—COS-1 cells, grown to 75–95% confluence, were transfected with 2 μg of pcDNA3, pcDNA3-GRK5, pcDNA3-GRK55, pcDNA3-GFP, or pcDNA3-GFP-β₂-adrenergic receptor/60-mm dish using FuGENE according to the manufacturer’s instructions. Cells were trypsinized and plated onto 12-mm glass coverslips in a 24-well plate at 8 h after addition of the DNA/FuGENE transfection mix. The cells were rinsed twice with phosphate-buffered saline (PBS) 24 h after transfection and then fixed with PBS containing 4% formaldehyde at 22 °C for 30 min. Fixed cells were subsequently rinsed three times with PBS and permeabilized with PBS containing 0.2% Triton X-100 for 10 min at 22 °C. These cells were blocked for 30 min with PBS containing 0.5% bovine serum albumin and then incubated for 30 min at 37 °C with PBS containing 5% nonfat dried milk and subsequently incubated with polyclonal GRK5(489–590) antisem diluted 1:200 in the same buffer for 1 h at 37 °C. Cells were then rinsed three times with PBS/Triton and incubated at 37 °C for 30 min. The cells were then incubated with a fluorescein isothiocyanate-conjugated goat anti-rabbit antibody diluted 1:100 in PBS/Triton and 5% nonfat dried milk for 1 h at 37 °C. Cells were rinsed four additional times with PBS/Triton and then incubated for 30 min at 37 °C with PBS containing 4% formaldehyde for 5 min. Cells were then rinsed with PBS and mounted on a slide with SlowFade mounting medium. Preparation of cells expressing green fluorescent protein (GFP) or GFP-β₂-adrenergic receptor was identical to that described above with the exception that primary and secondary antibodies were not included in the PBS/Triton/nonfat dried milk solution. Fluorescence microscopy was performed on a Nikon Eclipse E800 fluorescence microscope using a Plan Fluor ×40 objective.

Radioactive Labeling and Immunoprecipitation of Purified GRK5—Human GRK5 was overexpressed and purified from SF9 cells as described (5). Purified GRK5 was 35S-labeled via autophosphorylation. Autophosphorylation reactions contained 40 pmol (2.7 μg) of GRK5 in 40 μl of 20 mM Tris·HCl, pH 8.0, 4 mM MgCl₂, 0.2 mM CaCl₂, 1 mM EDTA, 0.1 mM [γ-32P]ATP (1000 cpm/μmol), and either 0.85 mg/ml soybean phosphatidylcholine or 2 μM calmodulin. Reactions were incubated at 30 °C for 15 min. After phosphorylation GRK5 was purified by batch-wise chromatography on SP-Sepharose as described (9). Typically, 70–80% of the GRK was recovered by this procedure, whereas calmodulin did not bind to SP-Sepharose. Total aliquots of ~0.18 μg of protein were mixed with 20 μl of buffer A with or without 4 ng of trypsin. The reactions were incubated for 20 min at 30 °C before they were placed on ice, and trypsin inhibitors were added (0.1 mM leupeptin, 0.02 mg/ml soybean trypsin inhibitor). The reactions were then added to 70 μl of Tris-buffered saline with 1.5 μg of polyclonal antibodies against either GRK5 residues 571–590 or GRK5 residues 525–544. Samples were
incubated on ice for 15 min, 20 μl of a 50% protein A-agarose suspension were added, and the samples were incubated an additional 30 min. The resin was washed twice with 0.5 ml of Tris-buffered saline. The amount of 32P bound was determined by counting the final washed pellets.

RESULTS

Role of the C-terminal Region of GRK5 in Substrate Interaction—Previous studies have implicated the C-terminal region of GRKs in membrane association (4). Although lipid modifications and phospholipid binding domains have been identified in most of the GRKs, specific regions of GRK5 involved in membrane interaction have not been defined. A polybasic domain within the C terminus of GRK5 has been proposed to play a role in phospholipid association (5). Interestingly, the C-terminal region of GRK5 has also been identified as the major site of phosphorylation by protein kinase C, a modification that results in significant inhibition of GRK5 activity (9). To further define the role of the C-terminal region of GRK5 in phospholipid binding and substrate interaction, we generated a series of C-terminal truncation mutants of GRK5. GRK5 mutants with 5, 12, 28, 39, or 50 residues deleted from the C terminus were readily expressed in COS-1 cells and had detectable activity (Fig. 1, A and B).

Interestingly, the various deletions had a differential effect on the ability of GRK5 to phosphorylate different substrates. Phosphorylation of the soluble protein phosvitin was unaffected by deletion of up to 50 residues of GRK5 (Fig. 1, A and B). In contrast, phosphorylation of light-activated rhodopsin was increased 30–70% when 5–28 residues were deleted from the C terminus (GRK51–585, GRK51–578, and GRK51–562), whereas the deletion of 11 or 22 additional residues (GRK51–551 and GRK51–540) resulted in significant inhibition of rhodopsin phosphorylation (Fig. 1, A and B).

A more detailed kinetic analysis of rhodopsin phosphorylation revealed that deletion of only 5 C-terminal residues increased the affinity of GRK5 for rhodopsin ~3-fold ($K_m$ decreased from 3.2 μM for wild-type GRK5 to 1.0 μM for GRK51–551) and had no effect on the $V_{max}$, whereas deletion of 38 residues (GRK51–562) resulted in no additional change in $K_m$ and an ~25% increase in $V_{max}$ (Fig. 1C). The deletion of 11 additional residues (GRK51–551) caused an ~6-fold loss in affinity for rhodopsin ($K_m$ of 5.8 μM) and ~2.5-fold lower $V_{max}$ compared with GRK51–562.

The increased affinity of the GRK5 C-terminal truncation mutants for rhodopsin was also evident when we tested their ability to directly bind to ROS membranes. Under conditions in which only ~25% of wild-type GRK5 co-sedimented with ROS, ~75% of GRK51–551 (Fig. 1D) and GRK51–562 (data not shown) was pelleted. In contrast, deletion of 11 additional residues (GRK51–551) caused a significant decrease in kinase binding to ROS (down to ~25% bound; Fig. 1D).

Residues 552–562 of GRK5 Constitute a Phospholipid Binding Domain—Because deletion of the C-terminal domain of GRK5 had a significant effect on phosphorylation of the membrane-bound substrate rhodopsin but had no effect on phosphorylation of the soluble substrate phosvitin, we assessed whether the various deletions affected GRK5 interaction with phospholipids. We and others have shown that GRK5 can directly associate and co-sediment with phospholipid vesicles (5, 9, 17). In our initial experiments we used liposomes generated from crude soybean PC, a preparation that contains ~17% PC as well as numerous other phospholipids. We found that the...
Role of the C Terminus in GRK5 Regulation

Because previous studies have suggested an important role of an N-terminal binding domain in GRK5 interaction with PIP₂ (6), we next investigated the effects of PIP₂ on GRK5 binding to crude PC vesicles. The addition of 5% PIP₂ to the crude PC enhanced the affinity of wild-type GRK5 and GRK5₁₅₅₁,5₆₂₆ by 3–4-fold (EC₅₀, values, −0.02 and −0.004 mg/ml, respectively; Fig. 2C). Strikingly, addition of PIP₂ resulted in an 13-fold increase in the affinity of GRK5₁₅₅₁ (EC₅₀, −0.13 mg/ml). This finding confirms a PIP₂ binding site in GRK5 located upstream of the C-terminal phospholipid binding domain identified in this study.

The interaction of the C-terminal residues of the kinase with phospholipids was further demonstrated using peptides. A peptide corresponding to GRK5 residues 547–565 effectively competed for interaction with phospholipids and inhibited GRK5 binding to liposomes with an IC₅₀ of −15 μM (Fig. 3, A and B). A peptide containing only a portion of the putative phospholipid binding site (residues 556–571) had no effect on GRK5 binding to phospholipids at concentrations up to 0.4 mM (Fig. 3A). Consistent with the ability of peptide 547–565 to inhibit GRK5 membrane association, this peptide effectively inhibited the phosphorylation of rhodopsin, whereas peptide 556–571 had no effect on the kinase activity (Fig. 3C). A more detailed kinetic analysis of rhodopsin phosphorylation revealed that the presence of 0.3 mM peptide 547–565 decreased the affinity of GRK5 for rhodopsin −3.1-fold and reduced the Vₘₐₓ −1.6-fold (data not shown). Neither of these peptides had a significant effect on phosphatidylinositol phosphorylation (Fig. 3C).

The ability of residues 552–562 of GRK5 to promote interaction with phospholipids was also shown using a series of GST-GRK5 fusion proteins. A fusion protein containing residues 489–551 of GRK5 (GST-GRK5₄₈₉–₅₅₁) displayed no ability to bind phospholipid vesicles (<5% bound), whereas the addition of 11 residues (GST-GRK5₄₈₉–₅₆₂) resulted in substantial phospholipid binding (>70% protein bound; Fig. 3D). The binding of GRK5 to phospholipid appears to be predominantly attributable to ionic interactions, because sodium chloride concentrations >300 mM significantly inhibit phospholipid binding of both GRK5 and GST-GRK5₄₈₉–₅₆₂ (data not shown).

Role of the C-terminal Phospholipid Binding Domain in GRK5 Localization—To assess the potential role of the C-terminal phospholipid binding domain in the intracellular localization of GRK5, GRK5 was transiently expressed in COS-1 cells and visualized by immunofluorescence microscopy. The distribution of wild-type GRK5 is consistent with a significant fraction of the kinase being associated with the plasma membrane (Fig. 4, A and B). Indeed, the distribution of GRK5 appears similar to that of an epitope-tagged β₄-adrenergic receptor (Fig. 4G, GFP-β₄AR), a transmembrane protein that is primarily localized at the plasma membrane. GRK5₁₅₅₁ had a distribution virtually identical to that of wild-type GRK5 (Fig. 4, C and D), confirming the ability of this protein to associate with the phospholipid bilayer. In contrast, the expression pattern of GRK5₁₅₅₁ displayed a distribution consistent with a cytosolic localization (Fig. 4, E and F). Indeed, the distribution of GRK5₁₅₅₁ was similar to that of GFP (Fig. 4H), a soluble protein that when overexpressed localizes to the cytosol and nucleus. These results demonstrate that the C-terminal phospholipid binding domain of GRK5 (residues 552–562) plays a critical role in the intracellular localization of this kinase.

Role of the C-terminal Domain in GRK5 Regulation—Previous studies have demonstrated that PKC phosphorylation of GRK5, primarily within residues 565–572, results in significant inhibition of GRK5 activity (9). Because calmodulin-stimulated autophosphorylation also significantly inhibits GRK5 activity (13), we examined the aforementioned deletion mutants to determine whether the major site autophosphorylated in the presence of calmodulin also resided within the C-terminal domain of GRK5. Although the deletion of 5 C-terminal...
residues (GRK51–585) had no effect on calmodulin-stimulated autophosphorylation, the deletion of 7 additional residues (GRK51–578) nearly abolished autophosphorylation (Fig. 5A).

This finding suggests that residues autophosphorylated in the presence of calmodulin lie within amino acids 579–585 of GRK5. This region includes Ser\textsuperscript{579}, Ser\textsuperscript{583}, and Ser\textsuperscript{584} and is
calmodulin. GRK5 expressed in Sf9 cells and purified was labeled with $^{32}$P via autophosphorylation in the presence of phospholipids. The cells were harvested 48 h after transfection, and protein extracts enriched in GRKs were then used for autophosphorylation. We recently identified a C-terminal site within GRK5 that also interacts with calmodulin (residues 540–578) (18). However, a mutation within the N-terminal calmodulin binding domain, W30A/K31Q, which caused a >10-fold decrease in affinity of this site for calmodulin, had no effect on calmodulin inhibition of rhodopsin phosphorylation by GRK5 (18). Similarly, the W30A/K31Q mutation had no effect on calmodulin-stimulated autophosphorylation of GRK5 (Fig. 5A). This finding suggests that calmodulin interaction with the C-terminal binding domain on GRK5 likely plays the major role in promoting GRK5 autophosphorylation.

To further define the role of the C-terminal calmodulin binding domain of GRK5, we tested the effect of calmodulin on the various C-terminal deletion mutants of GRK5. Calmodulin inhibited rhodopsin phosphorylation by wild-type GRK5 with an $IC_{50}$ of $0.25 \mu M$ (Fig. 5, C and D). GRK5$_{1–578}$ and GRK5$_{1–562}$ were inhibited in a manner similar to that of the wild-type kinase. In contrast, the deletion of residues 552–562 caused an ~3.5-fold reduction in calmodulin inhibition of GRK5 ($IC_{50}$, ~0.9 $\mu M$), whereas the deletion of residues 541–551 had no additional effect. These results suggest that the C-terminal calmodulin binding domain within GRK5 (residues 552–562) substantially overlaps with the phospholipid binding domain on GRK5.

**DISCUSSION**

GRKs play an important role in regulating numerous G protein-coupled receptors. Not surprisingly, the activity of

**FIG. 5.** **Mapping of calmodulin-stimulated autophosphorylation and calmodulin binding sites in GRK5.** A, COS-1 cells were transiently transfected with either vector pcDNA3 (vect) or expression constructs containing cDNAs for wild-type GRK5 (wt) or the indicated mutant forms of GRK5. The cells were harvested 48 h after transfection, and protein extracts enriched in GRKs were then used for autophosphorylation reactions in the presence or absence of calmodulin (CaM). Proteins were immunoprecipitated with polyclonal GRK5 antiserum, separated on a 10% SDS-polyacrylamide gel, and visualized by autoradiography. B, immunoprecipitation of GRK5 peptide autophosphorylated in the presence of calmodulin. GRK5 expressed in Sf9 cells and purified was labeled with $^{32}$P via autophosphorylation in the presence of phospholipids (CaM). The reactions were then split into two equal aliquots. One aliquot was incubated with trypsin for 20 min at 30 °C before trypsin inhibitors were added (filled bars), whereas the second aliquot was incubated without trypsin (open bars). Reactions were then used for immunoprecipitation with polyclonal antibodies against GRK5 residues 571–590 (GRK5 Ab) or GRK6 residues 525–544 (GRK6 Ab). The amount of $^{32}$P bound was determined by counting pellets and expressed as a percentage of the amount precipitated with GRK5 antibody in the absence of trypsin treatment. The GRK5 peptide sequence used to generate the antibodies is shown at the bottom. C and D, calmodulin interaction with the wild-type and truncated forms of GRK5. COS-1 cells were transiently transfected with the expression vector pcDNA3 containing cDNAs for wild-type (1–590) and truncated forms of GRK5. The cells were harvested 48 h after transfection, and protein extracts enriched in GRKs were used to phosphorylate ROS membranes (8 $\mu$g rhodopsin) in the presence of the indicated concentrations of calmodulin (CaM). Proteins were separated on a 10% SDS-polyacrylamide gel and visualized by autoradiography, and $^{32}$P incorporation was determined by excising and counting the radioactive bands. The activity of GRK5 in the presence of calmodulin is expressed as a percentage of the rhodopsin phosphorylation in the absence of calmodulin.

distinct from the primary sites phosphorylated by PKC.

To confirm that the major calmodulin-stimulated autophosphorylation site is located at the extreme C terminus of GRK5, we used an antibody generated against residues 571–590 that is capable of immunoprecipitating the kinase. $^{32}$P-Labeled GRK5 was initially generated via autophosphorylation in the presence of either phospholipids or calmodulin. Each sample was then divided in half, incubated with or without trypsin for 20 min at 30 °C, quenched with trypsin inhibitors, and subjected to immunoprecipitation. Trypsin treatment should result in cleavage after lysine 570 and thus release the C-terminal 20-amino acid peptide recognized by the antibody. Indeed, trypsin treatment significantly reduced the amount of $^{32}$P immunoprecipitated from the reaction with GRK5 autophosphorylated in the presence of phospholipids (Fig. 5B). The major sites phosphorylated under these conditions were determined to be Ser$^{548}$ and Thr$^{546}$ (5, 17). In contrast, trypsin digestion had no effect on the amount of $^{32}$P immunoprecipitated from the reaction with GRK5 autophosphorylated in the presence of calmodulin (Fig. 5B). This demonstrates that the calmodulin-stimulated autophosphorylation sites lie within the antibody recognition sequence (i.e. amino acids 571–590).

Previously we reported that GRK5 has a calmodulin binding domain at the N terminus of the protein (residues 20–39) (13). We recently identified a C-terminal site within GRK5 that also interacts with calmodulin (residues 540–578) (18). However, a
GRKs themselves are also regulated. Although some regulatory mechanisms appear common among GRK subtypes, others are unique. Because GRKs are cytoplasmic proteins, they require a mechanism enabling their recruitment to the plasma membrane where GPCRs reside. Different GRKs use distinct mechanisms to regulate their membrane association (4). GRK1 possesses a C-terminal CAAX motif (CVLS) that directs farnesylation (15-carbon isoprenoid) and carboxylmethylation of the kinase (19, 20), which are critical for GRK1 membrane association and activity (20, 21). GRK2 and GRK3 contain a C-terminal pleckstrin homology domain that directs association of these kinases with both Gβγ subunits and acidic phospholipids (22–27). GRK4 and GRK6 are palmitoylated at C-terminal cysteine residues (28, 29). Mutagenesis of the palmitoylation sites in GRK6 results in a reduced affinity for lipid membranes and reduced receptor phosphorylation (30). Although no lipid modifications have been identified for GRK5, the protein readily associates with phospholipid membranes. The C-terminal polybasic 102-amino acid domain has been implicated in phospholipid binding (5). However, simultaneous mutation of 5 basic residues within this domain has no apparent effect on GRK5-promoted receptor phosphorylation (6). In this study we provide a detailed analysis of the role of the C-terminal domain of GRK5 in receptor phosphorylation and association with phospholipid membranes.

The deletion of 39 C-terminal residues of GRK5 (amino acids 552–590) had no direct effect on the catalytic activity of the kinase, inasmuch as phosphorylation of a soluble substrate such as phosvitin was unchanged. However, this deletion produced a ~30-fold decrease in the affinity of GRK5 for phospholipids. The phospholipid binding properties of this region of GRK5 appear to be primarily attributed to an 11-amino acid region of the kinase (residues 552–562). Indeed, addition of this domain to a GST-GRK5 construct was sufficient to promote phospholipid binding of the resulting fusion protein, and the peptide containing this sequence inhibited kinase interaction with liposomes. These data provide compelling evidence that residues 552–562 constitute a phospholipid binding domain of GRK5 critical for its interaction with membranes.

The binding of GRK5 to phospholipids likely involves an electrostatic interaction between negatively charged phospholipid head groups and the positively charged basic residues of the electrostatic nature of this interaction. The C-terminal phospholipid binding domain of GRK5 contains several basic residues (Arg553, Lys556, Arg557, and His559), with Lys547, Lys548, and Lys564 also possibly involved in phospholipid interaction. A recent review speculated that this region of GRK5 might be involved in interaction with phosphatidylinerine (4). However, the addition of 5% phosphatidylserine to the crude phospholipid vesicles used in our assays had no effect on GRK5 binding (data not shown). Thus, although our data clearly demonstrate that residues 552–562 of GRK5 constitute a phospholipid binding domain, the nature of the lipids interacting with this domain remains to be determined.

Interestingly, similar regions of GRK4 (residues 554–566) and GRK6 (residues 553–565) display a significant level of homology with the phospholipid binding domain of GRK5, although they also contain cysteine residues that appear to be palmitoylated (Fig. 6A). In contrast, the comparable C-terminal region of GRK1 retains little homology with the other GRKs, although it possesses a CAAX motif for farnesylation (Fig. 6A). Thus, the C-terminal regions of GRK1, 4, 5, and 6 appear involved in phospholipid interaction. One might speculate that this region evolved as a direct phospholipid binding domain.

That GRK5 interaction with PIP2 may involve residues 22–27 (6). Our data confirm that PIP2 enhances GRK5 binding to membranes, because the addition of 5% PIP2 to crude phospholipids results in an ~3-fold increase in the affinity of GRK5 for phospholipid vesicles. PIP2 has an even larger effect on GRK51–551 (which lacks the C-terminal lipid binding domain), increasing affinity for liposomes ~13-fold. It is possible that the binding of GRK51–551 to crude phospholipids is attributable to the presence of trace amounts of PIP2 in the crude lipid preparation. However, the exact location of the PIP2 binding site remains to be confirmed, because GST-GRK51–200 displayed significantly weaker association with lipid vesicles compared with GST-GRK5489–590 and this association was not enhanced by the presence of 5% PIP2 (data not shown).

The C-terminal phospholipid binding domain also appears to be critical for the intracellular localization of GRK5 (Fig. 4). Wild-type GRK5 expressed in COS-1 cells displays a high degree of localization at the plasma membrane, suggesting that GRK5 is primarily membrane bound in intact cells. In contrast, the deletion of residues 552–562 results in a primarily cytosolic distribution of the kinase. These data suggest that interaction with PIP2 is not sufficient for GRK5 binding to the native membrane, and that the C-terminal lipid binding domain is critical for constitutive membrane association. PIP2 interaction might be involved in targeting GRK5 to specific regions of the cell with high local concentrations of this lipid.

Not surprisingly, the C-terminal phospholipid binding domain also appears important for the function of GRK5. Although deletion of residues 552–562 had no effect on phosphorylation of the soluble protein phosvitin, it resulted in a 6-fold higher $K_v$, and 2.5-fold lower $V_{max}$ for phosphorylation of membrane-bound rhodopsin. The higher $V_{max}$ of GRK51–562 versus GRK51–551 for rhodopsin phosphorylation indicates that interaction of the C-terminal domain with phospholipids not only enhances kinase association with membrane but also increases the catalytic activity of GRK5 for GPCR phosphorylation. Interestingly, the deletion of residues 552–562 had a significantly larger effect on the affinity of GRK5 for phospholipid vesicles compared with ROS membranes. Compared with GRK51–562, GRK51–551 had a >30-fold lower affinity for liposomes and only an ~6-fold lower affinity for rhodopsin. Similarly, the inhibition of phospholipid interaction by the peptide 547–565 resulted in only an ~3-fold reduction in the affinity of GRK5 for rhodopsin. This implies that other regions of GRK5 contribute to kinase binding to ROS membranes, most likely via interaction with rhodopsin, and is consistent with previous findings demonstrating GRKs can interact with cytosolic loops of GPCRs in the absence of lipids (31, 32).

Recent evidence shows that GRK activity is regulated by calcium-binding proteins. GRK1 is specifically inhibited by the Ca$^{2+}$-binding protein recoverin (10), whereas the other GRKs can be selectively inhibited by calmodulin (11–13). Calmodulin binds to GRK5 with high affinity and inhibits both 1) GRK5 binding to ROS membranes and phospholipid vesicles and 2)
GRK5 phosphorylation of rhodopsin (11, 13). Calmodulin appears to bind to two distinct domains on GRK5, one in the N terminus (residues 20–39) (13) and one in the C terminus (residues 540–578) (18). In the present study we demonstrate that GRK5−−662 is as sensitive to calmodulin as is GRK51−−578 and wild-type GRK5, whereas GRK51−−551 has reduced sensitivity to calmodulin. These results suggest that the C-terminal calmodulin binding site resides within residues 552–562, the same domain involved in phospholipid binding. Thus, calmodulin binding to the C-terminal site would be predicted to inhibit both GRK5 interaction with phospholipid and, consequently, phosphorylation of receptor substrates. This overlap of calmodulin and phospholipid binding domains may serve as a general mechanism for calmodulin-dependent regulation of proteins. Several calmodulin-binding proteins, such as GAP-43 (33), MARCKS (34), and nitric oxide synthase (35), have calmodulin binding sites that are directly involved in phospholipid interaction.

The other manner in which calmodulin affects GRK5 activity is via kinase autophosphorylation (13). Calmodulin-stimulated autophosphorylation directly inhibits GRK5 phosphorylation of rhodopsin and binding to ROS membranes. The sites autophosphorylated in the presence of calmodulin lie within the sequence 578−−585 of GRK5, downstream of the calmodulin-binding domain (residues 552–562). Calmodulin binding may induce a conformational change in the C-terminal domain of GRK5, positioning it close to the catalytic site. The calmodulin-stimulated autophosphorylation sequence is also located next to the region phosphorylated by PKC (amino acids 565–572). The close proximity of these two sites may explain why calmodulin-stimulated autophosphorylation and PKC phosphorylation have almost identical inhibitory effects on GRK5 activity. Based on these results, we can speculate about the role of the extreme C-terminal domain (residues 563–590) in GRK5 regulation (Fig. 6A). It seems unlikely that this sequence is involved in a direct interaction with either phospholipids or substrates, because deletion of this domain has no inhibitory effect on the kinase. In fact, deletion of this region results in a 3–4-fold increased affinity of GRK5 for both phospholipids and rhodopsin. Thus, we postulate that this region of GRK5 may function as an autoinhibitory domain to inhibit the basal activity of GRK5. Deletion of only 5 residues (amino acids 586–590) is sufficient to enhance GRK5 interaction with phospholipids and rhodopsin. The region upstream (residues 565–584) seems to work as an “enhancer” of autoinhibitory function. When calcium concentrations inside the cell increase, this results in increased phosphorylation of GRK5 either through activation of PKC or via calmodulin-stimulated autophosphorylation (Fig. 6B). The resulting phosphorylated residues might then be able to interact with upstream basic residues, “locking” the C-terminal peptide into an inhibitory conformation.

Amino acid alignment of the GRK4 subfamily reveals that GRK5 has a 17-residue extension that is not present in GRK4 or GRK6 (Fig. 6A). Thus, the presence of this autoinhibitory domain may be unique to GRK5. GRK4 and GRK6 are palmitoylated, a modification that has been demonstrated to be important for GRK6 function (30). Because palmitoylation is a reversible modification, one might speculate that depalmitoylation would promote GRK4 and GRK6 dissociation from the membrane, effectively turning these kinases off. Because
GRK5 appears to interact with phospholipids via basic residues and displays an apparent constitutive membrane association, it likely requires some other mechanism of regulation. It is possible that the autoinhibitory domain has evolved to decrease phosphorylation of nonactivated receptors and to provide a way to turn GRK5 off in a Ca\(^{2+}\)-dependent manner. Fig. 6B summarizes the mechanisms that could lead to inhibition of GRK5 upon activation of receptors linked to phospholipases.

In conclusion, our studies describe two important structural domains in the C terminus of GRK5 that significantly contribute to kinase function. Residues 552–562 form a phospholipid binding domain, which overlaps with a calmodulin binding site, and is important for the intracellular localization of the GRK5. Residues 563–590 constitute an autoinhibitory domain, the efficacy of which is enhanced by PKC phosphorylation or calmodulin-stimulated autophosphorylation.

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