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Role of the Amino Terminus of G Protein-Coupled Receptor Kinase 2 in Receptor Phosphorylation[†]

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ABSTRACT: G protein-coupled receptor kinases (GRKs) specifically phosphorylate activated G protein-coupled receptors. While the X-ray crystal structures of several GRKs have been determined, the mechanism of interaction of GRK with GPCRs is currently unknown. To further characterize the role of the GRK2 amino terminus in receptor interaction and phosphorylation, we generated a series of point mutations within the first 10 amino acids of GRK2 and tested their ability to phosphorylate receptor and nonreceptor substrates. Although all mutants exhibited some impairment in receptor phosphorylation, three of the mutants, D3K, L4A, and D10A, were the most severely affected. Using the β_2-adrenergic receptor and rhodopsin as receptor substrates and tubulin as a nonreceptor substrate, we demonstrated that the kinase activity toward the receptors was severely decreased in the mutants, while they fully retained their ability to phosphorylate tubulin. Moreover, the amino-terminal mutants were able to bind to the receptor but, in contrast to wild-type GRK2, were not activated by receptor binding. A synthetic peptide containing residues 1−14 of GRK2 served as a noncompetitive inhibitor of receptor phosphorylation by GRK2, while a comparable peptide from GRK5 had no effect on GRK2 activity. Secondary structure prediction and circular dichroism suggest that the GRK2 amino-terminal peptide forms an amphipathic α-helix. Taken together, we propose a mechanism whereby the extreme amino terminus of GRK2 forms an intramolecular interaction that selectively enhances the catalytic activity of the kinase toward receptor substrates.

G protein-coupled receptors (GPCRs [1]) constitute a large class of plasma membrane proteins that respond to a wide variety of stimuli, including hormones, odorants, peptides, and lipids (*1*). Agonist binding transduces the signal into the cell by promoting interaction of the receptor with heterotrimeric G proteins, which subsequently activate effectors such as adenylyl cyclase, phosphodiesterases, phosphatidylinositol 3-kinase, and various ion channels. Cellular responsiveness to external stimuli is tightly regulated. In GPCR-mediated signaling, the waning of receptor responsiveness to agonist is a key regulatory mechanism known as desensitization (*2*). Additional regulatory processes such as receptor endocytosis and downregulation limit the cellular response to continued stimuli by reducing the number of receptors on the cell surface (*3*, *4*). Some of these events are regulated by a family of serine/threonine protein kinases called GPCR kinases (GRKs) that specifically phosphorylate agonist-occupied receptors (*5*, *6*). Receptor phosphorylation by GRKs promotes the binding of arrestins, which effectively uncouple the receptor from the G protein and terminate signaling (*7*).

The seven mammalian members of the GRK family can be divided into three subfamilies based on their structural differences: (i) GRK1 and GRK7, (ii) GRK2 and GRK3, and (iii) GRK4−GRK6. All GRKs share a common topological structure that includes an N-terminal regulator of G protein signaling homology (RH) domain, a central kinase catalytic domain, and a C-terminal membrane targeting domain. GRK2 is the most extensively characterized member of the family and is ubiquitously expressed in mammals. GRK2 phosphorylates a variety of GPCRs and can be activated by numerous signaling molecules, including activated GPCRs, G$\beta\gamma$ subunits, and phospholipids (*8*−*12*).

The X-ray crystal structures of GRK2 (*13*), GRK2/G$\beta_1\gamma_2$ (*13*, *14*), GRK2/G$\beta_1\gamma_2$/Gαq (*15*), GRK6 (*16*), and GRK1 (*17*) have provided significant insight into GRK function. Interestingly, the crystal structure of GRK2 reveals that the N-terminal RH domain, central catalytic domain, and C-terminal pleckstrin homology domain form an equilateral triangle that is ∼80 Å on a side. The RH domain contacts both the kinase and PH domains and consists of two discontinuous regions with the characteristic nine-helix bundle in the N-terminal region and two additional helices following the kinase domain. The interaction surface of GRK2 with Gαq primarily resides on the α5 and α6 helices of the RH domain (*15*, *18*). The kinase domain is most similar to that of PKA, PKB, and PDK1 but appears to be in an inactive conformation in the crystal structure. This inactive conformation is also observed in the structure of GRK6 even when bound to AMPPNP and GRK1 when bound to ATP (*16*, *17*).

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[1]Abbreviations: α_{2A}AR, α_{2A}-adrenergic receptor; β_2AR, β_2-adrenergic receptor; CD, circular dichroism; GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; RH domain, regulator of G protein signaling homology domain; Rho*, light-activated rhodopsin; TFE, trifluoroethanol.

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Interestingly, the structures of the RH and kinase domain cores of GRKs appear to have similarities to the inactive structure of Src with the α10 helix of the RH domain potentially functioning to regulate GRK activation (*14*, *16*). Similarly, the interface between the α4−α5 loop of the RH domain and the αJ helix of the kinase large lobe might also modulate GRK activity by influencing the orientation of the large and small kinase lobes. It is worth noting that the crystal structures of GRK2 and GRK6 reveal that the extreme amino terminus and the nucleotide gate in the kinase domain are disordered (*14*, *16*), although residues 5−30 are observed in GRK1·$(Mg^{2+})_2$·ATP (*17*). The nucleotide gate is predicted to make contacts with the active site cleft, promoting the active conformation of the kinase, while the extreme amino terminus is required for efficient receptor phosphorylation and may be involved in the structural transition from the inactive to the active state of the kinase (*19*, *20*).

We and others have previously shown that GRK2 can interact with several domains in the receptor (*21*−*23*); however, the sites on GRK2 that are critical for receptor interaction remain poorly defined. Previous studies have suggested that the amino terminus of GRK is required for efficient receptor phosphorylation. For example, antibodies specifically targeting the amino terminus of GRK1 blocked phosphorylation of light-activated rhodopsin but did not disrupt phosphorylation of a peptide substrate (*24*). In addition, amino-terminal truncation of GRK1, GRK2, and GRK5 resulted in the loss of receptor phosphorylation, although the GRK1 mutant was still able to bind to the receptor (*19*, *20*). Studies from Ferguson and co-workers suggested a role for the GRK2 RH domain in binding the metabotropic glutamate receptor and reported that mutation of Asp-527 in GRK2 disrupted receptor binding (*25*). In another study, a role for the 15 GRK1 amino-terminal residues in receptor phosphorylation was suggested by the ability to bind to recoverin and sterically block the interaction of GRK1 with rhodopsin (*26*). Taken together, these studies suggest a regulatory role for the GRK amino terminus in receptor phosphorylation.

Recent studies have also started to identify some of the GRK-specific residues within the catalytic domain that contribute to receptor phosphorylation. For example, a V477D mutation in the AGC kinase C-tail of GRK2 was defective in receptor phosphorylation and receptor-mediated activation, although it also had reduced activity against nonreceptor substrates (*27*). Similarly, Huang et al. identified a number of residues in the GRK1 catalytic domain, including Phe-190, Arg-191, Leu-212, Tyr-274, Val-476, and Val-484, that when mutated were defective in receptor phosphorylation and also had decreased activity against peptide substrates (*28*). Interestingly, the GRK1-R191A mutant also had a >10-fold increase in its K_m for rhodopsin phosphorylation, suggesting an important role for this residue in receptor binding. Since similar catalytic defects were observed with an N-terminal deletion, the authors proposed a model in which the amino terminus interacts with a surface on the GRK1 catalytic domain that stabilizes the closed active conformation of the kinase (*28*).

In this study, we further delineate the role of the amino terminus of GRK2 in receptor phosphorylation and propose that the GRK2 N-terminal region forms an intramolecular interaction that regulates kinase activity toward receptor substrates. To test this, we generated a series of amino-terminal GRK2 mutants and found that three of the mutants (D3K, L4A, and D10A) were severely defective in both receptor-promoted activation and receptor phosphorylation. Interestingly, these mutants were fully capable of binding to the β_2-adrenergic receptor (β_2AR) and phosphorylating a nonreceptor substrate with activities comparable to that of wild-type GRK2. In addition, we show that a peptide composed of the first 14 residues of GRK2 forms an amphipathic α-helix and that this peptide inhibits receptor phosphorylation and enhances the binding of GRK2 to phospholipids. These studies provide insight into the mechanism of GRK2 activation by GPCRs and suggest that the amino terminus of GRK2 forms an intramolecular interaction that modulates the catalytic activity of the kinase toward receptor substrates.

MATERIALS AND METHODS

Plasmid Construction. Amino-terminal mutants generated by PCR were digested with EcoRI and KpnI, purified, and inserted into EcoRI- and KpnI-digested pcDNA3-GRK2. DNA sequences were verified by dideoxy chain sequence analysis. D3K, L4A, and D10A were also cloned into the pFastBac vector for expression in Sf9 insect cells.

GRK2 Expression and Purification. GRK2 and amino-terminal mutants were overexpressed and purified from Sf9 insect cells as previously detailed (*29*). Briefly, cells were harvested by low-speed centrifugation 48 h after infection, and the pellet was homogenized in 20 mM Hepes (pH 7.2), 250 mM NaCl, 10 mM EDTA, 1 mM phenylmethanesulfonyl fluoride, 10 μg/mL leupeptin, 0.2 mg/mL benzamidine, and 0.02% Triton X-100 followed by high-speed centrifugation. The supernatant was diluted and applied to an SP-Sepharose column, and the column was washed and eluted with a 50 to 300 mM NaCl gradient. Peak fractions were pooled, loaded onto a heparin-Sepharose CL-6B column, and eluted with a 100 to 600 mM NaCl gradient. Peak fractions were pooled, aliquoted, and stored at −80 °C. Protein purity was determined by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and Coomassie blue staining.

GRK2 Substrate Phosphorylation. GRK2 (20 nM) was incubated at 30 °C for the indicated time with substrate [1−2 μM light-activated rhodopsin (Rho*), 50 nM β_2AR in mixed micelles in the absence or presence of 10 μM isoproterenol, or 0.1−5 μM tubulin] in 20 mM Tris-HCl (pH 7.5), 2 mM EDTA, 5 mM $MgCl_2$, 0.2 mM ATP, and 1−2 μCi of [γ-^{32}P]ATP in a final volume of 20 μL. Reactions were stopped by the addition of 5 μL of SDS sample buffer and incubation at room temperature for 30 min. Samples were then electrophoresed on a 10% polyacrylamide gel; the gel was dried and autoradiographed, and ^{32}P-labeled proteins were excised and counted by scintillation.

Receptor Binding Assay. A tagged human β_2AR was expressed in Sf9 insect cells and purified as previously described (*30*). Purified phosphatidylinositol (PI) was resuspended in 20 mM Tris-HCl (pH 7.4) and 2 mM EDTA to a final concentration of 10 mg/mL, and vesicles were formed by sonicating the sample on ice three times for 1 min. The purified β_2AR (10 pmol) in 10 μL of buffer containing 20 mM Hepes (pH 7.5), 100 mM NaCl, 1 mM EDTA, 100 μM FLAG peptide, 15% glycerol, and 0.1% dodecyl maltoside was added to PI vesicles (2 μL of a 10 mg/mL solution) and incubated on ice for 20 min to allow insertion of the receptor into the phospholipid/detergent mixed micelles as previously described (*9*). This preparation of the β_2AR was used in both receptor binding and phosphorylation assays. GRK2 (20 nM) was added to the β_2AR/PI mixture in a buffer containing 20 mM Tris-HCl (pH 7.5) and 50 mM NaCl

and then incubated at room temperature for 15 min. The total volume of the incubations was 40 μL, and the final concentrations of all key components in the assay were 20 mM Tris-HCl (pH 7.5), 60 mM NaCl, 0.25 μM β_2AR, 20 nM GRK2, 0.5 mg/mL PI, and 0.025% dodecyl maltoside. The sample was centrifuged at 100000*g* for 20 min, and equal portions of the supernatant and pellet were resuspended in sample buffer. Equal volumes of the supernatant and pellet fractions were assessed via SDS−PAGE, transferred to nitrocellulose, and detected by immunoblotting using a GRK2/3 monoclonal antibody (Upstate Biotechnology) at a 1:10000 dilution.

Activation of GRK2 by Rhodopsin. The peptide RRRA-SAAASAA was synthesized by the solid state Merrifield method on an Applied Biosystems automated synthesizer and purified by reverse phase high-performance liquid chromatography. Activation assays (20 μL) were conducted by incubating 1 mM synthetic peptide with 0.2 μM GRK2 and 2 μM Rho* for 10 min at 30 °C in a buffer containing 20 mM Tris-HCl (pH 7.5), 2 mM EDTA, 5 mM $MgCl_2$, 0.2 mM ATP, and 1−2 μCi of [γ-^{32}P]ATP. Control incubations were performed in the absence of the peptide. Reactions were quenched by the addition of trichloroacetic acid to a final concentration of 15% and then mixtures centrifuged for 10 min at 14000*g* to remove phosphorylated rhodopsin. Supernatants (10 μL) were spotted onto P81 paper and washed six times with 75 mM phosphoric acid. GRK2 activity was defined as the amount of phosphate incorporated into the peptide.

Synthesis of GRK Peptides and Peptide Inhibition Studies. The wild-type (Wt) GRK2 peptide corresponds to the first 14 residues of GRK2 (MADLEAVLADVYSL), while the Pro peptide has a proline at position 7 in place of valine (MADLEAPLADVYSL). The GRK5 peptide corresponds to the first 13 residues of GRK5 (MELENIVANTLLK). Peptides were synthesized by the solid state Merrifield method and purified by reverse phase high-performance liquid chromatography. Various concentrations of peptide (0−200 μM) were tested in phosphorylation and binding assays, and kinetic studies used 1−15 μM rhodopsin in the presence or absence of 60 μM GRK2 peptide.

Circular Dichroism. The circular dichroism (CD) spectra of the GRK2 Wt and Pro peptides were recorded on a Jasco-810 spectrapolarimeter at 4 °C in the range of 200−280 nm using cuvettes with a path length of 1.0 cm. The acquisition parameters were 1 nm/min with a 4 s response and a 1 nm bandwidth. Peptides were diluted in a phosphate-buffered saline solution (pH 7.0) to 16 μM with various amounts of trifluoroethanol (TFE). Molar ellipticities were determined using the formula $[\theta] = [\theta]_{obs}(MRW)/(10lc)$, where $[\theta]_{obs}$ is the observed ellipticity in millidegrees, MRW is the mean residue weight, *l* is the cell path length in centimeters, and *c* is the peptide concentration in milligrams per milliliter. The relative helix content was deduced according to the method of Chen et al. (*31*) as the percentage of helix $= [\theta]_{222}/[\theta]^{max}_{222}(1 - k/n)$ (in degrees square centimeters per decimole), where $[\theta]_{222}$ is the observed mean residue ellipticity at 222 nm, $[\theta]^{max}_{222}$ is the theoretical mean residue ellipticity for a helix of infinite length, *n* is the number of residues, and *k* is a wavelength-dependent constant (2.57 for 222 nm).

RESULTS AND DISCUSSION

The Amino-Terminal Domain of GRK2 Is Important for Receptor Phosphorylation. The amino terminus of GRKs has been implicated as playing an important role in receptor phosphorylation. A previous study used a yeast bioassay to screen for GRK mutations that disrupt receptor phosphorylation and found multiple mutations within the first 10 residues of GRK5 (*20*), while another study showed that conserved glutamic acids in GRK1 (Glu-7) and GRK2 (Glu-5) were critical for receptor phosphorylation (*19*). The first 14 residues in the GRKs exhibit significant sequence conservation with an overall level of similarity of 50% between all GRKs, 100% within the GRK2 subfamily (GRK2 and GRK3), and 86% within the GRK4 subfamily (GRK4−GRK6) (Figure 1A). To gain a better understanding of how the amino terminus mediates receptor phosphorylation, we introduced several point mutations within the first 10 amino acids of GRK2. The mutants were expressed in COS-1 cells and then screened for the ability to phosphorylate rhodopsin. The results obtained for three mutants, D3N, D3K, and D10A, are shown in Figure 1B. The mutants were expressed to comparable levels in COS-1 cells, although D10A was somewhat lower, and D3K and D10A were found to be completely defective in their ability to phosphorylate Rho* compared to Wt GRK2 (Figure 1B). Five additional point mutations in GRK2 were also generated, and their ability to phosphorylate Rho* was assessed. All of the mutants tested exhibited a significant defect in mediating receptor phosphorylation (Figure 1C). Interestingly, mutation of Asp-3 to asparagine resulted in an ∼60% decrease in the level of receptor phosphorylation, while mutation to either a neutral amino acid (alanine) or an oppositely charged residue (lysine) resulted in 85 and 95% reduced phosphorylation activity, respectively. This suggests that both the side chain and negative charge of Asp-3 contribute to mediating GPCR phosphorylation. Leu-4 mutations also had ∼95% reduced activity when mutated to either alanine or lysine. The E5A mutant exhibited an ∼50% decrease in the level of Rho* phosphorylation compared to Wt GRK2, while those of the V7A/L8A and D10A mutants were decreased by ∼95%.

Characterization of GRK2 Amino-Terminal Mutants. The β_2AR is readily phosphorylated by GRK2 and has been extensively used as a model receptor for studying GRKs (*32*). To further characterize the mutants with the largest defect in receptor phosphorylation, we expressed GRK2-D3K, -L4A, and -D10A in Sf9 insect cells, purified them to near homogeneity (Figure 2A), and assessed their ability to phosphorylate the purified β_2AR in the presence of the agonist isoproterenol. All three mutants had significantly reduced activity compared to that of wild-type GRK2, with L4A and D10A having ∼90−95% lower activity and D3K ∼60% lower activity (Figure 2B).

To address whether the defect in receptor phosphorylation was due to impaired receptor binding, we developed a GRK2−β_2AR binding assay. Purified β_2AR in phosphatidylinositol/detergent mixed micelles was incubated with purified GRK2. Samples were subjected to high-speed centrifugation, and equal volumes of supernatant and pellet fractions were immunoblotted for GRK2. In the absence of β_2AR, the supernatant fraction contained the vast majority of the GRK2 (Figure 2C). Addition of β_2AR resulted in a significant increase in the level of wild-type GRK2 in the pellet fraction, demonstrating that GRK2 is able to directly bind to the receptor. Activation of the receptor with the agonist isoproterenol had little effect on the ability of GRK2 to interact with the receptor with either no change or an ∼2-fold increase in the level of binding (Figure 2C and data not shown). Thus, GRK2 appears to be able to bind to the β_2AR in the absence of agonist, perhaps reflecting the high concentration of receptor (0.25 μM) used in the binding assay. The D3K, L4A, and D10A

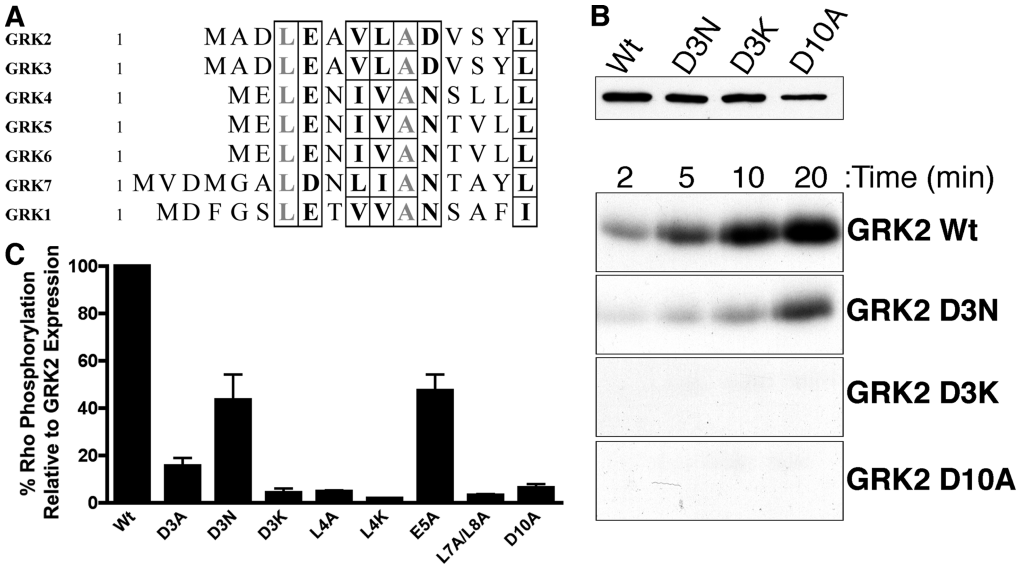

FIGURE 1: First 10 amino acids are critical for receptor phosphorylation. (A) Sequence alignment of the amino-terminal region of GRK1−GRK7. Highlighted in gray are residues that are identical and in black residues that are conserved within the extreme amino terminus. (B) GRK2 mutants were expressed in COS-1 cells, and lysates were used to phosphorylate Rho*. COS-1 cell lysates were separated by SDS−PAGE, transferred to nitrocellulose, and immunoblotted for GRK2 expression. Phosphorylation reactions were performed in a total volume of 20 μL containing 3 μg of COS-1 cell lysate, 2 μM ROS, 20 mM Tris-HCl (pH 7.5), 2 mM EDTA, 5 mM $MgCl_2$, 0.2 mM ATP, and ~1−2 μCi of [γ-^{32}P]ATP. The reaction mixtures were exposed to light to activate rhodopsin, incubated at 30 °C for 2−20 min, and then analyzed via SDS−PAGE and autoradiography. (C) Rhodopsin phosphorylation by GRK2 point mutants was performed as described above and analyzed at the 10 min time point. After autoradiography, bands were excised and counted by scintillation. Data shown are the mean ± the standard deviation of three experiments. The amount of phosphorylation by each mutant relative to that of the wild type was adjusted on the basis of the expression of the constructs as assessed using an Odyssey Infrared Imaging System (level of expression varied < 2-fold).

GRK2 mutants were also able to effectively bind to the β_2AR. Interestingly, the L4A and D10A mutants demonstrated enhanced association with the pellet fraction compared to wild-type GRK2 (Figure 2D). We also used a GST construct containing the third intracellular loop of the α_{2A}-adrenergic receptor (α_{2A}AR) to test binding of the purified GRK2 mutants, since this construct was previously shown to directly bind GRK2 (*22*). The GRK2 mutants were all able to bind to the α_{2A}AR third loop as well as wild-type GRK2 (data not shown). These data suggest that the GRK2 mutants are able to efficiently bind to GPCRs; however, they are impaired in terms of receptor phosphorylation. Thus, the observed defect in receptor phosphorylation does not appear to be caused by deficient receptor binding.

We next tested the kinase activity of these mutants using the nonreceptor substrate tubulin (*33*). All mutants were able to phosphorylate tubulin in a manner comparable to that of Wt GRK2, demonstrating that the mutants are catalytically active and selectively deficient in receptor phosphorylation (Figure 2E). Tubulin provides a good control for these studies since it is a bona fide GRK substrate that reflects both the functional binding and catalytic activity of the kinase (*33*, *34*). In fact, the K_m for the phosphorylation of tubulin by GRK2 (0.4 μM) is comparable to that observed for GRK2 phosphorylation of the β_2AR (0.25 μM), and in striking contrast to the K_m observed for peptide substrates (0.2−2 mM) (*29*, *33*). However, tubulin is not a particularly good substrate for GRK2 (V_{max} = 17−26 nmol min^{-1} mg^{-1}) compared to the β_2AR (1−2 μmol min^{-1} mg^{-1}) and, in fact, is comparable to a good peptide substrate (11 nmol min^{-1} mg^{-1}) (*29*, *33*). This suggests that tubulin likely contains domains that bind GRKs with an affinity comparable to that of a GPCR but that this binding does not promote closure and activation of the catalytic domain.

GRK activity can be modulated by interaction with activated receptors. For example, GRK2 is activated upon binding to agonist-occupied β_2AR or Rho* (*35*). To test whether the GRK2 amino-terminal mutants are activated by rhodopsin, we assayed the ability of the mutants to phosphorylate the peptide substrate RRRASAAASAA (*35*). This assay showed that light-activated rhodopsin was able to activate Wt GRK2 100-fold whereas it had minimal effect on the D3K, L4A, and D10A mutants (Figure 2F). Taken together, these data suggest that despite being capable of binding to the receptor, the GRK2 mutants are not activated by receptor binding and are deficient in phosphorylating receptors.

Residues 1−14 of GRK2 Form an Amphipathic Helix. The X-ray crystal structure of the inactive form of GRK2 has been determined, revealing an equilateral triangle formed by the RH, kinase, and pleckstrin homology domains (*13*, *14*). While the first 29 residues of GRK2 were not observed in the X-ray structure (*13*, *14*), analysis of residues 2−14 of GRK2 using secondary structure prediction algorithms suggests an amphipathic α-helix, where one side is rich in hydrophobic residues and the other side rich in polar residues (Figure 3A). The amino-terminal regions of GRK1 and GRK5 have also been predicted to be α-helices (*20*, *26*). The ability of this region to form an α-helix was examined by circular dichroism using GRK2 peptides corresponding to the first 14 residues of GRK2 (Wt peptide) or the first 14 residues with proline in place of valine at position 7 (Pro peptide). Since the Wt and Pro peptides exhibited minimal helical formation in solution (Figure 3B), trifluoroethanol (TFE), a solvent known to promote and stabilize α-helix formation (*36*, *37*), was added to the peptides. The addition of 25% TFE to the Wt peptide induced an α-helix, as characterized by two minima displayed at 208 and 222 nm (Figure 3C). Increasing the TFE concentration to 50% increased the level of α-helix formation of the peptide to ~44% helicity, while no helix formation was observed for the Pro peptide in the presence of TFE. Thus, the GRK2 N-terminal 14-amino acid region has a propensity to form an α-helix.

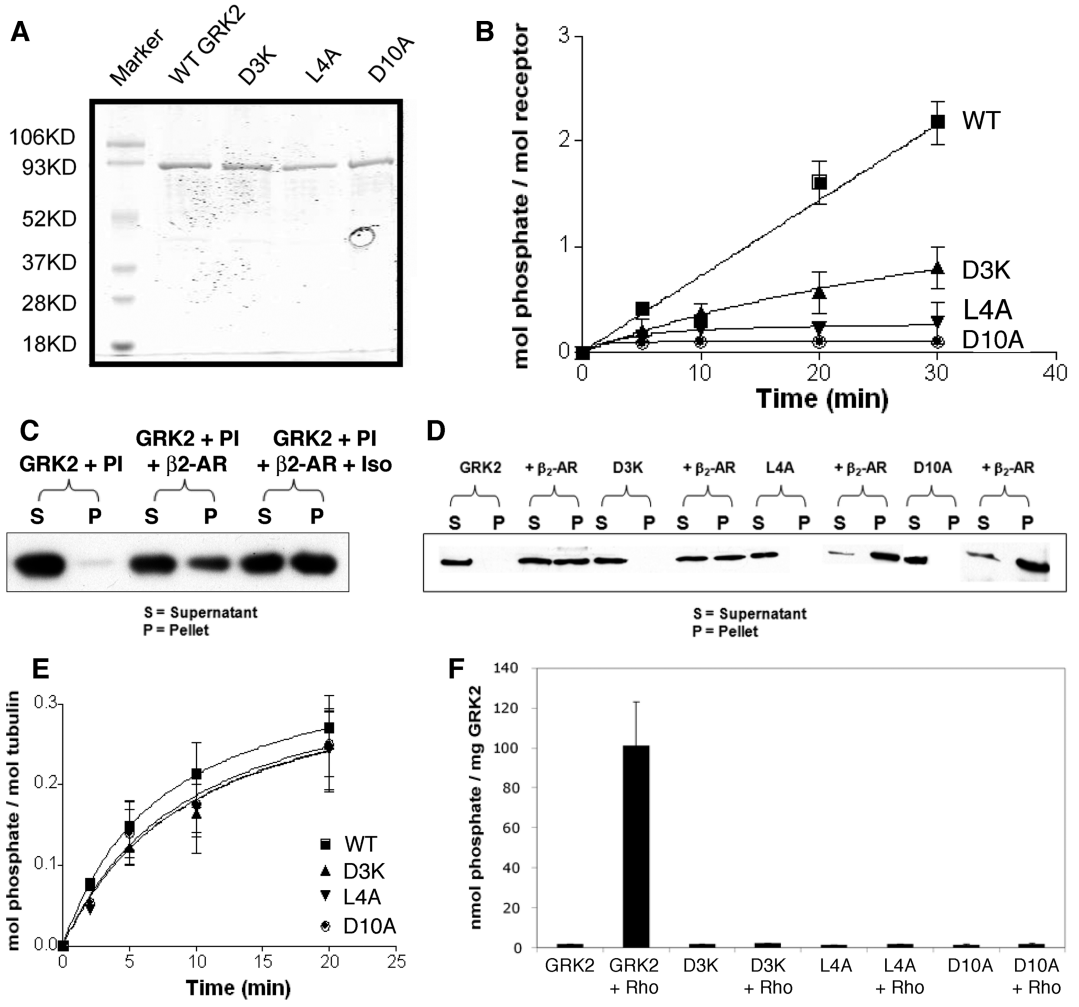

FIGURE 2: GRK2 mutants are specifically defective in receptor phosphorylation. (A) SDS−PAGE of wild-type and mutant GRK2 expressed and purified from Sf9 insect cells. Wild-type GRK2 and the GRK2 mutants D3K, L4A, and D10A were cloned and expressed using the Bac to Bac expression system in Sf9 insect cells and then purified as described in Materials and Methods. One microgram of each purified protein was run on SDS−PAGE and stained with Coomassie blue. (B) Activity of the mutants compared with that of wild-type GRK2. Phosphorylation reactions were performed in a total volume of 20 μL containing 50 nM purified β_2AR in mixed micelles, 20 nM wild-type or mutant GRK2, 10 μM isoproterenol, 20 mM Tris-HCl (pH 7.5), 2 mM EDTA, 5 mM $MgCl_2$, 0.2 mM ATP, and ~1−2 μCi of [γ-^{32}P]ATP. The reaction mixtures were incubated at 30 °C for the indicated times and analyzed by SDS−PAGE and autoradiography. Bands were excised and counted by scintillation. Shown are the means ± the standard deviation from three experiments. (C) Wild-type GRK2 (20 nM) was incubated with mixed micelles, 0.25 μM β_2AR in mixed micelles, or β_2AR in mixed micelles in the presence of isoproterenol. Reaction mixtures were centrifuged at 100000*g* for 15 min. Pellets and supernatants were separated, and equal amounts from each fraction were run on SDS−PAGE and immunoblotted for GRK2 using a monoclonal GRK2/3 antibody. Data are representative of three to five independent experiments. (D) GRK2 mutants bind to β_2AR. Wild-type GRK2 and its mutants (20 nM) were incubated with mixed micelles with or without β_2AR and processed as described above. (E) Phosphorylation reactions were performed in a total volume of 20 μL containing 0.5 μM tubulin, 20 nM wild-type or mutant GRK2, 20 mM Tris-HCl (pH 7.5), 2 mM EDTA, 5 mM $MgCl_2$, 0.2 mM ATP, and ~1−2 μCi of [γ-^{32}P]ATP. The reaction mixtures were incubated at 30 °C for the times indicated and analyzed by SDS−PAGE and autoradiography, and the bands were excised and counted by scintillation. The graph shows the means ± the standard deviation of three experiments. (F) Activation of wild-type or mutant GRK2 by rhodopsin. The synthetic peptide RRRASAAASAA (1 mM) was incubated with 0.2 μM wild-type or mutant GRK2 and 2 μM rhodopsin in a total volume of 20 μL in 20 mM Tris-HCl (pH 7.5), 2 mM EDTA, 5 mM $MgCl_2$, 0.2 mM ATP, and ~1−2 μCi of [γ-^{32}P]ATP. Reaction mixtures were exposed to light to activate rhodopsin, incubated at 30 °C for 10 min, quenched by the addition of 15% trichloroacetic acid, and centrifuged for 10 min at 14000*g*. Supernatants (10 μL) were spotted on P-81 paper and washed six times with 75 mM phosphoric acid. Peptide phosphorylation was assessed by scintillation counting. Data are the means ± the standard deviation from three experiments performed in duplicate.

We hypothesize that the hydrophobic face of the helix, which includes three leucines, two valines, and a serine in GRK2, might be involved in phospholipid binding. The charged face of the helix contains two aspartic acids, one glutamic acid, three alanines, and a tyrosine (Tyr) at position 13 that can be phosphorylated by c-Src (*38*) and would likely contribute to the polarity of the helix. Since Asp-3 and Asp-10 on the polar side of the amphipathic helix do not appear to be involved in receptor binding (Figure 2B), this region might be involved in an intramolecular interaction in the kinase. One region that might interact with the polar face of the N-terminus is the surface of the catalytic domain recently identified to be critical for receptor phosphorylation (*28*). This region includes a number of basic residues with Arg-195 being particularly important in GRK2 function (*28*).

A GRK2 Amino-Terminal Peptide Inhibits Receptor Phosphorylation. To further characterize the GRK2 amino-terminal peptide, we tested whether it could affect GRK2-mediated receptor phosphorylation. We found that the GRK2 peptide effectively inhibited isoproterenol-stimulated β_2AR

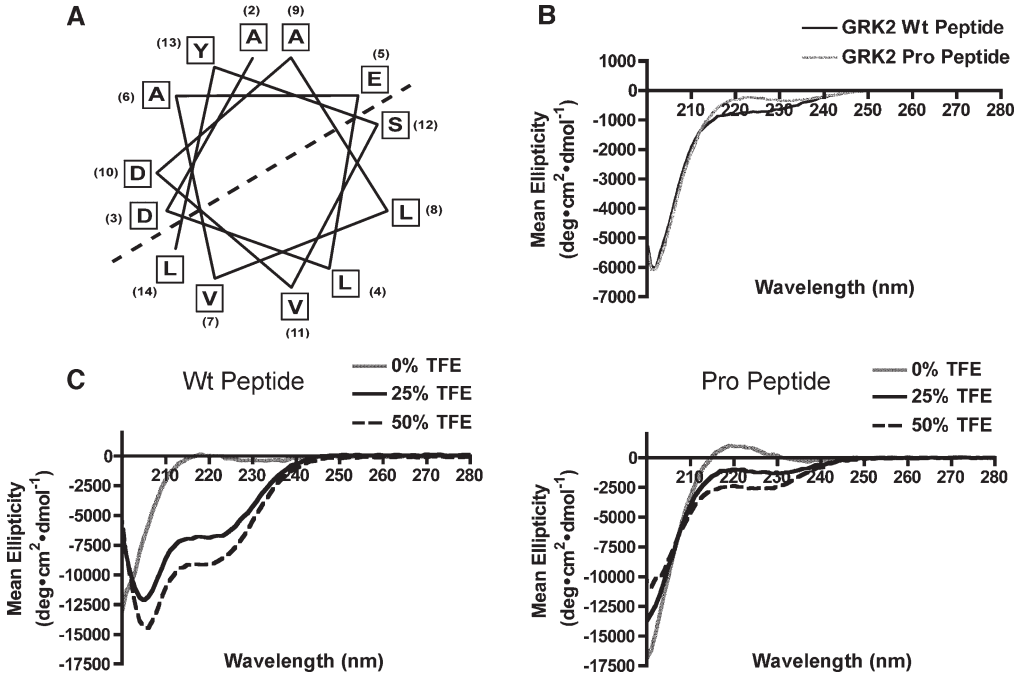

FIGURE 3: First 14 amino acids form an amphipathic α-helix. (A) Secondary structure prediction programs revealed that the first 14 amino acids of GRK2 form an amphipathic α-helix, where one face is rich in polar residues and the other rich in hydrophobic residues as delineated by the dashed line. (B) Representative CD spectra of the GRK2 Wt (black line) and Pro (gray line) amino-terminal peptides were recorded in the range of 200−280 nm at 4 °C using a spectrapolarimeter. (C) Increasing concentrations of the monohydric alcohol TFE increased the helicity of the GRK2 peptide. Data for 0% TFE are shown in gray representing a random coil, data for 25% TFE (solid black line) ~20% helicity, and data for 50% TFE (dashed black line) ~44% helicity.

phosphorylation with an IC_{50} of ~50 μM (Figure 4A). We also used Rho* as a substrate and found a similar pattern of inhibition by the peptide (Figure 4B). In contrast, the peptide had no effect on GRK2 phosphorylation of the nonreceptor substrate tubulin (Figure 4C). Kinetic studies revealed that the peptide inhibited receptor phosphorylation noncompetitively, with no change in the K_m and a decrease in the V_{max} (Figure 4D). These data suggest that the GRK2 amino-terminal peptide may be binding to the kinase itself, since it does not appear to compete for kinase binding to the receptor.

Our previous work using a GRK5 amino-terminal peptide showed that it inhibited phosphorylation of rhodopsin by GRK5 with an IC_{50} of 20 μM but had no effect on GRK2-mediated phosphorylation of rhodopsin (*20*). Here, we verified that the GRK5 peptide had no significant effect on GRK2-mediated phosphorylation of the β_2AR while the GRK2 peptide effectively inhibited β_2AR phosphorylation (Figure 4E). Taken together, these results suggest that the amino-terminal GRK peptides are specific for their respective kinases, lending further support for this region of the kinase being involved in an intramolecular interaction.

Interestingly, addition of the GRK2 peptide to the GRK2−β_2AR binding assay led to an increase in the amount of GRK2 in the pellet fraction, with ~90% in the pellet at 100 μM peptide (Figure 4F, top panel). To test whether the GRK2 peptide enhances the interaction of GRK2 with phosphatidylinositol, the peptide was added to GRK2 and purified phosphatidylinositol in the absence of the receptor (Figure 4F, middle panel). At 50 μM peptide, there was significant association of GRK2 with the pellet fraction, suggesting that the GRK2 amino-terminal peptide enhanced binding of GRK2 to the phospholipid even in the absence of the receptor. When combined with previous findings demonstrating that the GRK5 amino-terminal peptide may directly bind to phospholipids (*20*), our studies suggest an important role for the amino terminus of GRKs in membrane binding.

Proposed Role of the Amino Terminus of GRK2 in Receptor Phosphorylation. In this study, we have shown that mutations in the amino terminus of GRK2 do not affect the catalytic activity or the ability of the kinase to bind to the receptor; however, these mutations effectively inhibit GRK2 activation and receptor phosphorylation (Figures 1 and 2). We also demonstrated that a peptide containing the first 14 residues of GRK2 forms an amphipathic α-helix, inhibits GRK2-mediated receptor phosphorylation, and promotes binding of GRK2 to phosphatidylinositol vesicles (Figures 3 and 4). The amino-terminal region of GRK2 appears to be flexible and unable to anchor to a specific site while the enzyme is in its inactive state as shown in the crystal structure (*13*, *14*). In light of these findings, we propose a novel regulatory mechanism whereby receptor binding regulates the ability of the amphipathic amino-terminal α-helix of GRK2 to anchor GRK2 on the inner leaflet of the cytoplasmic membrane, with the hydrophobic side binding to phospholipids and the polar side forming an intramolecular interaction with GRK2. This intramolecular interaction anchors the flexible amino-terminal region, promoting a conformational change in the catalytic domain, resulting in an active kinase. We further hypothesize that the interaction with the amino-terminal region takes place within the kinase domain of GRK2. This hypothesis is supported by the recent studies identifying a surface on the kinase domain that appears to be critical for receptor phosphorylation (*28*). In these studies, mutation of Arg-191 in GRK1 or Arg-195 in GRK2 results in an ~1000-fold decrease in k_{cat}/K_m. The GRK1-R191A mutant also has a >10-fold increase in the K_m for rhodopsin phosphorylation, suggesting an important role for this residue in receptor

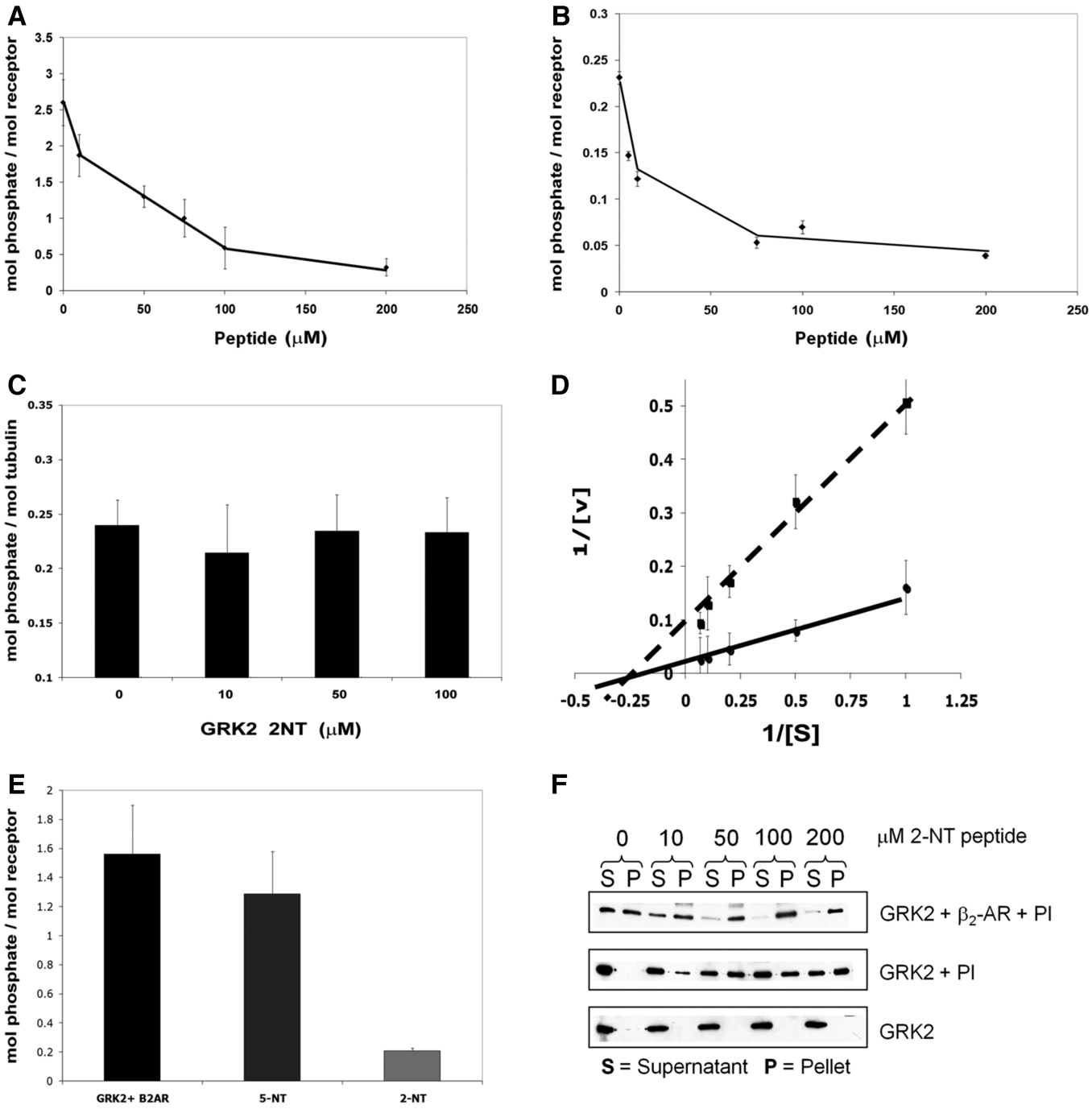

FIGURE 4: GRK2 amino-terminal peptide inhibits β_2AR phosphorylation. (A) Increasing amounts of the GRK2 peptide were used in a phosphorylation reaction mixture containing 50 nM purified β_2AR in mixed micelles and 10 μM isoproterenol. The reaction mixture also contained 20 nM GRK2, 20 mM Tris-HCl (pH 7.5), 2 mM EDTA, 5 mM $MgCl_2$, 0.2 mM ATP, and ~1−2 μCi of [γ-^{32}P]ATP. The reaction mixtures were incubated at 30 °C for 10 min and analyzed by SDS−PAGE and autoradiography. Shown are the means ± the standard deviation from three experiments. (B) Phosphorylation reactions were performed as described above using 2 μM light-activated rhodopsin as the substrate. (C) Phosphorylation reactions were performed in a total volume of 20 μL containing 0.5 μM tubulin, 20 nM GRK2, 0−100 μM GRK2 peptide, 20 mM Tris-HCl (pH 7.5), 2 mM EDTA, 5 mM $MgCl_2$, 0.2 mM ATP, and ~1−2 μCi of [γ-^{32}P]ATP. The reaction mixtures were incubated at 30 °C for 10 min and analyzed by SDS−PAGE and autoradiography. Bands were excised and counted by scintillation. Shown are the means ± the standard deviation from three experiments. (D) Noncompetitive inhibition by the GRK2 amino-terminal peptide. Phosphorylation of 1−15 μM rhodopsin was performed in the absence (—) or presence (− − −) of 60 μM GRK2 peptide using conditions as described in panel A. Data were plotted using Graphpad Prism and are the means ± the standard deviation of three independent experiments. (E) The GRK2 peptide specifically inhibits GRK2 phosphorylation of β_2AR. A synthetic peptide of residues 1−14 of GRK5 was generated. Phosphorylation reactions were performed as described above in the absence or presence of either 200 μM GRK2 or GRK5 amino-terminal peptide. The mean ± standard deviation from three experiments is shown. (F) The GRK2 peptide enhances binding of GRK2 to phosphatidylinositol. Binding assays were performed as described in Materials and Methods with the addition of increasing concentrations of the GRK2 peptide (0−200 μM). In the top panel, reaction mixtures included purified GRK2, β_2AR, and phosphatidylinositol. The middle panel contains GRK2 and phosphatidylinositol in the absence of β_2AR, and the bottom panel serves as a control with only GRK2 present. Data shown are representative of three independent experiments.

binding. The GRK1·$(Mg^{2+})_2$·ATP structure is also informative since residues 12−23 of the N-terminus appear to be α-helical (*17*) while residues 4−16 form an amphipathic α-helix when an N-terminal GRK1 peptide is bound to recoverin (*39*).

The GRK1, GRK2, and GRK6 crystal structures reveal that there is a bipartite interaction between the RH and kinase domains (*14*, *16*, *17*). This interaction suggests several roles for the RH domain in terms of kinase activation. First, the RH domain could play a role in stabilizing the small lobe of the kinase domain in its active state by stabilizing the hydrophobic motif. Second, the RH domain could act to bridge the small and large lobes of the kinase domain. The crystal structure reveals that there is an extensive interface between the kinase domain and the α10 helix of the RH domain, mainly through salt bridges and hydrogen bonds (*14*, *16*). It is possible that binding of the amino-terminal region to the kinase domain could cause a conformational change, thereby bringing the small and large lobes together. This conformation would form the active state of the kinase, which could then phosphorylate its receptor substrate. Other studies have implicated such intramolecular interactions in regulating kinase activity. For example, in the Src-family kinases, the SH2 domain interacts with a phosphorylated tyrosine residue in the regulatory domain of the kinase, thereby stabilizing the inactive conformation of the kinase.

In conclusion, we propose a novel mechanism for the activation of GRK2, in which the polar face of the α-helical amino terminus is involved in an intramolecular interaction with the kinase domain. We hypothesize that this interaction is regulated by binding of kinase to an activated GPCR and that this causes a conformational change that activates the kinase and enhances its ability to bind to phospholipids and phosphorylate the receptor. While this proposed mechanism would likely regulate all GRKs, cocrystallization of a GPCR−GRK complex may be needed to more fully understand the conformation of an activated GRK.

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