A Predicted Amphipathic Helix Mediates Plasma Membrane Localization of GRK5*

Received for publication, September 29, 2003, and in revised form, January 23, 2004
Published, JBC Papers in Press, February 19, 2004, DOI 10.1074/jbc.M310738200

Manimekalai M. Thiagarajan, RoseAnn P. Stracquatanio, Alexey N. Pronin‡, Daniel S. Evanko§, Jeffrey L. Benovic, and Philip B. Wedegaertner¶

From the Department of Microbiology and Immunology and Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, Pennsylvania 19107

G protein-coupled receptor kinases (GRKs) specifically phosphorylate agonist-occupied G protein-coupled receptors at the inner surface of the plasma membrane (PM), leading to receptor desensitization. GRKs utilize a variety of mechanisms to bind tightly, and sometimes reversibly, to cellular membranes. Previous studies demonstrated the presence of a membrane binding domain in the C terminus of GRK5. Here we define a mechanism by which this short C-terminal stretch of amino acids of GRK5 mediates PM localization. Secondary structure predictions suggest that a region contained within amino acids 546–565 of GRK5 forms an amphipathic helix, with the key features of the predicted helix being a hydrophobic patch of amino acids on one face of the helix, hydrophilic amino acids on the opposite face, and a number of basic amino acids surrounding the hydrophobic patch. We show that amino acids 546–565 of GRK5 are sufficient to target the cytoplasmic green fluorescent protein (GFP) to the PM, and the hydrophobic amino acids are necessary for PM targeting of GFP-546–565. Moreover, full-length GRK5-GFP is localized to the PM, but mutation of the hydrophobic patch or the surrounding basic amino acids prevents PM localization of GRK5-GFP. Last, we show that mutation of the hydrophobic residues severely diminishes phospholipid-dependent autophosphorylation of GRK5 and phosphorylation of membrane-bound rhodopsin by GRK5. The findings in this report thus suggest the presence of a membrane binding motif in GRK5 and define the importance of a group of hydrophobic amino acids within this motif in mediating its PM localization.

Cell surface localized G protein-coupled receptors (GPCRs) detect a large variety of extracellular stimuli and initiate numerous intracellular signaling pathways. Proper regulation of GPCR signaling is maintained in part by a process known as desensitization (1), which refers to the turning off of a GPCR-initiated signaling event in the continuous presence of agonist. A key mechanism of GPCR desensitization is phosphorylation of agonist-occupied receptor by the G protein-coupled receptor kinases (GRKs) (2, 3). This phosphorylation promotes binding of arrestin proteins to the GPCR and results in subsequent uncoupling of the GPCR from G protein.

To function properly GRKs need to be localized to the cytoplasmic face of the plasma membrane (PM) where their GPCR substrates are located. Different members of the GRK family utilize a variety of mechanisms to bind to cellular membranes (2, 3). GRK2 and GRK3 contain well characterized C-terminal pleckstrin homology domains (4) that mediate translocation of the kinases from the cytoplasm to the PM in response to GPCR activation (5, 6). The pleckstrin homology domains allow PM binding by interacting with both phospholipids and free G protein βγ subunits. In contrast to GRK2/3, other GRK family members exhibit a more constitutive association with cellular membranes. GRK1 and GRK7 contain a C-terminal CAAAX motif, which directs covalent modification by a farnesyl or geranylgeranyl isoprenoid (1, 7). The isoprenyl lipid contributes to membrane binding by providing a hydrophobic anchor to insert into membranes. Similarly, covalent attachment of palmitate to C-terminal cysteines of GRK4 and GRK6 contributes to their PM localization (8–11).

The remaining GRK, GRK5, does not appear to undergo a lipid modification, yet it binds tightly to membranes. Interestingly, a short region, amino acids 552–562, in the C terminus of GRK5 has been defined as critical for binding of GRK5 to phospholipids in vitro and localization of GRK5 to the PM in cells (12). However, the mechanism by which this C-terminal sequence of GRK5 contributes to membrane binding has not been defined. It has been noted that this region is rich in basic amino acids, and thus it has been proposed that the positively charged C-terminal region of GRK5 mediates binding to negatively charged phospholipid membranes (12–14). In this report, we propose and test a refined mechanism for membrane targeting mediated by the short C-terminal stretch of amino acids of GRK5. We provide evidence that is consistent with a novel prediction that this region of GRK5 forms an amphipathic helix in which a hydrophobic patch of amino acids is surrounded by basic residues. Our results demonstrate a major role for a sequence of hydrophobic residues in mediating PM localization and function of GRK5.

EXPERIMENTAL PROCEDURES

Plasmids—GRK2-GFP and GRK5-GFP were constructed by subcloning the coding sequences of bovine GRK2 and human GRK5, respectively, into pEGFP-N1 (Clontech). Mutants of full-length GRK5-GFP...
were generated using QuikChange (Stratagene) site-directed mutagenesis. GFP-546–565 was generated by inserting the complementary oligos, 5′-gatt ctc cca aag ggc tcc tgc aag gac gac gac aca aca att cca aga gtt a-3′ and 5′-agc tta act ctt gga att gtt ctc cgg cct gaa gag tct gta gag ccc ttt ctt ggg a-3′ and then directly subcloning into the BglII and Hind III restriction sites of pEGFP-C1 (Clontech). Mutants of GPF-546–565 were generated using QuikChange (Stratagene) site-directed mutagenesis.

Confocal Microscopy—HEK293 cells were grown to 50–90% confluence on 6-well dishes in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. The cells were transfected with 1 μg of total DNA using FuGene 6 (Roche Applied Science) according to the manufacturer's instructions. Cells were analyzed for GFP fluorescence in live cells using a Bio-Rad MRC-600 laser scanning confocal microscope (Kimmel Cancer Center Bioimaging Facility) running CoMos 7.0 software and interfaced to a Zeiss Plan-Apo 63×1.40 oil immersion objective. Samples were analyzed using excitation at 488 nm. Images of “x-y” sections through the middle of the cell were recorded.

Confocal microscopy images of single cells were performed using the Plot Profile tool of NIH ImageJ software. The relative magnitude of GFP distribution along linear slices of the cell was quantitated as described previously (15). Appropriate linear slices were chosen to maintain a standard distance of 100 pixels from one edge of a cell to an edge on the opposite side of the same cell. The average pixel intensity in 15–45 traces from n = 5 cells for each protein was determined and plotted. Gray values of the distribution curves were normalized to 100. Histograms of the different GRK5 constructs are presented together to reveal quantitative differences in plasma membrane localization. The specific percent plasma membrane fluorescence for GRK5-GFP and GFP-546–565 was determined by quantitating the total pixel intensity in a 10-pixel-wide region at the cell periphery (562 of GRK5 as critical for membrane targeting) and then dividing the total of the two peaks by the total pixel intensity under the entire histogram trace. To remove contributions to the PM signal not attributable to plasma membrane localization, the percent of fluorescence at the 10-pixel-wide edge was determined for GRK5(4A)-GFP and GFP-546–565(4A), and then these values were subtracted from the values for GRK5-GFP and GFP-546–565, respectively.

Preparation of COS-1 Cell Lysates—COS-1 cells were grown at 37°C to 50–90% confluence on 10-cm dishes in DMEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. The cells were transfected with 10 μg of total DNA (pcDNA3 alone or wt or mutant GRK5-GFP in pEGFP-N1) using FuGene 6 (Roche Applied Science) according to the manufacturer's instructions. The cells were harvested after 48 h, washed twice in ice-cold phosphate-buffered saline, and lysed in 1 ml buffer (20 mM HEPES, pH 7.2, 250 mM NaCl, 10 mM EDTA, 0.02% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, and 5 μg/ml benzamidine) by Polytron homogenization (two 15-s bursts at 2500 rpm). Lysates were centrifuged for 10 min at 40,000 × g to remove particulate matter, and supernatants were then assayed. Levels of protein expression were equivalent based on Western blot analysis.

Substrate Phosphorylation—To test for the ability of the wt and mutant GRKs to phosphorylate rhodopsin, 2 μl of COS-1 cell lysates was incubated with 20 nm Tris-HCl, pH 7.5, 2 mM EDTA, 5 mM MgCl2, 100 μM ATP, −1 μCi of [γ-32P]ATP with or without 20 μg of crude soybean phosphatidyicholine (Sigma) for 2 min at 30°C. Reactions were quenched by addition of SDS sample buffer and incubation for 10 min at room temperature. Samples were then electrophoresed on a 10% SDS-polyacrylamide gel, and gels were fixed in 0.7M trichloroacetic acid, 0.14 M acetic acid for 10 min, washed twice in 50% ethanol, 16% acetic acid for 10 min, dried, and subjected to autoradiography. Tubulin bands were excised and counted in a liquid scintillation counter. Data were analyzed and plotted using GraphPad Prism.

For both rhodopsin and tubulin phosphorylation, cell lysates containing GRK5-GFP were directly compared with lysates containing non-tagged GRK5 to demonstrate that the C-terminally fused GFP does not drastically inhibit kinase activity. Cell lysates containing GRK5-GFP and GRK5 phosphorylated rhodopsin at 11.9 ± 1.1 (n = 8) pmol of phosphate/min/mg of protein and 16.7 ± 1.9 (n = 8) pmol of phosphate/min/mg of protein, respectively. Cell lysates containing GRK5-GFP and GRK5 phosphorylated tubulin at 18.6 ± 2.4 (n = 3) pmol of phosphate/min/mg of protein and 35.3 ± 3.1 (n = 3) pmol of phosphate/min/mg of protein, respectively. Thus, the relative activity of GRK5-GFP to non-tagged GRK5 is 0.71 and 0.53 for phosphorylation of rhodopsin and tubulin, respectively.

Autophosphorylation of GRK5—To determine the ability of wt and mutant GRK5-GFP to undergo autophosphorylation, 2 μl of COS-1 cell lysates was incubated with 20 μl Tris-HCl, pH 7.5, 2 mM EDTA, 8 mM MgCl2, 100 μM ATP, −1 μCi of [γ-32P]ATP with or without 20 μg of crude soybean phosphatidyicholine (Sigma) for 2 min at 30°C. Reactions were quenched by addition of SDS sample buffer and incubation for 10 min at room temperature. Samples were then electrophoresed on a 10% SDS-polyacrylamide gel, and gels were fixed in 0.7 M trichloroacetic acid, 0.14 M 5-sulfosalicylic acid for 10 min, washed twice in 50% ethanol, 16% acetic acid for 10 min, dried, and subjected to autoradiography.

RESULTS

Prediction of an Amphipathic Helical Membrane Binding Motif in the C Terminus of GRK5—Previous deletion mutagenesis identified amino acids 552–562 of GRK5 as critical for phospholipid binding and PM localization (12). Moreover, this C-terminal region of GRK5 (Fig. 1A) is rich in basic amino acids that may function to interact with negatively charged phospholipid membranes (12, 13). In this study, we note that all secondary structure programs tested (16) strongly predict an alpha-helical structure for this region, minimally amino acids 549–557 of GRK5 (Fig. 1A). Analysis of the predicted helical region by a helical wheel or helical net projection (17) (Fig. 1B and C) reveals an amphipathicity for this membrane-targeting region. A hydrophobic patch, consisting of Leu554, Leu555, and Phe556, lies on one face of the helix (Figs. 1B and 1C). The other side of the helix consists mostly of hydrophilic residues, and a number of the basic residues are predicted to surround the hydrophobic patch. Thus, an amphipathic helical membrane-binding motif, similar to that proposed recently for several other proteins (17–20), is predicted to exist within amino acids 546–565 in GRK5. The hydrophilic residues can directly insert into membrane lipids, while the surrounding positively charged amino acids would further enhance membrane binding by interacting with acidic phospholipids.

Amino Acids 546–565 of GRK5 Are Sufficient for PM Localization—To examine the C-terminal membrane-targeting domain of GRK5 in more detail, amino acids 546–565 were fused to GFP, and subcellular localization of GFP-tagged proteins was determined by confocal microscopy of live cells (Fig. 2). When GFP was expressed alone in HEK293 cells, fluorescence was observed throughout the cytoplasm and nucleus (Fig. 2A). As described previously (6), GRK2-GFP is localized exclusively to the plasma membrane (Fig. 2B). In contrast, full-length GRK5 fused to GFP is predominantly localized at the PM with very little in the cytoplasm, as seen by strong fluorescence at the cell periphery (Fig. 2C). Importantly, GFP-546–565, in which amino acids 546–565 of GRK5 are fused to the C terminus of GFP, shows PM localization (Fig. 2D), as clearly distinguished by sharp fluorescence at edges of the cells and at interfaces between cells, indicating that this amino acid sequence can function as an autonomous membrane-targeting signal to direct PM localization of a normally cytoplasmic protein. GFP-546–565 also displays prominent...
staining throughout the cytoplasm and nucleus, and we cannot rule out that other regions of GRK5 contribute to PM localization. Although PM localization of GFP-546–565 is substantially weaker compared with GRK5-GFP, PM localization of GFP-546–565 is clearly distinguishable from the absence of PM staining observed with GFP alone (Fig. 2A) or mutants of GFP-546–565 described below (Fig. 2, E–H). Significant PM localization of GFP-546–565 is also revealed by quantitative analysis of confocal images (Fig. 3E), as discussed below.

**Mutation of Hydrophobic Patch and Basic Residues Prevents PM Localization of GRK5**—To test the prediction of membrane binding mediated by a patch of hydrophobic residues within the 546–565 domain of GRK5 (Fig. 1), subcellular localization of several mutants of GFP-546–565 were examined. When Leu550, Leu551, Leu554, and Phe555 were all mutated together to alanines in the context of GFP-546–565, to generate GFP-546–565(4A), PM localization was abolished (Fig. 2E); GFP-546–565(4A) was only observed in the cytoplasm and nucleus of cells (Fig. 2E). Moreover, the loss of only two hydrophobic side chains was sufficient to disrupt PM localization of GFP-546–565, GFP-546–565(AALF) (Fig. 2F), in which both Leu550 and Leu551 were replaced with alanines, and GFP-546–565(LLAA) (Fig. 2G), in which both Leu554 and Phe555 were replaced with alanines, failed to show any detectable PM localization when expressed in HEK293 cells. Last, Leu550, Leu551, Leu554, and Phe555 were all replaced simultaneously with hydrophobic glutamates, and the resulting mutant, GFP-546–565(Q), again failed to display any PM localization (Fig. 2H). Thus, the results are consistent with the prediction that a patch of hydrophobic amino acids is essential for membrane binding of GRK5.

The above results utilized the short 546–565 sequence fused to GFP, but it is important to test whether mutation of the hydrophobic patch can disrupt localization of full-length GRK5. In contrast to the almost complete PM localization of GRK5-GFP (Fig. 3A), GRK5(4A)-GFP, in which Leu550, Leu551, Leu554, and Phe555 were all mutated together to alanines in the context of full-length GRK5-GFP, was diffusely distributed throughout the cytoplasm with no detectable PM localization (Fig. 3B). When Gln552 and Arg553, which in the linear sequence reside within the stretch of hydrophobic residues (Fig. 1), were both replaced with alanines in full-length GRK5-GFP, no effect on subcellular localization was observed; GRK5(4A)-GFP retained strong PM localization (Fig. 3C). However, when four basic residues, Lys547, Lys548, Lys556, and Arg557, that are predicted to surround the hydrophobic patch (Fig. 1C) were changed to alanines, the resulting GRK5(basic)-GFP was located throughout the cytoplasm of cells with no detectable PM association (Fig. 3D). Thus, disruption of either the hydrophobic patch or surrounding basic amino acids in the C-terminal 546–565 membrane-targeting sequence is sufficient to prevent PM localization of GRK5. Moreover, the results with full-length GRK5-GFP (Fig. 3) are consistent with membrane binding mediated by an amphipathic helix.

PM localization of GRK5-GFP (Figs. 2C and 3A) and GFP-546–565 (Fig. 2D) was confirmed by quantitative analysis of confocal images. The pixel intensity along linear slices of images of single cells was determined and plotted as a histogram to indicate distribution across the cell (Fig. 3E). GRK5-GFP showed sharp peaks at the extremities of the distribution graph with very little pixel intensity in the middle, consistent with predominant localization at the PM and much lesser localization in the interior of the cell. Sharp peaks at the edges were also evident in the histogram trace for GFP-546–565 (Fig. 3E), but a large amount of pixel intensity was also observed in the middle portion of the trace, consistent with the qualitative view of GFP-546–565 localization (Fig. 2D) showing both PM and substantial cytoplasmic and nuclear localization. Analysis of the PM peaks indicated that 56% of overexpressed GRK5-GFP and 9% of overexpressed GFP-546–565 was at the PM in these linear profiles. GFP-546–565(4A) and GFP alone displayed almost identical traces; peaks at the edges corresponding to PM localization were completely absent, and virtually all pixel intensity was found in the middle of the histogram, corresponding to a cytoplasmic and nuclear distribution. GRK5(4A)-GFP also lacked sharp peaks at the extremities of the distribution plot; instead, GRK5(4A)-GFP displayed wide peaks corresponding to a cytoplasmic distribution and a central dip corresponding to a lack of the protein in the nucleus. Note that
the histogram traces clearly show a difference in subcellular distribution between GFP-546–565 and GFP-546–565(AA); GFP-546–565 is partially present at the PM, but GFP-546–565(AA) is not present at the PM.

The Hydrophobic Patch Is Necessary for Phosphorylation of Rhodopsin by GRK5—Previous deletion mutagenesis indicated that a GRK5(1–551) mutant, in which the C-terminal amino acids 552–590 were deleted, showed a substantial decrease compared with full-length GRK5 in its ability to phosphorylate membrane-bound rhodopsin in vitro (12), a widely used model for GPCR phosphorylation by GRKs (14). Cell lysates from COS-1 cells expressing GRK5-GFP or its mutants were prepared and assayed for their ability to phosphorylate rhodopsin. GRK5-GFP displayed a time-dependent increase in phosphorylation of light-activated rhodopsin, and GRK5(QR)-GFP, a mutant that retained strong PM localization (Fig. 3C), showed a virtually identical profile of rhodopsin phosphorylation (Fig. 4). Mutation of the four basic residues, Lys547, Lys548, Lys556, and Arg557, in GRK5(basic)-GFP, resulted in a reduction in rhodopsin phosphorylation (Fig. 4). Strikingly, GRK5(4A)-

GFP, in which amino acids in the predicted hydrophobic patch were replaced with alanines, was decreased by 90% in rhodopsin phosphorylation compared with wt GRK5-GFP (Fig. 4).

Mutation of the Hydrophobic Patch Reduces GRK5 Autophosphorylation and Phosphorylation of Tubulin—The functional consequences of mutations in the C-terminal membrane binding domain were examined further by phosphorylation of the soluble substrate tubulin (21). GRK5(QR)-GFP was slightly reduced compared with wt GRK5-GFP in its ability to phosphorylate tubulin (Fig. 5A). However, both mutation of the hydrophobic patch and of the basic residues substantially inhibited tubulin phosphorylation. Tubulin phosphorylation by GRK5(4A)-GFP and GRK5(basic)-GFP was reduced 57 and 46%, respectively, compared with wt GRK5-GFP (Fig. 5A).

Because previous studies demonstrated that autophosphorylation of GRK5 is promoted by phospholipid binding, the autophosphorylation of the GRK5-GFP mutants was examined. As described for GRK5 (14, 22), autophosphorylation of GRK5-GFP is also dependent upon the addition of phospholipids (Fig. 5B), crude soybean phosphatidylcholine in these experiments. Similarly, phospholipids stimulated the in vitro autophosphorylation of both GRK5(QR)-GFP and GRK5(basic)-GFP (Fig. 5B). In contrast, GRK5(4A)-GFP displayed a significant defect in phospholipid-dependent autophosphorylation (Fig. 5B), although weak autophosphorylation could be detected. These results are consistent with the rhodopsin phosphorylation results (Fig. 4) in that GRK5(4A)-GFP is more severely impaired compared with GRK5(basic)-GFP in catalytic activity that depends on phospholipid binding.

**DISCUSSION**

Although it was recognized previously that the GRK5 C-terminal membrane-targeting sequence contains a number of basic amino acids (12, 14), the data presented here reveal novel features of this C-terminal sequence and suggest a mechanism by which this sequence mediates membrane binding. Secondary structure prediction (Fig. 1) suggests that the GRK5 membrane-targeting sequence forms a helix, and the key features of the helix are a patch of hydrophobic amino acids surrounded by a number of basic amino acids. In this model, the hydrophobic amino acids insert into the membrane bilayer, and the basic amino acids are poised to interact with acidic phospholipid head groups.

Here we tested the amphipathic helix model for membrane targeting by examining the subcellular localization of GRK5 mutants. Whereas expressed GRK5-GFP was predominantly found at the PM (Figs. 2C and 3A), the GRK5(4A)-GFP mutant, in which the hydrophobic amino acids Leu550, Leu551, Leu554, and Phe555 were all changed to alanines, was not detected at the PM but instead was found throughout the cytoplasm (Fig. 3B). Likewise, substituting the basic amino acids Lys547, Lys548, Lys556, and Arg557 with alanines prevented PM localization of GRK5(basic)-GFP (Fig. 3D). These results indicate that both the hydrophobic patch and the surrounding positive charges contribute to membrane binding of GRK5.

Moreover, we demonstrated that the region containing the predicted amphipathic helix of GRK5 is sufficient to function as a membrane-targeting motif; when amino acids 546–565 were fused to GFP, fluorescence was observed at the PM (Fig. 2D). In agreement with this result, previous work (15) demonstrated that replacing the N-terminal palmitoylation site of the G protein αs with amino acids 546–565 of GRK5 was sufficient to restore PM localization of αs, and a peptide corresponding to 547–565 blocked binding of full-length GRK5 to phospholipids in vitro (12). We also demonstrated that mutation of only two of the hydrophobic residues in the predicted hydrophobic patch is sufficient to prevent the membrane targeting by 546–565 when...
fused to GFP (Fig. 2, F and G), further confirming the importance of the hydrophobic patch in this membrane-targeting motif. Interestingly, GFP-546–565 displayed substantially weaker PM localization compared with full-length GRK5; for GFP-546–565, cytoplasmic and nuclear localization was observed in addition to PM localization (Figs. 2D and 3E). Weak PM localization of GFP-546–565 suggests that additional sequences in GRK5 contribute to strong PM localization. Consistent with this proposal, additional binding sites for phospholipids have been identified in the N terminus of GRK (13, 23).

Alternatively, it is possible that when removed from the context of the full-length GRK5 sequence and fused to GFP, only a fraction of 546–565 is properly folded and thus able to direct PM localization.

Amphipathic helix membrane-targeting motifs have been identified in several other signaling proteins, including the regulator of G protein signaling (RGS) proteins RGS2, RGS4, and RGS16 (17–19) and the small GTPase ARF1 (20). For the RGS proteins, helical models predict a hydrophobic face and a hydrophilic face rich in positive charged amino acids, and, similar to the results described here for GRK5, both the hydrophobic and basic residues contribute to membrane binding. For ARF1, structural studies have verified the amphipathic nature of its N-terminal helix (24–26), where hydrophobic residues in the ARF1 helix lie on one side thus presenting a hydrophobic patch for membrane interaction. The similarity of the predicted amphipathic helix of GRK5 and that of ARF1 is underscored by the presence of a similar hydrophobic amino acid sequence motif, LLXXLF in GRK5 (Fig. 1) and IFXXLFXXLF in ARF1 (20). Moreover, similar to GRK5 and RGS2, RGS4 and RGS16, the ARF1 N-terminal amphipathic helix contains several basic residues, which have been proposed to contribute to membrane binding (20). Interestingly, the N-terminal amphipathic helix of ARF1 mediates guanine nucleotide-dependent reversible membrane targeting. In soluble ARF1-GDP, the amphipathic helix binds intramolecularly to a hydrophobic groove; however, upon GTP binding, conformational changes abolish the intramolecular binding site, allowing the amphipathic N-terminal helix to interact with cellular membranes. We can speculate that conformational changes in GRK5 or interacting proteins (22) might affect the accessibility of its membrane binding amphipathic helix and provide a mechanism for regulating GRK5 membrane binding.

Examination of the C-terminal amino acid sequences of

**Fig. 3.** Mutation of the hydrophobic patch or surrounding basic residues prevents PM localization of GRK5-GFP. Plasmids expressing full-length GRK5-GFP (A), full-length GRK5(4A)-GFP (B), full-length GRK5(QR)-GFP (C), or full-length GRK5(basic)-GFP (D) were transfected into HEK293 cells. 48 h later GFP fluorescence was visualized by confocal microscopy of live cells, as described under “Experimental Procedures.” E, the histogram shows the distribution of GRK5-GFP (red), GRK5(4A)-GFP (peach), GFP-546–565 (blue), GFP-546–565(4A) (dark green), and GFP (light green) across linear slices of images from single cells. The traces shown represent the average pixel intensity in 15–45 traces from n = 5 cells for each protein.

**Fig. 4.** Time course of rhodopsin phosphorylation by GRK5. The ability of lysates from COS-1 cells expressing wt or mutant GRK5-GFP to phosphorylate rhodopsin is shown. Reactions were quenched with SDS sample buffer after 2, 5, 10, and 20 min and then subjected to SDS-PAGE. Rhodopsin bands were excised and counted via liquid scintillation to quantitate pmol of phosphate transferred per pmol of rhodopsin substrate. Mean ± S.E. of three experiments is displayed.
GRK4 and GRK6 suggests that they also each contain an amphipathic helix membrane binding domain. As previously noted, GRK4 and GRK6 have numerous basic amino acids in similar positions to those found in the membrane binding domain of GRK5 (12). Here, we note that GRK4 and GRK6 also contain the critical hydrophobic motif (Fig. 1A), consistent with the proposal that GRK4 and GRK6 utilize an amphipathic C-terminal helix for membrane binding. Like GRK5, GRK6 has the hydrophobic motif LLXXLF, whereas FFXXLF is found in the predicted amphipathic helix of GRK4 (Fig. 1A). Interestingly, the sequence identity between GRK4, GRK5, and GRK6 diverges immediately after the predicted C-terminal amphipathic helices, and this divergence is coincident with exon boundaries (12). After the amphipathic helix, GRK4 and GRK5 diverge, and GRK6A, one of three GRK6 splice variants (27, 28), contain the critical hydrophobic motif (Fig. 1A). Here, we note that GRK4 and GRK6 also contain the critical hydrophobic motif (Fig. 1A), consistent with the proposal that GRK4 and GRK6 utilize an amphipathic C-terminal helix for membrane binding. Like GRK5, GRK6 has the hydrophobic motif LLXXLF, whereas FFXXLF is found in the predicted amphipathic helix of GRK4 (Fig. 1A). Interestingly, the sequence identity between GRK4, GRK5, and GRK6 diverges immediately after the predicted C-terminal amphipathic helices, and this divergence is coincident with exon boundaries (12). After the amphipathic helix, GRK4 and GRK6A, one of three GRK6 splice variants (27, 28), contain sites for palmitoylation (Fig. 1A), whereas GRK5 contains numerous serine sites of phosphorylation. On the other hand, the two additional splice variants of GRK6, GRK6B and GRK6C (Fig. 1A), lack cysteine sites of palmitoylation in their C termini (27) yet retain the predicted amphipathic helix motif. One would predict that palmitoylation combined with a membrane binding helix, as is found in GRK4 and GRK6A, would result in tighter membrane binding compared with GRK5, GRK6B, and GRK6C, which all appear to lack lipid modification, and possibly would promote localization to different membrane microdomains.

Last, we demonstrated here that GRK5 mutants that are defective in PM localization also display defects in function. Previous work has led to a model for GRK5 activation in which binding to phospholipid membranes stimulates the autophosphorylation of GRK5, and then both autophosphorylation and membrane binding are required for GRK5 to phosphorylate agonist-activated GPCRs (12–14). In agreement with this model, the hydrophobic site mutant GRK5(4A)-GFP was severely inhibited in its ability to autophosphorylate (Fig. 5B) and to phosphorylate light-activated rhodopsin (Fig. 4). Surprisingly, the basic mutant GRK5(basic)-GFP displayed no detectable defect in phospholipid-stimulated autophosphorylation (Fig. 5B) and only a partial inhibition of its ability to phosphorylate membrane-bound rhodopsin (Fig. 4), even though GRK5(basic)-GFP failed to localize to the PM in cells (Fig. 3). Thus, GRK5(4A)-GFP and GRK5(basic)-GFP are similarly defective in PM localization, but the functional analyses demonstrate a difference between GRK5(4A)-GFP and GRK5(basic)-GFP. This may indicate that GRK5(basic)-GFP is able to interact, at least transiently, with phospholipids in vitro sufficiently to promote autophosphorylation and subsequent receptor phosphorylation, and thus the hydrophobic patch may play a greater role than the basic residues of the amphipathic helix in membrane binding of GRK5.

Interestingly, both GRK5(4A)-GFP and GRK5(basic)-GFP displayed a decreased ability compared with wt GRK5-GFP to phosphorylate the soluble substrate tubulin (Fig. 5A). The amphipathic helix domain may be positioned to affect the catalytic activity of GRK5 or substrate binding, in addition to mediating interaction with membranes. Consistent with this idea, a previous report noted that phosphorylation of GRK5 by protein kinase C, which likely occurs immediately adjacent to the amphipathic helix membrane binding domain at multiple serines between 562 and 572, did not affect binding of GRK5 to phospholipids but significantly inhibited phosphorylation of soluble substrates by GRK5 (29). The C terminus of GRK5 appears to be positioned to detect a variety of regulatory inputs that affect GRK5 function and localization (12).

In summary, our results have defined a mechanism by which GRK5 binds to cellular membranes. The findings presented here suggest that GRK5 utilizes an amphipathic C-terminal helix consisting of a hydrophobic face and a hydrophilic face to mediate proper localization at the PM. It will be important to determine whether physiological stimuli modulate the ability of this membrane binding domain to direct subcellular localization of GRK5 and the related subfamily members, GRK4 and GRK6.

Acknowledgments—We thank So-Young Heo for assistance in plasmid construction and immunofluorescence analysis and Steven Luke for dedicated assistance with confocal microscopy.

REFERENCES

1. Kohout, T. A., and Lefkowitz, R. J. (2003) Mol. Pharmacol. 63, 9–18
2. Penn, R. B., Pronin, A. N., and Benovic, J. L. (2000) Trends Cardiovasc. Med. 10, 18–19
3. Pitcher, J. A., Freedman, N. J., and Lefkowitz, R. J. (1998) Annu. Rev. Biochem. 67, 653–692
4. Carman, C. V., Barak, L. S., Chen, C., Liu-Chen, L. Y., Onorato, J. J., Kennedy, S. P., Caron, M. G., and Benovic, J. L. (2000) J. Biol. Chem. 275, 10443–10452
5. Pitcher, J. A., Inglese, J., Higgin, J. B., Arrieta, J. L., Casey, P. J., Kim, C., Benovic, J. L., Kwatra, M. M., Caron, M. G., and Lefkowitz, R. J. (1992) Science 257, 1264–1267
6. Barak, L. S., Warabi, K., Feng, X., Caron, M. G., and Kwatra, M. M. (1999) J. Biol. Chem. 274, 7565–7569
7. Inglese, J., Koch, W. J., Caron, M. G., and Lefkowitz, R. J. (1992) Nature 359, 147–150
8. Fremont, R. T., Maerz, A. D., Stoffel, R. H., Chung, N., Pitcher, J. A.,...
Membrane Targeting of GRK5

Ambrose, C., Inglese, J., MacDonald, M. E., and Lefkowitz, R. J. (1996) J. Biol. Chem. 271, 6403–6410
9. Stoffel, R. H., Inglese, J., Macrae, A. D., Lefkowitz, R. J., and Premont, R. T. (1998) Biochemistry 37, 16553–16559
10. Stoffel, R. H., Randall, R. R., Premont, R. T., Lefkowitz, R. J., and Inglese, J. (1994) J. Biol. Chem. 269, 27791–27794
11. Loudon, R. F., and Benovic, J. L. (1997) J. Biol. Chem. 272, 27422–27427
12. Pronin, A. N., Carman, C. V., and Benovic, J. L. (1998) J. Biol. Chem. 273, 31510–31518
13. Pitcher, J. A., Frederick, Z. L., Stone, W. C., Premont, R. T., Stoffel, R. H., Koch, W. J., and Lefkowitz, R. J. (1995) J. Biol. Chem. 271, 24967–24973
14. Kanapuli, P., Gurevich, V. V., and Benovic, J. L. (1994) J. Biol. Chem. 269, 10209–10212
15. Thiyagarajan, M. M., Bigras, E., Van Tol, H. H., Hebert, T. E., Evanko, D. S., and Wedegaertner, P. B. (2002) Biochemistry 41, 9470–9484
16. Combet, C., Blanchet, C., Geourjon, C., and Deleage, G. (2000) Trends Biochem. Sci. 25, 147–150
17. Hexemer, S. P., Lim, H., Bernard, J. L., and Blumer, K. J. (2001) J. Biol. Chem. 276, 14195–14203
18. Chen, C., Snow, K. T., Guo, K., Yaw, L. P., and Lin, S. C. (1999) J. Biol. Chem. 274, 19709–19806
19. Bernstein, L. S., Grillo, A. A., Loranger, S. S., and Linder, M. E. (2000) J. Biol. Chem. 275, 18520–18526
20. Antony, B., Beraud-Dufour, S., Chardin, P., and Chabre, M. (1997) Biochemistry 36, 4675–4684
21. Carman, C. V., Son, T., Kim, C. M., and Benovic, J. L. (1998) J. Biol. Chem. 273, 20308–20316
22. Pronin, A. N., Satpae, D. K., Slepk, V. Z., and Benovic, J. L. (1997) J. Biol. Chem. 272, 18273–18280
23. Noble, B., Kallal, I. A., Paasch, M. H., and Benovic, J. L. (2003) J. Biol. Chem. 278, 47466–47476
24. Losonczi, J. A., Tian, F., and Prestegard, J. H. (2000) Biochemistry 39, 3804–3816
25. Noble, B., Kallal, I. A., Paasch, M. H., and Benovic, J. L. (2003) J. Biol. Chem. 278, 47466–47476
26. Greasley, S. E., Juthi, H., Teahan, C., Solari, R., Pennise, A., Thomas, G. M., Cockcroft, S., and Bax, B. (1996) Nat. Struct. Biol. 3, 797–806
27. Hall, R. A., Spurney, R. F., Premont, R. T., Rahman, N., Blitzer, J. T., Pitcher, J. A., and Lefkowitz, R. J. (1999) J. Biol. Chem. 274, 24328–24334
28. Firsov, D., and Elalouf, J. M. (1997) Am. J. Physiol. 273, C953–C961
29. Pronin, A. N., and Benovic, J. L. (1997) J. Biol. Chem. 272, 3806–3812
A Predicted Amphipathic Helix Mediates Plasma Membrane Localization of GRK5
Manimekalai M. Thiagarajan, RoseAnn P. Stracquatanio, Alexey N. Pronin, Daniel S. Evanko, Jeffrey L. Benovic and Philip B. Wedegaertner

J. Biol. Chem. 2004, 279:17989-17995.
doi: 10.1074/jbc.M310738200 originally published online February 19, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M310738200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 29 references, 18 of which can be accessed free at http://www.jbc.org/content/279/17/17989.full.html#ref-list-1