Plasma Membrane and Nuclear Localization of G Protein–coupled Receptor Kinase 6A[□]

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G protein–coupled receptor (GPCR) kinases (GRKs) specifically phosphorylate agonist-occupied GPCRs at the inner surface of the plasma membrane (PM), leading to receptor desensitization. Here we show that the C-terminal 30 amino acids of GRK6A contain multiple elements that either promote or inhibit PM localization. Disruption of palmitoylation by individual mutation of cysteine 561, 562, or 565 or treatment of cells with 2-bromopalmitate shifts GRK6A from the PM to both the cytoplasm and nucleus. Likewise, disruption of the hydrophobic nature of a predicted amphipathic helix by mutation of two leucines to alanines at positions 551 and 552 causes a loss of PM localization. Moreover, acidic amino acids in the C-terminus appear to negatively regulate PM localization; mutational replacement of several acidic residues with neutral or basic residues rescues PM localization of a palmitoylation-defective GRK6A. Last, we characterize the novel nuclear localization, showing that nuclear export of nonpalmitoylated GRK6A is sensitive to leptomycin B and that GRK6A contains a potential nuclear localization signal. Our results suggest that the C-terminus of GRK6A contains a novel electrostatic palmitoyl switch in which acidic residues weaken the membrane-binding strength of the amphipathic helix, thus allowing changes in palmitoylation to regulate PM versus cytoplasmic/nuclear localization.

INTRODUCTION

G protein–coupled receptor kinases (GRKs) phosphorylate agonist-activated G protein–coupled receptors (GPCRs) at the cytoplasmic surface of the plasma membrane (PM), and this phosphorylation is a critical and initial step in the rapid “turning-off” or desensitization of GPCR signaling. GPCR phosphorylation by GRKs provides a binding site for the recruitment of arrestin proteins and causes subsequent uncoupling of the GPCR from the heterotrimeric G protein. Phosphorylation of GPCRs and arrestin binding also promote other events, including internalization of the GPCR and recruitment of additional proteins to form novel GPCR signaling complexes (Penn *et al.*, 2000; Penela *et al.*, 2003; Reiter and Lefkowitz, 2006; Smith and Luttrell, 2006).

To phosphorylate PM-localized GPCRs, GRKs must be positioned at the PM. Membrane binding by GRKs appears to function not only to localize the GRK in the proximity of the GPCR, but also as a requirement for the activation of the GRK (Penn *et al.*, 2000). The seven members of the GRK family have evolved a number of diverse mechanisms for interacting with cellular membranes, although all GRKs appear to share the common feature that key membrane-tar-

geting determinants are located in the C-terminal portion of the protein. GRK1 and GRK7 are covalently modified by isoprenylation at a cysteine within a C-terminal CaaX motif; the isoprenyl group provides a hydrophobic anchor to tether the GRK to membranes. On the other hand, GRK2 and GRK3 localize in the cytoplasm but are recruited to the cellular PM upon GPCR activation. The translocation to the PM is mediated by the C-terminal PH domain of GRK2 and GRK3, which binds to both acidic membrane phospholipids and free G protein $\beta\gamma$ subunits. The third subfamily of GRKs consists of GRK4, GRK5, and GRK6. Recently, we demonstrated that GRK5 is constitutively localized to the PM when overexpressed in cells, and its membrane localization is mediated by a C-terminal stretch of ~20 amino acids that is predicted to form an amphipathic helix with hydrophobic and basic character (Thiyagarajan *et al.*, 2004). The amphipathic helix membrane-binding motif is also conserved in GRK4 and GRK6 and thus predicted to contribute to membrane localization of these GRKs. In addition, GRK4 and GRK6A, one of three GRK6 splice variants, are also palmitoylated, but it remains to be tested whether both the amphipathic helix motif and palmitoylation are required for membrane localization of these GRKs (Stoffel *et al.*, 1994, 1998; Premont *et al.*, 1996; Loudon and Benovic, 1997).

In addition to their well-described role in phosphorylating and regulating GPCRs, increasing evidence suggests that GRKs have additional cellular functions. Although GRKs are highly selective for agonist-activated GPCRs as substrates, other substrates has been identified. For example, GRK2 and GRK5 can efficiently phosphorylate synucleins (Pronin *et al.*, 2000), and GRK6A phosphorylates the Na⁺/H⁺ exchanger regulatory factor (Hall *et al.*, 1999). Moreover, novel functions for GRKs are predicted by recent demonstrations of unique subcellular localizations. In particular, GRK5 can exist as a nuclear protein in various cell lines and cardiac myocytes (Yi *et al.*, 2002, 2005; Johnson *et al.*, 2004), and a recent report predicted that other members

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Abbreviations used: G protein, guanine nucleotide-binding protein; GPCR, G protein–coupled receptor; GRK, GPCR kinase; PM, plasma membrane; HEK293 cells, human embryonic kidney cells; COS-1, African green monkey kidney cells; GFP, green fluorescent protein; 2-BP, 2-bromopalmitate.

of GRK4/5/6 are also localized in the nucleus (Johnson *et al.*, 2004). Such results suggest novel though not understood functions for GRKs in the nucleus. In this report, we focused on understanding the mechanisms of subcellular localization of GRK6 and addressing whether GRK6 is a nuclear protein. We define critical elements in the C-terminal 30 amino acids of GRK6A that mediate and regulate PM localization. Our results indicate that both the predicted amphipathic helix motif and cysteine sites of palmitoylation are essential for PM localization of GRK6A. In addition, we identify two regions of negative charge that function to inhibit PM localization and show that these stretches of acidic amino acids are necessary to allow changes in palmitoylation to regulate localization of GRK6A. Moreover, we demonstrate that depalmitoylation of GRK6A promotes its translocation from the PM to both the cytoplasm and nucleus.

MATERIALS AND METHODS

Plasmids

The human pcDNA3-GRK6A and GRK5 plasmids have been described (Benovic and Gomez, 1993; Kunapuli and Benovic, 1993). Mouse pBK(Δ)-GRK6B and GRK6C were a generous gift from Dr. Richard Premont (Duke University). All GRK6A mutants described in this report were created using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA).

Cell Culture and Transfection

Human embryonic kidney (HEK) 293 and COS-7 (African green monkey kidney) cells were obtained from the American Type Culture Collection (Manassas, VA) and maintained as previously described in DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μ g/ml streptomycin. Transfections were performed using FuGene 6 (Roche, Indianapolis, IN) according to the manufacturer's instruction. For establishment of stable cell lines, cells were subjected to G418 (500 μ g/ml) treatment after 48 h of transfection. Surviving colonies were selected and expanded into cell lines.

Immunofluorescence Microscopy

Cells transfected with indicated plasmids were grown on coverslips. The immunofluorescence microscopy was performed as described previously (Grabocka and Wedegaertner, 2005). Briefly, cells were fixed with 3.7% formaldehyde for 15 min and permeabilized with blocking buffer (2.5% nonfat milk and 1% Triton X-100 in Tris-buffered saline [TBS]) for 20 min. Cells were then incubated with anti-GRK 4–6 mouse mAb (Upstate Biotechnology, Lake Placid, NY) for 1 h. After washing five times with blocking buffer, cells were incubated in a 1:1000 dilution of a goat anti-mouse conjugated with Alexa 488 for 30 min. The coverslips were washed with TBS, counterstained with DAPI (100 ng/ml), rinsed in distilled water, and mounted on glass slides with Prolong Gold antifade reagent (Molecular Probes, Eugene, OR). Images were recorded with an Olympus BX-61 microscope (Melville, NY) and 60 $\times$ PlanApo objective with an ORCA-ER (Hamamatsu, Bridgewater, NJ) cooled charge-coupled device camera controlled by Slidebook version 4.0 (Intelligent Imaging Innovation, Denver, CO). Images were transferred to Adobe Photoshop (San Jose, CA) for digital processing.

Cell Fractionation Assay

Soluble and particulate fractions were isolated as described previously (Evanko *et al.*, 2000; Takida and Wedegaertner, 2003). Briefly, 48 h after transfection, HEK293 cells were washed in ice-cold phosphate-buffered saline (PBS) and lysed in hypotonic lysis buffer (50 mM Tris-HCl, pH 8, 2.5 mM MgCl₂, 1 mM EDTA, 1 mM dithiothreitol [DTT]) with protease inhibitors. Cells were passed through a 27-gauge needle 10 times. Lysed cells were centrifuged at 400 $\times$ g for 5 min to remove nuclei and debris. The supernatant was centrifuged at 150,000 $\times$ g for 20 min at 4°C. Fractions were analyzed by SDS-PAGE and immunoblotting using the indicated antibody.

Palmitoylation Assay

Palmitoylation assays were done as described (Takida *et al.*, 2005). Briefly, 36 hours after transfection, the cells were metabolically labeled with [³H]palmitic acid (Perkin Elmer-Cetus Life Science, Norwalk, CT) for 3 h and then lysed. GRK6A and its mutants were immunoprecipitated using an anti-GRK6 antiserum (Kunapuli *et al.*, 1994). The samples were separated by SDS-PAGE and transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA). The membrane was sprayed with EnHance (PerkinElmer Life and Analytical Sciences, Boston, MA) and exposed to Hyperfilm MP (Amersham Biosciences) at –80°C. A duplicate membrane was subjected to immu-

noblotting using the anti-GRK 4–6 mAb (Upstate Biotechnology) as described in *Immunoblotting*.

Nuclear Extract Preparation

Cells were collected by trypsinization and centrifugation. After a wash with cold PBS and determination of the cell count, cells were gently resuspended in an ice-cold lysis buffer (320 mM sucrose, 10 mM HEPES, pH 7.4, 5 mM MgCl₂, 1 mM DTT, 1 mM PMSF) containing 1% Triton X-100 to a concentration of 1 $\times$ 10⁶ cells per ml and incubated on ice for 5 min. In an Eppendorf tube, 1 ml of the suspension was layered onto a cold 0.5-ml sucrose cushion (1.8 M sucrose, 10 mM HEPES, pH 7.4, 5 mM MgCl₂, 1 mM DTT, 1 mM PMSF). Nuclei were then pelleted on a bench-top Biofuge (Heraeus Instrument, Sayreville, NJ) centrifuge (13,200 rpm for 15 min at 4°C). The nuclear extracts (pellets) were resuspended in the lysis buffer lacking Triton X-100 and saved.

Immunoblotting

Equal amounts of samples were resolved on 8% SDS/PAGE and transferred to PVDF membranes (Millipore). The membranes were incubated with a block buffer (TBS with 0.1% Tween 20 and 5% dry milk) for 1 h at room temperature. Primary antibody incubations with a mouse anti-GRK4–6 antibody (1:2000, Upstate) or a rabbit anti-SP1 antibody (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA) were performed in the block buffer overnight at 4°C. After washing with TBS buffer containing 0.1% Tween 20, the membranes were incubated with the appropriate secondary peroxidase-conjugated antibody (1:10 000, Promega, Madison, WI) at room temperature for 1 h. Immunoreactive proteins were visualized by using an enhanced chemiluminescence system from Pierce (Rockford, IL). To quantify expression level of proteins, the Western blot bands were measured by densitometry analysis using a Scion Image freeware software (Frederick, MD; <http://www.scioncorp.com/frames/fr.download.now.htm>). The relative density of the protein bands was calculated in the area encompassing the immunoreactive protein band and subtracting the background of an adjacent nonreactive area in the same lane of the protein of interest.

Materials

Protein A/G agarose was from Santa Cruz Biotechnology. 2-bromopalmitate (2-BP) was from Aldrich Chemical Co., and leptomycin B (LMB) from Biomol (Plymouth Meeting, PA).

RESULTS

The C-Terminus of GRK6A Is Required for PM Localization

Previous results demonstrated that a predicted amphipathic helix in the C-terminus of GRK5 was essential for PM localization of GRK5 (Thiyagarajan *et al.*, 2004). Inspection of the extreme C-termini of all members of the GRK4/5/6 family (Figure 1A) reveals that the amphipathic helix motif is highly conserved. Thus, we sought to determine whether the amphipathic helix motif played a similar critical role in subcellular localization of GRK6A and to understand the role for palmitoylation of GRK6A.

GRK6 consists of four splice variants (Firsov and Elalouf, 1997; Moepps *et al.*, 1999; Premont *et al.*, 1999), three of which differ only at the extreme C-terminus (Figure 1A). Very few studies have addressed the subcellular localization of GRK6 splice variants (Vatter *et al.*, 2005), and thus initially we examined the subcellular localization of GRK6A, GRK6B, and GRK6C. Immunofluorescence microscopy of HEK293 cells stably expressing one of each of the three GRK6 variants demonstrated that all three are strongly targeted to the PM (Figure 1B), as evidenced by intense staining at the cell periphery, consistent with a recent report utilizing transient expression in COS-7 cells (Vatter *et al.*, 2005).

To confirm that the C-terminal region of GRK6A is critical for its PM localization, we examined the subcellular localization of GRK6A Δ 30, in which the last 30 amino acids were deleted. Although GRK6A displayed strong localization at the PM in transiently transfected HEK293 cells, GRK6A Δ 30 failed to localize at the PM but instead was found throughout the cytoplasm (Figure 1C). GRK6A Δ 30 also displayed a surprising accumulation in nucleus (Figure 1C), and, in fact,

Figure 1. C-terminus is required for plasma membrane localization of GRK6A. (A) The C-terminal amino acid sequences of GRK4, GRK5, and three splice variants of GRK6 are shown. Cysteines that are likely sites of palmitoylation are underlined (C), and the conserved predicted amphipathic helix motif present in all family members is indicated with the bar. (B) GRK6A, GRK6B, and GRK6C each were stably expressed in HEK293 cells and visualized by immunofluorescent staining using an anti-GRK4-6 mAb as described in *Materials and Methods*. (C) GRK6A wild type (Wt) and the C-terminal deletion mutant GRK6A $\Delta 30$ were transiently expressed in HEK293 cells and visualized by immunofluorescent staining using an anti-GRK4-6 mAb. Bar, 10 μ m. (D) Forty-eight hours after transfection with empty vector (pcDNA3, lanes 1 and 2) or pcDNA3 expression vectors containing GRK6A wild type (Wt, lanes 3 and 4) or GRK6A $\Delta 30$ (lanes 5 and 6), HEK293 cells were lysed, and the cell lysates were separated by high-speed centrifugation into soluble (S) and particulate (P) fractions. Cell fractions were subjected to Western blotting with the anti-GRK4-6 mAb.

all C-terminal mutants (described below) that lose PM localization were found in the nucleus, in addition to the cytoplasm. Fractionation of cell lysates by high-speed centrifugation into soluble (cytosol) or particulate (membrane) fractions was consistent with the immunofluorescence results. Although GRK6A was found almost exclusively in the particulate fraction, GRK6A $\Delta 30$ shows a dramatic shift to the soluble fraction (Figure 1D). We consistently observe that ~50% of GRK6A $\Delta 30$, and other PM localization-defective C-terminal GRK6A mutants (described below), remained in the particulate fraction. GRK6A C-terminal mutants may localize to endomembranes, although we do not observe any clear localization to Golgi or endoplasmic reticulum membranes (Supplementary Figure 1). The apparent membrane binding by GRK6A $\Delta 30$ may represent contributions to membrane binding from regions of GRK6A beyond the C-terminus. Indeed, the recently solved crystal structure of GRK6A revealed a large positively charged surface in the kinase region of the protein (Lodowski *et al.*, 2006). We found that GRK6A $\Delta 30$ was released from the particulate fraction when 0.5 M NaCl was added to the lysate, suggesting that ionic interactions are responsible for the partial membrane binding of GRK6A $\Delta 30$; however, wild-type GRK6A remained predominantly in the particulate fraction

in the presence of high salt (Supplementary Figure 2), consistent with a role for hydrophobic palmitoylation contributing to membrane binding of wild-type GRK6A. We also note that PM localization-defective C-terminal GRK6A mutants were more efficiently expressed than wild-type GRK6A, consistent with earlier observations showing higher expression levels of a nonpalmitoylated mutant of GRK6A (Loudon and Benovic, 1997).

Both Palmitoylation and the Amphipathic Helix Are Required for Plasma Membrane Localization of GRK6A

Within the C-terminal 30 amino acids of GRK6A, at least two potential membrane-targeting regions are present: the amphipathic helix and three cysteine sites of palmitoylation (Figure 1A). To determine whether both motifs play a role in PM localization of GRK6A and to examine the importance of individual cysteine residues, the C-terminal region was subjected to further mutagenesis and analysis. First, cysteines at positions 561, 562, and 565 were individually changed to serines. Previous studies mutated all three cysteines together (Stoffel *et al.*, 1994, 1998; Loudon and Benovic, 1997), but individual cysteine mutants have not been analyzed. When expressed in HEK293 cells, each of the cysteine point mutants showed a complete loss of PM localization, as determined by immunofluorescence microscopy (Figure 2A). GRK6A-C561S, GRK6A-C562S, and GRK6A-C565S were distributed throughout the interior of the cell in contrast to the sharp PM staining observed with wild-type GRK6A. Consistent with the immunofluorescence results, fractionation experiments demonstrated an increase in the amount of soluble GRK6A upon cysteine to serine mutation (Figure 2C, lanes 5–10). It was also confirmed that individual mutation of the cysteine residues inhibited radiolabeled palmitate incorporation into GRK6A (Figure 2D). GRK6A can incorporate radiolabeled palmitate (Figure 2D, lane 2), as described previously (Stoffel *et al.*, 1994, 1998; Loudon and Benovic, 1997), but GRK6A-C561S and GRK6A-C562S show no detectable palmitoylation (Figure 2D, lanes 3 and 4). GRK6A-C565S displays a decreased but detectable level of palmitoylation (Figure 2D, lane 5) compared with wild-type GRK6A. Collectively, these results indicate that all three cysteine residues are required for efficient palmitoylation and PM localization of GRK6A.

To further address the requirement for palmitoylation in PM targeting of GRK6A, cells expressing wild-type GRK6A were treated with the palmitoylation inhibitor, 2-BP (Webb *et al.*, 2000). Incubation of cells expressing GRK6A with 100 μ M 2-BP caused a shift in subcellular localization of GRK6A from the PM to the cytoplasm and nucleus (Figure 2B). This effect was specific for palmitoylated GRK6A, because PM localization of GRK6B and GRK6C, GRK6 variants that do not have palmitoylatable C-terminal cysteines (Figure 1A) was not disrupted by 2-BP treatment (Figure 2B). Moreover, treatment with 2-BP resulted in a shift of GRK6A into the soluble fraction (Figure 2C, lanes 3 and 4). These results confirm the importance of palmitoylation in PM targeting of GRK6A.

Next, we examined whether the predicted amphipathic helix region of GRK6A is important for PM localization as has been shown for GRK5 (Thiyagarajan *et al.*, 2004). The hydrophobic LLXXLF sequence was demonstrated to be necessary for PM localization of GRK5 (Thiyagarajan *et al.*, 2004), and thus we generated a mutant GRK6A, termed GRK6A-LL551,552AA, in which both leucines at position 551 and 552 (Figure 1A) were mutated to alanines to remove the hydrophobic side chains. When expressed in cells, GRK6A-LL551,552AA showed a loss of PM localization (Fig-

Figure 2. Both palmitoylation and the amphipathic helix are required for plasma membrane localization of GRK6A. (A) immunofluorescence staining using the anti-GRK4-6 mAb shows subcellular distribution of transiently transfected GRK6A, GRK6A-C561S, GRK6A-C562S, GRK6A-C565S, and GRK6A-LL551,552AA (LL→AA). (B) HEK293 cells stably expressing the three GRK6 splicing variants were treated with 100 μ M 2-bromopalmitate (2-BP) for 16 h, and immunofluorescent staining was carried out. Bar, 10 μ m. (C) HEK293 cells were transiently transfected with expression vectors for GRK6A (lanes 1-4) and the indicated mutants (lanes 5-12). Thirty-two hours after transfection, cells expressing GRK6A were treated with 100 μ M 2-BP for 16 h (lanes 3 and 4). Cells were lysed, and the lysates were separated by high-speed centrifugation into soluble (S) and particulate (P) fraction. The expressed proteins were detected by Western blotting with the anti-GRK4-6 mAb. (D) Cos-7 cells transfected with empty vector (lane 1) and GRK6A (lane 2), and the indicated GRK6A mutants (lanes 3-6) were labeled with [3 H]palmitate and then immunoprecipitated and subjected to SDS-PAGE and fluorography (top panel, 90-d exposure at -80° C), as described in *Materials and Methods*, and Western blotting using anti-GRK4-6 mAb (bottom panel).

ure 2A) and a shift to the soluble fraction (Figure 2C, lanes 11 and 12) identical to that observed for the cysteine mutants described above. Consistent with the localization results, the mutation of the two leucines to alanines inhibited the palmitoylation of GRK6A (Figure 2D, lane 6), even though all three C-terminal cysteines are present in GRK6A-LL551,552AA. Thus, the results in Figure 2 demonstrate that both the amphipathic helix motif and sites of palmitoylation are critical for PM localization of GRK6A.

Acidic Residues in the C-Terminus of GRK6A Negatively Regulate PM Localization and Allow Changes in Palmitoylation To Regulate Subcellular Localization of GRK6A

Interestingly, the results in Figure 2 presented an apparent paradox. Although GRK6A contains the amphipathic helix motif like other members of the family (Figure 1A), disruption of palmitoylation prevents PM localization. In other words, GRK5, GRK6B, and GRK6C do not require palmitoylation for PM localization, so why is the amphipathic helix motif of GRK6A not sufficient for its PM localization? An analysis of a GRK6A deletion mutant, GRK6A Δ 16, in which the C-terminal 16 residues are removed, reinforced this paradox and suggested that the C-terminus of GRK6A contains negative elements that function to decrease the strength of membrane binding. Thus, when GRK6A's C-terminal 16 amino acids, encompassing all three cysteine sites of palmitoylation, were removed (Figure 1A), only a partial defect in membrane targeting was observed (Supplementary Figures 2 and 3). By immunofluorescence GRK6A Δ 16 showed little or no loss of PM localization compared with wild-type GRK6A (Supplementary Figure 3). Cell fractionation demonstrated that GRK6A Δ 16 is increased compared with wild type in the soluble fraction, but the majority of GRK6A Δ 16 remained in the membrane-bound particulate fraction (Supplementary Figure 2). These results were in contrast to the localization of the individual cysteine mutants (Figure 2, A and C) that were no longer detected at the PM and showed a much stronger shift to the soluble fraction. In other words, mutation of a

single cysteine causes a much more severe defect in membrane localization than deletion of a C-terminal region that includes all three cysteines. Thus, to explain this conundrum we hypothesized that the C-terminal region must contain elements that inhibit membrane binding, in addition to the palmitoylation and amphipathic helix motifs that promote membrane binding.

The cysteine sites of palmitoylation are surrounded by acidic amino acids that could potentially play a negative membrane binding role by repelling interactions with negatively charged lipid head groups. Indeed, as described herein, substituting key acidic residues in the C-terminus of GRK6A rescued plasma membrane localization of the non-palmitoylated GRK6A-C561S mutant. Mutation of aspartic acid 560 to arginine in the background of the GRK6A-C561S mutant resulted in strong PM localization (Figure 3A) of this mutant, termed GRK6A-C561S/D560R, and a recovery of the majority of the protein in the particulate fraction (Figure 3B). Likewise, substitution of a stretch of three glutamic acids at position 569-571 with three arginines in the GRK6A-C561S mutant caused a strong recovery of PM localization (Figure 3A) and partitioning into membrane fraction (Figure 3B) for this mutant, termed GRK6A-C561S/3R. Moreover, merely disrupting the negative charge of amino acids 569-571 by changing the three glutamic acids to glutamines was sufficient to recover membrane targeting of GRK6A-C561S (not shown).

The GRK6A-C561S mutant retains two cysteine sites of palmitoylation; thus, we examined whether the acidic to basic mutations rescued PM localization in a palmitoylation-independent manner by testing the effect of 2-BP on PM localization of the GRK6A mutants. Although treatment with 2-BP caused a shift of wild-type GRK6A from the PM to the cytoplasm/nucleus (Figures 2B and 4), 2-BP did not change the strong PM localization of GRK6A-C561S/D560R, GRK6A-C561S/3R, or GRK6A-3R (Figure 4). These results suggest that under conditions where palmitoylation is disrupted, GRK6A remains tethered to the PM when key C-terminal acidic amino acids are replaced with basic residues. In addition, a palmitoylation assay indicated that very low

Figure 3. C-terminal acidic to basic residue mutations rescue plasma membrane localization of GRK6A-C561S. (A) Immunofluorescence staining using the anti-GRK4-6 mAb shows subcellular distribution of transiently transfected GRK6A, GRK6A-C561S, GRK6A-C561S/D560R, and GRK6A-C561S/3R in HEK293 cells. Bar, 10 μ m. (B) Cell fractionation assay of HEK293 cells transiently expressing GRK6A (lanes 1 and 2), GRK6A-C561S (lanes 3 and 4), GRK6A-C561S/D560R (lanes 5 and 6), and GRK6A-C561S/3R (lanes 7 and 8) was performed as described in *Materials and Methods*.

radiolabeled palmitate incorporation could be detected for GRK6A-C561S/D560R or GRK6A-C561S/3R (not shown), similar to GRK6A-C561S (Figure 2D), consistent with the

Figure 4. 2-BP does not disrupt plasma membrane localization of GRK6A acidic to basic mutants. GRK6A and the indicated mutants were transiently expressed in HEK293 cells. 32 h after transfection, cells were treated with 100 μ M 2-BP for 16 h. Immunofluorescent staining was performed using the anti-GRK4-6 mAb as described in *Materials and Methods*, and resulting images were deconvolved using a "no neighbors" algorithm (Slidebook v.4). The 3R mutation of GRK6A indicates substitution of glutamic acids 569-571 with arginines. Bar, 10 μ m.

Figure 5. C-terminal acidic to basic residue mutations fail to rescue plasma membrane localization of amphipathic helix mutant of GRK6A. GRK6A, GRK6A-LL551,552AA (LL→AA), and GRK6A-LL551,552AA with the further glutamic acids 569-571 to arginines mutations (LL→AA/3R) were transiently expressed in HEK293 cells and visualized by immunofluorescent staining using the anti-GRK4-6 mAb 48 h after transfection. Bar, 10 μ m.

2-BP evidence (Figure 4) that the acidic to basic mutations in the C-terminus recovered PM localization of GRK6A-C561S independent of palmitoylation. In contrast, when the 3R mutations, i.e., substitution of glutamic acids 569-571 with arginines, were introduced into wild-type GRK6A, palmitoylation was observed similar to or slightly increased compared with wild-type GRK6A (not shown). Thus, these results in Figures 3 and 4 are consistent with a role for negatively charged amino acids at positions 560 and 569-571 in inhibiting the membrane binding of GRK6A.

Next, we asked whether C-terminal acidic to basic mutations were able to rescue PM localization of the GRK6A amphipathic helix mutant GRK6A-LL551,552AA (Figure 5). Interestingly, substitution of glutamic acids 569-571 with arginines in the background of the GRK6A-LL551,552AA failed to recover any PM localization; GRK6A-LL551,552AA/3R remained distributed throughout the cytoplasm and nucleus identical to GRK6A-LL551,552AA (Figure 5). Thus, even though all three potential cysteine sites of palmitoylation are present in GRK6A-LL551,552AA/3R, the hydrophobic side chains of the amphipathic helix motif appear to be absolutely essential for GRK6A membrane targeting.

Depalmitoylation and Nuclear Localization of GRK6A

A recent study characterized nuclear localization of GRK5 and suggested that GRK6A is also a nuclear protein (Johnson *et al.*, 2004). Although overexpressed GRK6A is predominantly PM localized in HEK293 (results herein) and COS-7 cells (not shown; Vatter *et al.*, 2005), we do detect some clear nuclear localization of GRK6A in a minority of cells (not shown). However, dramatic nuclear localization is observed in many cells when PM localization of GRK6A is disrupted by C-terminal mutations or by blocking palmitoylation with 2-BP (Figures 1–5). This suggested the possibility that nuclear localization of GRK6A could be regulated by changes in palmitoylation status, and we thus characterized GRK6A nuclear localization further. A clear shift from PM-localized to nuclear-localized GRK6A could be observed within 4 h of treatment with 2-BP (Figure 6A), and, as described for GRK6B and GRK6C, the PM localization of GRK5 is not perturbed by 2-BP treatment (Figure 6A). Analysis of immunofluorescence microscopy results from multiple experiments indicated that, in untreated cells, expressed GRK6A was observed in the nucleus in <5% of transfected cells, but after 4 h of incubation with 2-BP nuclear GRK6A was clearly observed in at least 75% of transfected cells.

To extend the above results on nuclear translocation of GRK6A, nuclear fractions were prepared from cell lysates, and the presence of GRK6A was determined by immunoblotting (Figure 6, B and C). GRK6A was weakly detected in the nuclear fraction from untreated cells; however, consis-

Figure 6. Depalmitoylation promotes plasma membrane to nucleus translocation of GRK6A. (A) HEK293 cells were transfected with GRK5 or GRK6A expression vectors. Cells were treated with 100 μ M 2-BP for 4 or 16 h, as indicated, before fixing the cells 48 h after transfection. Localization of GRK5 and GRK6A were visualized by immunofluorescence staining using the anti-GRK4-6 mAb (top), and nuclei were visualized with DAPI staining (middle). Merge images are also shown (bottom). Bar, 10 μ m. (B) HEK293 cells were transfected with GRK5 (lanes 2–4) or GRK6A (lanes 5–7) expression vectors or with empty vector pcDNA3 (lane 1). Cells were treated with 100 μ M 2-BP for 4 or 16 h, as indicated, before lysing the cells 48 h after transfection. Nuclei were prepared as described in *Materials and Methods*, and nuclear extracts were used for Western blotting with anti-GRK4-6 mAb (top) or anti-SP1 antibody (middle). SP1 Western blotting was used to indicate equal loading of nuclear extracts. Total cell lysates from the same experiment were used for Western blotting with anti-GRK4-6 mAb (bottom). (C) The relative amount of GRK5 and GRK6A accumulation in nuclei from B was quantified and normalized by Sp1 levels, as described in *Materials and Methods*. Data are presented as the fold increase above the basal condition (0 h) and expressed as mean $\pm$ SEs of the mean from three independent experiments.

tent with the immunofluorescence localization, the amount of GRK6A in the nuclear fraction was increased three- to fourfold after 4 and 16 h of incubation of cells with 2-BP. Nuclear fractionation also detected a low level of nuclear GRK5, but it was unaffected by 2-BP (Figure 6, B and C). Thus, the results in Figure 6 suggest that depalmitoylation of GRK6A not only releases it from the PM but is also a trigger for nuclear localization.

Not only is nonpalmitoylated GRK6A targeted to the nucleus, but it is also exported out of the nucleus as determined through the use of the nuclear export inhibitor LMB. When the palmitoylation-deficient mutant GRK6A-C561S was expressed in HEK293 cells, a majority of cells (65%) showed a relatively even distribution of GRK6A-C561S between the cytoplasm and nucleus (Figure 7); however, after incubation of the cells for 4 h with LMB, a majority of cells (65%) displayed a GRK6A-C561S localization pattern (C < N) in which the nuclear staining was clearly much more intense than the cytoplasm staining (Figure 7), and likewise cells with GRK6A-C561S localization patterns of even distribution between the cytoplasm and nucleus (C = N) and greater cytoplasmic versus nuclear staining (C > N) were reduced compared with cells not treated with LMB. Arrestin-3 was used as a control protein (Figure 7) that dramatically

changes localization from mostly cytoplasmic to strongly nuclear upon LMB inhibition of nuclear export (Scott *et al.*, 2002; Wang *et al.*, 2003). These results indicate that GRK6A is capable of shuttling in and out of the nucleus.

Lastly, we asked whether a specific nuclear localization sequence (NLS) was involved in the depalmitoylation-dependent nuclear localization of GRK6A. A recent report (Johnson *et al.*, 2004) described a NLS in GRK5 and postulated based on sequence similarity that a NLS exists in GRK6 at the same location, amino acids 387–395 of GRK6A. It was also noted (Johnson *et al.*, 2004) that a second potential NLS exists in GRK4-6, at amino acids 219–226 of GRK6A. We thus tested for a potential NLS in GRK6A by mutating basic residues in the predicted sequences. Importantly, we established three criteria by which to determine in our system whether a particular amino acid or group of amino acids might function in nuclear localization: 1) Mutation of the basic residue(s) of the potential NLS in the background of wild-type GRK6A must not affect the predominant PM localization; 2) When cells expressing a potential NLS mutant in the background of wild-type GRK6A are treated with 2-BP, the mutant GRK6A will shift from the PM to cytoplasm, but be excluded from the nucleus; and 3) A potential NLS mutant generated in the background of the palmitoyl-

Figure 7. Leptomycin B causes increased nuclear accumulation of GRK6A-C561S. HEK293 cells were transfected with expression vectors for GRK6A-C561S (C561S) or arrestin-3-GFP (Arr-3). Twenty-four hours after transfection, cells were treated with 10 ng/ml leptomycin B (LMB) for 8 h. Cells were fixed and localization was visualized by immunofluorescence staining using the anti-GRK4-6 mAb as described in *Materials and Methods* or by direct visualization of GFP for GFP-arrestin-3-expressing cells. Localization of GRK6A-C561S or arrestin-3 was scored as follows: C > N indicates mostly in the cytoplasm; C = N indicates equally distributed in cytoplasm and nucleus; C < N indicates predominantly in nucleus. Data are presented as the percent ($\pm$ SE) of expressing cells exhibiting the above criteria. More than 100 cells were counted in each of three separate experiments.

lation-defective GRK6A-C561S will localize in the cytoplasm and be excluded from the nucleus. We tested a number of potential mutants (data not shown), but only one mutant satisfied the above criteria. As shown in Figure 8A, introduction of a lysine to glutamine substitution at position 219 did not disrupt the strong PM localization of GRK6A. However, introduction of K219Q into GRK6A-C561S caused GRK6A-C561S, K219Q to localize predominantly in the cytoplasm compared with the cytoplasmic and nuclear localization of GRK6A-C561S (Figure 8, A and B). Moreover, when cells expressing GRK6A-K219Q were treated with 2-BP, its localization shifted from PM to cytoplasm but was excluded from the nucleus (Figure 8, C and D).

DISCUSSION

In this report, we demonstrated that the C-terminal extension of GRK6A contains multiple elements that regulate plasma membrane localization (Figure 9). Specifically, two positive elements, an amphipathic helix motif and cysteine sites of palmitoylation, are both required for membrane localization. On the other hand, several acidic amino acids function in a negative way to decrease GRK6A's ability to localize to plasma membranes. Thus, the presence of the acidic amino acids in the C-terminal tail allows changes in palmitoylation to control the subcellular localization of GRK6A, and we propose that the C-terminus of GRK6A functions as a novel electrostatic-palmitoyl switch. Moreover, we show that depalmitoylation of GRK6A is not only responsible for plasma membrane to cytoplasm translocation but also triggers nuclear localization of GRK6A.

Previously, we made the novel observation that a predicted amphipathic helix that functions in plasma membrane localization is present in the C-terminus of GRK5 (Thiyagarajan *et al.*, 2004), and now we extend the importance of the amphipathic helix motif to GRK6A. Decreasing the hydrophobic nature of this region of GRK6A by substi-

Figure 8. Mutation of K219 inhibits nuclear localization of GRK6A. (A) Expression vectors for wild-type GRK6A, GRK6A-K219Q, GRK6A-C561S and GRK6A-C561S/K219Q were transiently transfected into COS-7 cells. Localization of the expressed proteins was visualized by immunofluorescence staining using the anti-GRK4-6 mAb. Bar, 10 μ m. (B) Nuclear versus cytoplasmic distribution for GRK6A-C561S and GRK6A-C561S/K219Q was quantified using the criteria as described in the Figure 7 legend. (C) Expression vectors for wild-type GRK6A and GRK6A-K219Q were transiently transfected into COS-7 cells. Cells were treated with 100 μ M 2-BP for 16 h and subjected to the immunofluorescent staining. Bar, 10 μ m. (D) Nuclear versus cytoplasmic distribution for GRK6A and GRK6A-K219Q, after 2-BP treatment, was quantified using the criteria as described in the Figure 7 legend.

tuting two leucines at positions 551 and 552 with alanines was sufficient to cause a dramatic defect in the ability of GRK6A to localize at cellular plasma membranes and reside in a membrane-bound cell fraction (Figure 2). Moreover, disruption of the amphipathic helix also inhibited palmitoylation of GRK6A (Figure 2), suggesting that the membrane binding mediated by the amphipathic helix is a prerequisite for palmitoylation. The primary importance of the amphipathic helix motif was also emphasized by the observation that changing the acidic residues to basics at positions

Figure 9. Model for multiple elements in the C-terminus of GRK6A regulating membrane attachment. Both a predicted amphipathic region and cysteine sites of palmitoylation are proposed to function together for plasma membrane localization of GRK6A. In addition, five acidic residues are present between positions 560 and 571, and these are proposed to inhibit membrane binding. When GRK6A is depalmitoylated, the regions of negative charge would weaken the membrane-binding ability of the amphipathic helix such that GRK6A is able to redistribute into the cytoplasm and into the nucleus. In this way, the combination of elements in the C-terminus of GRK6A could function as an electrostatic palmitoyl switch.

569-571 failed to recover PM localization of GRK6A-LL551,552AA (Figure 5), even though such acidic to basic changes did rescue PM localization of palmitoylation defective GRK6A. The amphipathic helix motif is conserved in all members of the GRK4 subfamily of GRKs (Figure 1A). This conservation, together with our demonstrations of the importance of this motif in GRK5 (Thiyagarajan *et al.*, 2004) and GRK6A (this report), suggests that the amphipathic helix motif provides a mechanism of membrane binding utilized by all members of the GRK4 subfamily. The predicted amphipathic helix is composed primarily of basic and hydrophobic residues, and a recent report indicated that polybasic regions of many proteins facilitate PM localization by binding $\text{PI}(4,5)\text{P}_2$ and $\text{PI}(3,4,5)\text{P}_3$ (Heo *et al.*, 2006). It will be interesting to determine whether the C-terminal amphipathic helices of the GRK4 subfamily show a similar membrane lipid-binding specificity.

In addition to defining the importance and commonality of the amphipathic helix motif in GRK6A, the results presented here addressed the importance of C-terminal palmitoylation of GRK6A and provided an explanation for how both membrane-binding mechanisms contribute to the subcellular localization of GRK6A. Individual substitution of cysteines at positions 561, 562, or 565 caused a loss of PM localization and palmitoylation of GRK6A (Figure 2), and inhibition of palmitoylation with 2-BP also prevented PM localization of GRK6A. Thus, both the amphipathic helix motif and palmitoylation are required for proper PM localization of GRK6A. In other words, neither the amphipathic helix motif nor the sites of palmitoylation alone are sufficient to target GRK6A to the PM. The lack of PM localization of nonpalmitoylated cysteine mutants of GRK6A was in contrast to the strong PM localization of nonpalmitoylated members of the GRK4 subfamily, such as GRK5, 6B, and 6C. Particularly intriguing was the demonstration that GRK6C and a 1-562 mutant of GRK5 (Pronin *et al.*, 1998), in which the C-terminal 28 amino acids are deleted, are localized at the PM. Both GRK6C and (1-562)GRK5 terminate immediately after the conserved amphipathic helix motif. The strong membrane targeting of GRK6C and (1-562)GRK5 but lack of membrane targeting of the cysteine mutants of GRK6A strongly suggested that the C-terminal region of

GRK6A contained elements that inhibited membrane binding. Consistent with this idea our results show that a number of acidic residues in the extreme C-terminus fulfill this inhibitory role (Figures 1A, 3, and 4).

Our results suggest that the combination of positive and negative membrane-targeting motifs contained within the 30 amino acid C-terminus of GRK6A functions as an electrostatic-palmitoyl switch (Figure 9). Myristoyl- and farnesyl-electrostatic switches in myristoylated alanine-rich C kinase substrate (MARCKS; McLaughlin and Aderem, 1995) and K-Ras (Bivona *et al.*, 2006), respectively, have been previously described. MARCKS is targeted to membranes via the combination of N-terminal myristoylation and a polybasic/hydrophobic stretch of amino acids, while K-Ras utilizes C-terminal farnesylation and an adjacent polybasic stretch for PM localization. In both cases, protein kinase C (PKC)-mediated phosphorylation of serine residues within the polybasic regions promotes translocation off of the PM, and mechanistically the phosphorylation is thought to provide the electrostatic switch by neutralizing the ability of the surrounding basic amino acids to interact with acidic lipid headgroups. The myristoyl and farnesyl lipids only weakly bind membranes (Peitzsch and McLaughlin, 1993; Silvius and l'Heureux, 1994), and therefore when the polybasic regions are neutralized, MARCKS and K-Ras have a decreased affinity for membranes. Myristoyl- and farnesyl-electrostatic switches thus utilize a static hydrophobic lipid combined with reversible phosphorylation to affect electrostatic interactions. The electrostatic-palmitoyl switch proposed for GRK6A presents a novel twist in that the electrostatic portion of the switch, the acidic residues in GRK6A, is static, but the hydrophobic lipid is reversible. A key component of the GRK6A electrostatic-palmitoyl switch that differentiates it from other demonstrations of depalmitoylation/palmitoylation membrane translocations (Hancock *et al.*, 1990; Wedegaertner and Bourne, 1994; Drenan *et al.*, 2005) is that the acidic amino acids that we identified are essential for the C-terminal region to function as a palmitoylation-regulated switch. We confirmed the importance of the acidic residues as a key part of the switch by mutating several of them to arginines (Figures 3 and 4) or glutamines (not shown). For example, disruption of palmitoylation of GRK6A, either by mutation of C561S (Figure 3) or by treatment of cells with 2-BP (Figure 4), failed to induce release of GRK6A-3R from the PM. The reversibility of palmitoylation of proteins can be regulated (Smotrys and Linder, 2004); it will be important to determine the rate of palmitate turnover on GRK6A and to define physiological stimuli that affect changes in the palmitoylation status of GRK6A.

H-ras provides another example of a palmitoylated protein that utilizes a combination of positive and negative forces to modulate its PM localization (Rotblat *et al.*, 2004). The C-terminal tail of H-ras contains two attractive membrane-binding forces, a farnesylated and palmitoylated extreme C-terminus and a hypervariable region, and these attractive forces also promote interaction with lipid raft or nonraft domains, respectively. On the other hand, H-ras also possesses a repulsive force that decreases membrane binding, and this repulsive force is enhanced by H-ras activation (i.e., GTP binding). The mechanism of this negative contribution to membrane binding has not been defined for H-ras. Nonetheless, it seems likely that other signaling proteins will be shown to utilize a combination of positive and negative membrane-binding elements for regulated and reversible membrane localization.

A recent report also examined the importance of the C-terminus of GRK6A (Vatter *et al.*, 2005) in terms of ability to

phosphorylate the GPCR rhodopsin *in vitro*. In agreement with earlier reports (Loudon and Benovic, 1997; Stoffel *et al.*, 1998), a triple cysteine mutant of GRK6A was decreased in its ability to phosphorylate rhodopsin (Vatter *et al.*, 2005). However, deletion of the C-terminal nine amino acids, including the stretch of glutamic acids 569–571, strongly restored the ability of nonpalmitoylated GRK6A to phosphorylate rhodopsin to a level equal to or greater than that of wild-type GRK6A (Vatter *et al.*, 2005). It has been established that GRKs must be able to interact with membranes in order to efficiently phosphorylate membrane bound GPCRs, and thus the differing activities of the described C-terminal deletions of GRK6A to phosphorylate rhodopsin (Vatter *et al.*, 2005) may simply reflect differential membrane binding, as described herein. However, we cannot rule out that the C-terminus also has direct effects on the catalytic activity of GRK6A. Interestingly, a structure of GRK6A was recently solved using a triple cysteine mutant of GRK6A, but the structure of the C-terminus (amino acids 536–576) could not be determined and was thus likely disordered (Lodowski *et al.*, 2006). Structures of different conformations of GRK6A or in the presence of lipid may shed further light on the roles of the C-terminus in regulating membrane binding and catalytic activity.

The results presented herein also suggest that changes in palmitoylation of GRK6A can regulate its nuclear localization. We demonstrated that nonpalmitoylated forms of GRK6A not only are released from the PM into the cytoplasm but are also detected in the nucleus (e.g., Figure 6). Moreover, increased nuclear localization of GRK6A-C561S after treatment with the nuclear export inhibitor LMB (Figure 7) suggests that GRK6A is capable of shuttling into and out of the nucleus. Taken together, these results suggest a novel trafficking pathway for GRK6A in which regulated changes in palmitoylation can regulate cycles of PM to nuclear shuttling. A similar model has recently been described for the R7 family binding protein (R7BP) that binds to a subfamily of the regulator of G protein–signaling (RGS) proteins. R7BP is C-terminally palmitoylated, and this palmitoylation is necessary to target a R7BP/RGS7/G β 5 complex to the PM; nonpalmitoylated R7BP directs the complex to the nucleus (Drenan *et al.*, 2005, 2006; Song *et al.*, 2006). In addition, estrogen receptor α (Li *et al.*, 2003; Acconcia *et al.*, 2005; Rai *et al.*, 2005), and phospholipid scramblase 1 (Wiedmer *et al.*, 2003) have been demonstrated recently to localize to the PM when palmitoylated but to move to the nucleus when depalmitoylated. As discussed recently (Drenan *et al.*, 2006), regulated palmitoylation may turn out to be a common mechanism used by signaling proteins to control PM/nuclear shuttling.

A role for nuclear GRK6A remains to be elucidated, but numerous G protein–signaling components have been detected in the nucleus, and, in some cases, potential functions have been described (Willard and Crouch, 2000; Johnson *et al.*, 2004; Drenan *et al.*, 2005; Kang *et al.*, 2005; Kino *et al.*, 2005; Gobeil *et al.*, 2006; Neuhaus *et al.*, 2006; Song *et al.*, 2006). Strikingly, recent studies have shown that GRK5 partially localizes to the nucleus when expressed in several cell lines (Johnson *et al.*, 2004) and also endogenously (Yi *et al.*, 2002, 2005; Johnson *et al.*, 2004). In cardiac myocytes, treatment with the PKC activator tissue plasminogen activator caused a nuclear accumulation of GRK5 (Yi *et al.*, 2002), and Ca²⁺/calmodulin binding to the GRK5 N-terminus was implicated in promoting nuclear export of GRK5 in Hep2 cells (Johnson *et al.*, 2004). Thus, phosphorylation and Ca²⁺/calmodulin binding appear to regulate PM/nuclear shuttling of GRK5, instead of regulated palmitoylation, as is the

case for GRK6A. It seems likely that additional mechanisms, such as phosphorylation and protein–protein interactions, will provide additional layers of regulation of GRK6A's subcellular localization.

GRK5 was shown to bind to DNA (Johnson *et al.*, 2004), suggesting that nuclear GRK5 regulates gene expression; DNA binding by GRK6A has not been tested. In addition to a potential direct role for GRK6A in the nucleus, removal of GRK6A from the PM might be expected to lead to enhanced GPCR signaling simply because a negative regulator, GRK6A, has translocated to another subcellular compartment. Such possibilities will require further investigation. Another key question is the mechanism of nuclear shuttling of GRK6A. Sequence predictions, as described recently (Johnson *et al.*, 2004), indicate potential NLS at positions 219–226 and 387–395 of GRK6A. Both of these sequences form part of a basic surface of GRK6A surrounding the catalytic site that may be positioned to interact with the PM (Lodowski *et al.*, 2006). Although the extreme C-terminus is clearly necessary for PM localization of GRK6A, it is likely that other regions of the protein contribute. These basic regions of GRK6A may be positioned to serve as both additional membrane-binding determinants and NLSs. Our studies identified one mutation, K219Q, that inhibited nuclear accumulation of nonpalmitoylated GRK6A (2-BP treated wild-type GRK6A or GRK6A-C561S) yet did not disrupt PM localization of wild-type GRK6A (Figure 8). These results suggest that the 219–226 sequence participates in nuclear localization of GRK6A. Further investigation is needed to understand the detailed mechanisms of nuclear import of GRK6A. Sequence predictions of GRK6A also suggest a leucine-rich potential nuclear export sequence (NES) at positions 259–265. However, this region is buried in GRK6A (Lodowski *et al.*, 2006), and substantial structural rearrangements would be required for this potential NES to become available to interact with a cell's nuclear export machinery. In summary, our results have identified a novel combination of positive and negative membrane-targeting elements in the short C-terminal extension of GRK6A (Figure 9). The C-terminus appears to function as an electrostatic palmitoyl switch to regulate PM/nuclear shuttling of GRK6A. Further understanding the mechanisms of GRK6A trafficking should provide insight into novel functional roles for this protein.

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