G Protein-coupled Receptor Kinase 5 Is Localized to Centrosomes and Regulates Cell Cycle Progression*

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Background: G protein-coupled receptor kinases (GRKs) are important regulators of receptor function although little is known about their cellular localization.

Results: GRK5 is localized in the centrosome during interphase and regulates normal cell cycle progression.

Conclusion: GRKs appear to dynamically modulate regulation of the cell cycle.

Significance: These findings reveal an additional mechanism by which plasma membrane signaling can regulate the cell cycle.

G protein-coupled receptor kinases (GRKs) are important regulators of G protein-coupled receptor function and mediate receptor desensitization, internalization, and signaling. While GRKs also interact with and/or phosphorylate many other proteins and modify their function, relatively little is known about the cellular localization of endogenous GRKs. Here we report that GRK5 co-localizes with γ-tubulin, centrin, and pericentrin in centrosomes. The centrosomal localization of GRK5 is observed predominantly at interphase and although its localization is not dependent on microtubules, it can mediate microtubule nucleation of centrosomes. Knockdown of GRK5 expression leads to G2/M arrest, characterized by a prolonged G2 phase, which can be rescued by expression of wild type but not catalytically inactive GRK5. This G2/M arrest appears to be due to increased expression of p53, reduced activity of aurora A kinase and a subsequent delay in the activation of polo-like kinase 1. Overall, these studies demonstrate that GRK5 is localized in the centrosome and regulates microtubule nucleation and normal cell cycle progression.

Materials—Nocodazole, cytochalasin B, and thymidine were purchased from Sigma-Aldrich. Monoclonal antibodies used were anti-γ-tubulin (1:1000, Abcam, Cambridge, MA), anti-α-tubulin (1:1000, Sigma-Aldrich), anti-GRK4 – 6 (1:100 – 1:1000, Millipore), anti-PLK1-pT210 (1:1000, Biolegend), anti-pH2AX (1:1000, Cell Signaling), anti-aurora A kinase (1:10000, Sigma-

EXPERIMENTAL PROCEDURES

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Aldrich), and anti-aurora A kinase-pT288 (1:1000, Cell Signaling). Polyclonal antibodies used were anti-γ-tubulin (1:1000, Sigma-Aldrich), anti-α-tubulin (1:1000, Abcam), anti-pericentrin (1:2000, Abcam), anti-nucleophosmin-pS4 (1:2000, Cell Signaling Technology), anti-calnexin (1:10,000, Stressgen), and anti-GRK5 (1:50, a generous gift from Dr. Lan Ma, Fudan University). Secondary antibodies for immunofluorescence were from Invitrogen and included Alexa-Fluor 488-goat anti-mouse, Alexa-Fluor 594-goat anti-mouse, Alexa Fluor 488-conjugated goat anti-rabbit, Alexa Fluor 594-conjugated goat anti-rabbit and Alexa Fluor 633-conjugated goat anti-rabbit. Secondary antibodies for immunoblotting were obtained from Vector Laboratories (Burlingame, CA), and Alexa Fluor 680-conjugated goat anti-rabbit (Molecular Probes) and IRDye 800-conjugated goat anti-mouse (Rockland Immunochemicals) were also used.

Cell Culture—HeLa and HEK293 cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) (Mediatech, Inc., Manassas, VA) supplemented with 10 mM HEPES and 10% fetal bovine serum (FBS) (GIBCO, Invitrogen). MDA-MB-231 cells

![FIGURE 1](image-url)

**GRK5 localization in the centrosome.** A, expression of GRK5 in HEK293, HeLa, and MDA-MB-231 cells, as demonstrated by immunoblotting with anti-GRK4–6 monoclonal antibody. B, HEK293, HeLa, and MDA-MB-231 cells were fixed and stained with anti-GRK4–6 monoclonal and γ-tubulin polyclonal antibodies. Representative images of cells in interphase are shown (n ≥ 120 cells from at least two independent experiments). Arrow denotes inset regions and colocalization. Scale bar, 5 μm. C, quantitation of the percentage of HEK293, HeLa, and MDA-MB-231 cells displaying centrosomal localization. D, control-shRNA and GRK5-shRNA-transfected HeLa cells were fixed and stained with anti-GRK4–6 monoclonal or γ-tubulin polyclonal antibodies. Note the decrease in staining intensity in cells transfected with GRK5-shRNA. Representative images of cells in interphase are shown. Arrow denotes inset regions and colocalization. Scale bar, 5 μm. E, immunoblotting for GRK5, using the GRK4–6 antibody, and γ-tubulin in HeLa cell sucrose density fractions enriched in centrosomal proteins such as γ-tubulin (n = 3). F, control-shRNA and GRK5-shRNA transfected HeLa cells were fixed and stained with anti-GRK5 or γ-tubulin polyclonal antibodies. Note the decrease in staining intensity in cells transfected with GRK5-shRNA.
were grown in MEM (Mediatech, Inc.) supplemented with sodium bicarbonate, sodium pyruvate, non-essential amino acids, and 10% FBS. RPE1 cells were cultured in DMEM:F12 medium (Mediatech, Inc) with 10% FBS. HeLa cells with stable GRK knock-down were grown in DMEM supplemented with 10 mM HEPES, 10% FBS, and 2 μg/ml puromycin. HeLa cells stably expressing GFP-centrin were grown in DMEM supplemented with 10 mM HEPES, 10% FBS, and 800 μg/ml G418 while HeLa cells stably expressing GFP-H2B were cultured in DMEM with 10% FBS and 1% penicillin/streptomycin. 

To create stable cell lines, shRNA constructs specific to GRK5 along with a scrambled control shRNA (Sigma-Aldrich) were transfected into HeLa cells using Lipofectamine 2000 (Invitrogen). Cells were then selected in 2 μg/ml puromycin to obtain a stably expressing population. The sequence of the GRK5 shRNA oligo was 5′-ACGAGATGATAGAAACAGAAT-3′. For GRK5 siRNA transfection, four independent GRK5 siRNA oligos were used together as an siRNA pool: 5′-CCAGCACGGGUCUUG CGAAGAAGGAAGAAGGAC-3′, 5′-GGGAGAAAACCAUCCAC CGA-3′, 5′-CCAACCAUGUCAGCGCU CGA-3′, and 5′-GAUUAUUGGCCACAUAGGA-3′. Control siRNA-scrambled sequences were purchased from Dharmacon.

For cell synchronization, mitotic cell shake off and double thymidine block were performed. For mitotic cell shake off, HeLa and RPE1 cells were grown to 70% confluency and treated with 0.5 μg/ml nocodazole for 16 h. To isolate mitotic cells, plates were tapped gently and media collected. Plates were also washed with phosphate-buffered saline (PBS), and media collected to obtain remaining mitotic cells while the remaining adherent cells were used as the interphase preparation. Cells were pelleted by centrifugation, lysed, and processed for Western blotting. For double thymidine blocks, HeLa cells were plated at 25% confluency and blocked in media containing 2 mM thymidine for 16 h, cells were released for 9–12 h into fresh medium and then blocked a second time with 2 mM thymidine for 16 h. RPE1 cells were plated at 25% confluency and blocked in media containing 2.5 mM thymidine for 16 h, released into fresh medium for 8 h, and then blocked again with 2.5 mM thymidine for 16 h. Cells were then released into fresh medium and the appropriate time points collected. Samples were prepared and standard Western blotting procedures were followed. Centrosomal fractionation was performed as previously described (27).

**Western Blotting**—For Western blots, samples were loaded onto either 10% or 8% SDS-PAGE gels, transferred to nitrocellulose membranes, and blocked in 5% nonfat milk in TBS (20 mM Tris-HCl, pH 7.5, 150 mM NaCl) with 0.1% Tween-20. Blots were incubated with primary antibody overnight at 4 °C, washed in TBS with 0.1% Tween-20, and incubated with appropriate secondary antibody for 1 h at room temperature. Blots

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**FIGURE 2.** GRK5 co-localizes with centrin and pericentrin and its localization is not affected by microtubule disruption. **A**, upper panel. GFP-centrin expressing HeLa cells were fixed and stained for GRK5 using the anti-GRK4–6 monoclonal antibody. Representative images of cells in interphase are shown. **Lower panel.** HeLa cells were fixed and stained with both monoclonal GRK4–6 and polyclonal pericentrin antibodies. Representative images of cells in interphase are shown. **B**, quantitation of GRK5 localization with the mother versus the daughter centriole (n = 30 mother/daughter centriole pairs from two independent experiments). **C**, HeLa cells pretreated with nocodazole to depolymerize microtubules were fixed and stained for GRK5 using the anti-GRK4–6 monoclonal antibody and γ-tubulin (n ≥ 85 cells from two independent experiments). Note that colocalization remains even after microtubule depolymerization. **D**, quantitation of the number of cells displaying centrosomal localization following nocodazole treatment. For all images, arrows denote inset regions and colocalization. Scale bar, 5 μm.
were washed extensively and then developed with either West PICO or DURA Chemiluminescence kit (Pierce). For some loading controls, blots were incubated with the designated primary antibody and detected using either the secondary antibody Alexa Fluor 680-conjugated goat anti-rabbit (Molecular Probes) or IRDye 800-conjugated goat anti-mouse (Rockland Immunochemicals) and the ODYSSEY infrared imaging system (Li-Cor Biosciences). Densitometry was performed with ImageJ (NIH). p53 and p21 expression was quantitated with ImageJ and normalized to ERK2 for loading, which was quantitated by ODYSSEY infrared imaging system. For RPE1 cells, p53 expression was quantified by ODYSSEY infrared imaging and normalized to the expression of calnexin as a control.

For immunoprecipitations, antibodies were first crosslinked to beads using dimethyl pimelimidate and then incubated with lysates from nocodazole-treated cells overnight at 4 °C. The beads were collected, washed four times with PBS, and then eluted by addition of SDS sample buffer and boiling.

**Immunofluorescence Microscopy**—For all immunofluorescence studies, 24–72 h before fixation/staining, cells were split onto poly-l-lysine-coated coverslips. To costain for endogenous GRK5 and y-tubulin, cells were fixed in 4% paraformaldehyde in PBS, permeabilized with methanol, incubated with Quench buffer (TBS, 3% nonfat milk, 150 mM sodium acetate) and block buffer (TBS, 0.1% Tween-20, 3% nonfat milk) at room temperature, and the appropriate primary antibodies overnight at 4 °C. Slides were incubated with the designated secondary antibodies for 1 h at room temperature and washed with TBS with 0.1% Tween-20. DNA was stained with DAPI (Molecular Probes, Eugene, OR) and slides mounted with Prolong Antifade (Molecular Probes). To stain for endogenous pericentrin and a-tubulin, cells were fixed in 20 °C methanol for 8 min, rehydrated in PBS for 10 min at room temperature, washed with KB buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 1% BSA), and incubated with primary antibody for 1 h at room temperature. Following washing with KB buffer, cells were incubated with the appropriate secondary antibody for 1 h at room temperature. Slides were washed with KB buffer, stained with DAPI, and mounted with Prolong Antifade. Images were taken using a Carl Zeiss LSM 510 META confocal microscope with a Plan-Apo 63 × 1.4 oil immersion lens except for RPE1 cells, which were taken on a Nikon Eclipse E800 fluorescence microscope. All intensity and area analysis was performed using Metamorph software. For p-H2AX staining, cells were fixed in 4% paraformaldehyde, permeabilized with 0.2% Triton X-100 in PBS, blocked in 1% BSA in PBS for 1 h, stained with p-H2AX antibodies overnight, washed in PBS, incubated with the designated secondary antibody for 1 h, washed again, and
then mounted. The cells were examined by microscopy on a Nikon Eclipse E800 fluorescence microscope using a Plan Fluor 60/1.4 oil objective. Images were collected using QED Camera software and processed with Adobe Photoshop and Image Pro Plus. Total cells stained and foci per cell were manually counted, with % cells p-H2AX stained expressed as (# cells p-H2AX stained/total cells counted) × 100. Some studies were also performed using a mouse polyclonal GRK5 antibody that was previously used to show co-localization of GRK5 and F-actin (17). To stain with this antibody, cells were grown on cover slips for 24–48 h, treated with 1% Triton X-100/PBS for 30 s, fixed with 4% paraformaldehyde in PBS, permeabilized with methanol, and subsequently blocked in 1% BSA/PBS for 1 h. Cells were then incubated with primary antibody (1:50 dilution in 1% BSA/PBS) overnight at 4 °C, washed with PBS, incubated with mouse secondary antibody for 1 h, washed, and then stained with DAPI and mounted. The cells were examined on a Nikon Eclipse E800 fluorescence microscope.

**Microtubule Nucleation Assay**—Stable GRK5 knockdown HeLa cells were treated for 1 h with 10 µg/ml nocodazole to completely depolymerize microtubules. To assess microtubule re-growth, cells were washed twice with warm media to remove nocodazole and incubated at 37 °C for 5 min. Cells were then fixed and stained with pericentrin and α-tubulin antibodies. Images were taken of at least 75 cells from six independent experiments and each aster was analyzed independently. Only asters nucleated from either single or paired centrosomes were analyzed. Quantitation of aster area (µm²) was obtained using Metamorph Software by drawing a region of interest around the aster and measuring the area of this region.

**Fluorescence Automated Cell Sorting (FACS) Analysis**—Briefly, cells were collected at designated times post release from double thymidine block and fixed with 70% ethanol. Cells were rehydrated with PBS, centrifuged and stained with propidium iodide staining solution (2 mg/ml DNase-free RNase, 1 mg/ml propidium iodide and 0.1% Triton X-100 in PBS). Samples were analyzed on a Beckman Coulter Epics XL-MCL Flow Cytometer.

**Live Cell Imaging**—HeLa cells stably over-expressing GFP-H2B were plated at 20% confluency in 6-well dishes 24 h before transfection. Cells were transfected with 200 nm control or GRK5 siRNA (ThermoFisher-Dharmacon) using Hiperfect (Qiagen). A standard double thymidine block as described above was performed 4 h following transfection. Filming began 2 h post release from the second thymidine block to allow cells to progress through S-phase for at least 20 h, and parallel plates were harvested to verify knockdown efficiency. For rescue studies, stable GRK5 knock-down HeLa cells were transfected with GFP-H2B and either 25–100 ng of vector, WT GRK5, or catalytically inactive GRK5 (GRK5-K215R). In these studies, the first thymidine block was started 24 h post-transfection and a standard double thymidine block was performed as described above.
Statistics—Student’s t test using Graphpad Prism was used to determine statistical differences between groups. p values <0.05 (*), <0.01 (**), and <0.001 (***) were considered statistically significant. Two-tailed t-tests were performed unless otherwise specified.

RESULTS

Based on recent evidence that arrestins are localized to centrosomes (25), we evaluated if GRKs are also localized in centrosomes. GRK5 localization to these structures was specifically explored since GRK5 had been previously identified in large-scale proteomic screens as one of many proteins that affect cell cycle progression (28, 29). Fig. 1A demonstrates the expression of GRK5 and GRK6 in the three cell lines tested for centrosomal localization, human embryonic kidney (HEK) 293 cells, cervical carcinoma HeLa cells and the breast cancer cell line MDA-MB-231. HeLa and MDA-MB-231 cells predominantly express GRK5 while HEK293 cells express both GRK5 and GRK6. Immunofluorescence microscopy was used to analyze GRK5 in these cells (Fig. 1B), and it was found that GRK5 localized to the centrosome in ~65–95% of cells, dependent on the specific cell line examined (Fig. 1C). We knocked-down GRK5 expression using an shRNA to validate the antibody specificity. HeLa cells stably transfected with a GRK5 shRNA exhibited an 80–90% reduction in GRK5 compared with control shRNA cells, as assessed by Western blotting (Fig. 5A), and a specific reduction in GRK5 centrosomal staining (Fig. 1D). The presence of GRK5 in centrosomes was also validated in a centrosomal preparation.
from HeLa cells (Fig. 1E) as well as by immunofluorescence staining using a GRK5-specific antibody (17) with ~80% of the cells showing centrosomal staining (Fig. 1F).

We next sought to further characterize the GRK5 centrosomal localization. To explore if GRK5 is localized to the centrioles or the pericentriolar material, co-localization studies were performed with the centriolar and pericentriolar material markers, centrin and pericentrin, respectively (Fig. 2). GRK5 co-localized with both markers (Fig. 2A) and, interestingly, preferentially associated with the mother centriole (Fig. 2B), which displays higher centrin staining (30). The dependence of the centrosomal localization of GRK5 on microtubules was next determined. Cells were treated with nocodazole to depolymerize the microtubule network and GRK5 localization was examined by immunofluorescence microscopy. GRK5 remained associated with centrosomes after microtubule disruption, suggesting that localization was independent of microtubules (Fig. 2, C and D).

To determine if the centrosomal localization of GRK5 is constitutive or varies during the cell cycle, the average intensity of GRK5 centrosomal localization in HeLa cells was quantified during interphase, metaphase, and late anaphase-early telophase (Fig. 3). GRK5 associated with the centrosome predominately during interphase with a significant reduction during mitosis (Fig. 3, A and B). To assess if fluctuations of GRK5 expression may be the reason for these changes in centrosomal localization, the expression of GRK5 was examined during the cell cycle. Blotting for GRK5 after mitotic shake off (Fig. 3C) or at different times after release from a G1/S arrest (Fig. 3D) showed that GRK5 expression was unchanged from interphase through mitosis. Therefore, the observed changes in localization are not due to alterations in GRK5 expression during the cell cycle.

Because ablation or impaired function of core centrosomal components, such as γ-tubulin (31), can have detrimental effects on the microtubule nucleation capacity of the centrosome, we next evaluated whether GRK5 impacted this process by measuring microtubule nucleation (Fig. 4). We observed that the knockdown of GRK5 expression severely impaired the ability of the centrosomes to nucleate cytoplasmic asters (Fig. 4A). Asters on average were ~50% smaller in GRK5 shRNA cells as compared with control shRNA cells (Fig. 4B). Despite a change in microtubule nucleation efficiency, no effect on centrosome amplification or size was seen with GRK5 knockdown (Fig. 4C).

Generally, loss of expression or activity of proteins that localize to centrosomes have significant effects on cell cycle dynamics (32). To assess if loss of GRK5 displays similar effects, flow
cytometric analysis was performed on GRK5 siRNA-treated cells or GRK5 shRNA stable cells (Fig. 5). These experiments revealed that siRNA depletion of GRK5 (Fig. 5A) induced a G2/M arrest/delay in HeLa cells, as seen by the reduced number of cells in G1 and greater number of cells in G2/M at 10 h post release from thymidine block compared with control cells (Fig. 5B). Similar results were observed in GRK5 shRNA knockdown cells (Fig. 5A), with delays in G2/M to G1 progression at both the 9 and 10 h time points compared with control shRNA cells (Fig. 5C). Analysis of cyclin A and cyclin B expression levels during the cell cycle in GRK5 shRNA cells revealed delays in both cyclin A destruction and cyclin B expression compared with control transfected cells (Fig. 5D). Similar cyclin level changes were also observed in siRNA-transfected cells (data not shown).

To distinguish between a G2 versus M phase delay, live cell imaging was utilized to evaluate the progression of cells following release from a double thymidine block (Fig. 6). GRK5 siRNA depletion induced a significant delay (172 ± 20 min) before mitotic entry (Fig. 6, A and B). A reduced, albeit still significant, delay in G2 (64 ± 9 min) was also observed in GRK5 shRNA cells (Fig. 6C). The difference between the duration of delay between the stable (shRNA) and transient (siRNA) cells is potentially because shRNA-transfected cells may have compensated for G2/M delays during the stable selection process. Rescue experiments using the wild type or kinase dead form (GRK5-K215R) of GRK5 demonstrated that the reintroduction of GRK5 largely rescued the delay at G2 seen in GRK5 shRNA cells while reintroduction of GRK5-K215R did not rescue the G2 delay (Fig. 6, C and D). Therefore, the kinase activity of GRK5 appears to be essential for normal entry into mitosis.

DNA damage can activate various cell cycle checkpoints. To determine if the G2/M delay with GRK5 knockdown is mediated by increases in DNA damage, phospho-H2AX staining was performed on GRK5 knockdown and control cells (Fig. 7). The number of cells that show p-H2AX staining did not vary between control and GRK5 knockdown cells at 0, 5, or 10 h after release from thymidine block (Fig. 7, A and B). Furthermore, the number of foci staining did not vary between the control and GRK5 shRNA cells (Fig. 7C).

Because PLK1 controls progression of cells from G2 to M, we next analyzed the expression of PLK1 by immunoblotting as well as the activity of PLK1 by measuring the phosphorylation of nucleophosmin at serine 4, a direct PLK1 substrate (33) (Fig. 8A). Whereas no significant effect was observed in PLK1 expression during the cell cycle after GRK5 shRNA cells were released from a double thymidine block, the activation of PLK1, as determined by immunoblotting for pThr210, was signifi-

**FIGURE 7. No increase in DNA damage is observed in GRK5 knockdown cells.** A, p-H2AX staining on control and GRK5 shRNA cells. Representatives of n = 4 experiments are shown. B, quantification of the number of cells stained by p-H2AX at 0, 5, or 10 h time points post-thymidine block. C, upper panel, quantitation of the number of stained p-H2AX foci per cell at 0, 5, or 10 h time points post-thymidine block. Lower panel, representative control and GRK5 shRNA cells at t = 0 h.
GRK5 Regulates Cell Cycle Progression

FIGURE 8. AUKA activity is diminished and p53 levels increased in GRK5 knockdown cells. A, blotting for serine 4 phosphorylation of nucleophosmin, threonine 210 of PLK1, total PLK1, threonine 288 phosphorylation of AUKA, and total AUKA in whole cell lysates of cells 0–12 h post release from a double thymidine block. Calnexin was used as a loading control. B, blotting for serine 4 phosphorylation of nucleophosmin in cell lysates collected 8–10 h post double thymidine block from control shRNA cells or GRK5 shRNA cells, both transfected with pcDNA3 empty vector, or GRK5 shRNA cells transfected with wild type GRK5 or GRK5-KD. Calnexin was used as a loading control. C, phosphorylation of AUKA at threonine 288, detected by the corresponding phosphospecific antibody, at 8 and 9 h post-thymidine block was quantified in control shRNA and GRK5 shRNA cells (n = 3–4 experiments, p < 0.05). D, blotting for p53 and p21 in control or GRK5-siRNA treated HeLa cells collected 2 h post-release from double thymidine block. E, quantitation of p53 and p21 expression in GRK5-siRNA HeLa cells (n = 5 experiments).

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significantly delayed (by ~1 h) compared with control shRNA cells (Fig. 8A). This delay could be rescued by expression of wild type GRK5 in the GRK5 shRNA cells but not by expression of catalytically inactive GRK5 (Fig. 8B). Because the activity of PLK1 is regulated by aurora kinase A-mediated phosphorylation of threonine 210 (34), we evaluated the expression of AUKA dur-
FIGURE 9. Characterization of GRK5 localization and role in cell cycle regulation in RPE1 cells. A, expression of GRK5, detected by GRK4–6 antibody, in RPE1 cells transfected with control or GRK5 siRNA (representative from three experiments). B, (top) representative images of RPE1 cells in interphase stained for GRK5 and γ-tubulin are shown (n > 110 cells from three independent experiments). Arrow denotes inset regions and colocalization. (bottom) Control-siRNA and GRK5-siRNA-transfected RPE1 cells were fixed and stained with anti-GRK4–6 monoclonal or γ-tubulin polyclonal antibodies. Note the decrease in staining intensity in cells transfected with GRK5-siRNA. Representative images of cells in interphase are shown. C, decreased GRK5 expression results in G2 phase delay at 7 and 8 h post release from double thymidine block (n = 4). * denotes p < 0.05. D, knockdown of GRK5 expression leads to delayed phosphorylation of serine 4 of nucleophosmin and threonine 210 of PLK1 as well as blunted phosphorylation of threonine 288 of AUKA (n = 3). E, knockdown of GRK5 results in increased p53 expression (n = 3) in nocodazole-synchronized cells and decreased phosphorylation of threonine 55 of p53 (n = 3). F, proposed model of GRK5-mediated delay of G2-M transition. GRK5 interaction with p53 promotes its phosphorylation of Thr-55, leading to decreased p53 expression (step 1) [14]. This regulation is lost in GRK5-depleted cells leading to increased p53 levels, which inhibits AUKA function, as demonstrated by decreased autophosphorylation of aurora kinase A at threonine 288 (step 2) and thus a delay in PLK1 activation as assessed by phosphorylation at threonine 210 (step 3). This delay in PLK1 activation leads to a delay in G2/M transition.
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ing the cell cycle and found no significant change in AUKA levels occur between control and GRK5 shRNA cells (Fig. 8A). Similar results were observed in siRNA-transfected cell lines (data not shown). These results suggest that AUKA function is perturbed during the G2 phase in GRK5 knockdown cells. To see if the activity of AUKA was affected, phosphorylation of AUKA at threonine 288 was assessed (Fig. 8A). This site is an autophosphorylation site and decreased phosphorylation indicates diminished AUKA activity. In GRK5 shRNA cells, phosphorylation of threonine 288 was consistently decreased by ~50% compared with control cells (Fig. 8, A and C), suggesting that AUKA activity was significantly reduced with GRK5 knockdown. Because one inhibitor of AUKA activity is the tumor suppressor protein p53, the protein levels of p53 and its companion protein p21 were measured in GRK5 knockdown cells synchronized at late S/early G2 phase. Increased levels of both p53 and p21 were observed in these cells compared with control cells (Fig. 8, D and E).

To validate the localization and cell cycle function of GRK5 in a non-cancer cell, we studied the normal retinal pigment epithelial cell line RPE1 (Fig. 9). RPE1 cells contain endogenous GRK5 that can be effectively knocked-down by siRNA treatment (Fig. 9A). Centrosomal staining for GRK5 was observed in 93% of the RPE1 cells and staining was reduced in GRK5 siRNA-treated cells (Fig. 9B). Decreased expression of GRK5 resulted in a G2 delay at the 7 and 8 h time points post release from a double thymidine block, as determined by FACS analysis (Fig. 9C). Similar to HeLa cells, knockdown of GRK5 in RPE1 cells also resulted in delayed phosphorylation of nucleoplasmin at serine 4 and PLK1 at threonine 210 as well as blunted phosphorylation of AUKA at threonine 288 (Fig. 9D). In RPE1 cells synchronized at G2/M by nocodazole treatment, increased p53 levels were observed in GRK5-siRNA treated cells versus control cells, albeit not as large of an increase as seen in HeLa cells (Fig. 9E). Because GRK5 was previously shown to phosphorylate p53 at threonine 55 (14), we tested if this also occurred in RPE1 cells. p53 threonine 55 phosphorylation was analyzed in G2/M-synchronized control and GRK5 siRNA-treated cells and found to be decreased by 29 ± 4% (n = 3) in cells with reduced GRK5 (Fig. 9F).

DISCUSSION

In this report, we show that GRK5 is localized to centrosomes during interphase and is mainly associated with the mother centriole. Knockdown of GRK5 resulted in a G2 arrest, which was rescued by wild type but not catalytically inactive GRK5. This arrest appears to be due to increased expression of p53, inhibition of AUKA and a subsequent delay in PLK1 activation.

GRK5, in addition to roles in the cytoplasm and recently discovered activities in the nucleus (12), is now also reported herein to be functionally localized to centrosomes. The localization of this kinase within the centrosome suggests another avenue by which GRKs can affect cellular processes. The transient nature of this localization, which predominantly occurs during interphase, suggests that there are mechanisms by which GRK5 is transported to and from the centrosome. These yet to be defined mechanisms suggest that cells need to control GRK5 function at the centrosome to regulate processes such as microtubule nucleation. While nothing is currently known about the localization of other GRKs in centrosomes, centrosomal preparations from HeLa cells also appear to contain GRK6 (Fig. 1F) and previous studies have demonstrated that GRK6 can redistribute to the nucleus (23, 36). Furthermore, in addition to GRKs and arrestins in centrosomes, a GPCR (37) and Goα (38) are also localized in this compartment, suggesting that GPCR signaling in centrosomes may control centrosomal functions.

Similar to other centrosomal proteins, GRK5 also affects the G2/M transition although we have not established that the centrosomal localization contributes to its role in cell cycle regulation. Nevertheless, the centrosome plays a vital role in coordinating the cell cycle by correctly positioning proteins to a location in the cell that is vital to mitotic spindle assembly. Two of the protein kinases localized to centrosomes, PLK1 (32) and AUKA (39), have well-characterized roles in the cell cycle. PLK1 promotes G2/M transition by phosphorylating WEE1, cyclin B, and cdc25c phosphatase, promoting cyclin B activation (40). In addition, PLK1 also phosphorylates the nuclear- and centrosomal-localized protein nucleoplasmin during G2, regulating the assembly of the nucleus and mitotic spindles (33). AUKA initiates PLK1 activity in late G2 phase by phosphorylating, with assistance from a number of PLK1-interacting proteins (41), threonine 210 on PLK1, leading to increased PLK1 activity. The abolishment of AUKA or PLK1 activity both lead to G2/M arrest (32, 42).

The AUKA-PLK1 nexus that controls the G2/M transition is partially disrupted in cells lacking GRK5. Potentially, increased levels of p53, observed in GRK5 knockdown cells, attenuate this pathway because p53 directly binds to and inhibits AUKA catalytic activity (43). p53-activated factors, such as Gadd43a, which bind and inhibit AUKA activity (44), can also temper AUKA kinetics. There also exists interplays between p53 and AUKA, with p53 levels regulated by both AUKA (45) and PLK1 (35) phosphorylation, leading to its degradation. How GRK5 regulates p53 during the cell cycle is unclear although recent studies reveal that GRK5 phosphorylates p53 at threonine 55 in osteosarcoma cells, damaged by irradiation, and results in increased apoptosis caused by increased p53 levels (14). This phosphorylation was also observed to occur at G2/M of the cell cycle and could be contributing to the regulation of p53 expression. A proposed model of GRK5 regulation of the cell cycle is illustrated in Fig. 9G.

In this report, we show a novel role for GRK5 at the centrosome, where it is effectively positioned to modify G2/M transitions and subsequent cell division. Knockdown of GRK5 expression in cells leads to decreased AUKA activity, possibly through increased p53 expression, resulting in delayed PLK1 activation and subsequent G2/M arrest. These findings are important as it expands on the functionality of GRKs by demonstrating a novel avenue by which GRKs may regulate normal cell function.

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