G Protein-coupled Receptor Kinase 5 Phosphorylates Nucleophosmin and Regulates Cell Sensitivity to Polo-like Kinase 1 Inhibition*

Christopher H. So, Allison M. Michal, Rouzbeh Mashayekhi, and Jeffrey L. Benovic

From the Department of Biochemistry and Molecular Biology, Thomas Jefferson University, Philadelphia, Pennsylvania 19107

Background: G protein-coupled receptor kinases (GRKs) are important regulators of receptor signaling although little is known about their functions beyond their receptor modifying activities.

Results: GRK5 binds and phosphorylates nucleophosmin.

Conclusion: GRK5 and polo-like kinase 1 coordinately regulate nucleophosmin phosphorylation and cell sensitivity to inhibitor-induced apoptosis.

Significance: GRKs play an important role in regulating normal cell functions such as cell cycle regulation and apoptosis.

G protein-coupled receptor kinases (GRKs) phosphorylate activated G protein-coupled receptors, leading to their desensitization and endocytosis. GRKs have also been implicated in phosphorylating other classes of proteins and can localize in a variety of cellular compartments, including the nucleus. Here, we attempted to identify potential nuclear substrates for GRK5. Our studies reveal that GRK5 is able to interact with and phosphorylate nucleophosmin (NPM1) both in vitro and in intact cells. NPM1 is a nuclear protein that regulates a variety of cell functions including centrosomal duplication, cell cycle control, and apoptosis. GRK5 interaction with NPM1 is mediated by the N-terminal domain of each protein, and GRK5 primarily phosphorylates NPM1 at Ser-4, a site shared with polo-like kinase 1 (PLK1). NPM1 phosphorylation by GRK5 and PLK1 correlates with the sensitivity of cells to undergo apoptosis with cells having higher GRK5 levels being less sensitive and cells with lower GRK5 being more sensitive to PLK1 inhibitor-induced apoptosis. Taken together, our results demonstrate that GRK5 phosphorylates Ser-4 in nucleophosmin and regulates the sensitivity of cells to undergo apoptosis with cells having higher GRK5 levels being less sensitive and cells with lower GRK5 being more sensitive to PLK1 inhibitor-induced apoptosis. These additional activities may be important because GRK levels vary in many pathological conditions including cardiovascular disease (18–21), cancer (22–25), and various neurological disorders (26–29). Further expanding the scope of GRK function is the finding that GRK5 and GRK6 are found in the nucleus (13, 30, 31). Interestingly, the ability of GRK5 to localize in the nucleus may be regulated by Gα signaling (13, 30), whereas the nuclear localization of GRK6 may be regulated by palmitoylation (31). These studies suggest a novel, underevaluated, location within cells where GRKs may mediate their effects.

To identify potential nuclear targets for GRKs, we initially used in vitro approaches such as chromatography and mass spectrometry to identify potential substrates. Herein, we present our results identifying the nuclear protein nucleophosmin (NPM1), also known as B23, as a novel substrate for GRK5. NPM1 belongs to the nucleoplasmin family of proteins, made up of nucleophosmin, nucleoloplasmin (NPM2), and NPM3 (32). An N-terminal core structure, which is required for oligomerization, is shared within members of this family. There are 2 splice variants of NPM1, B23.1 and B23.2, with B23.1 containing an additional 35 amino acids in the C terminus (33). NPM1 is involved in a variety of functions, including the regulation of centrosomal duplication, the cell cycle, mitosis, apoptosis, and nuclear localization, is shared within members of this family. There are 2 splice variants of NPM1, B23.1 and B23.2, with B23.1 containing an additional 35 amino acids in the C terminus (33). NPM1 is involved in a variety of functions, including the regulation of centrosomal duplication, the cell cycle, mitosis, apoptosis, and DNA replication, and it also serves as a chaperone for proteins such as histones. NPM1 is also overexpressed in a number of cancers and, thus, is a potential target within the cancer field (34).

In this report we demonstrate that NPM1 is phosphorylated by GRK5 both in vitro and in cells, with Ser-4 being the major phosphorylation site. Interestingly, GRK5-depleted cells were more sensitive to undergoing cell death from polo-like kinase 1 (PLK1) inhibition, and this increased susceptibility corresponded to decreased NPM1 phosphorylation. Conversely, cells with higher GRK5 levels exhibited reduced sensitivity to PLK1 inhibition. Taken together, our results demonstrate that
GRK5 phosphorylates Ser-4 in nucleophosmin and regulates the sensitivity of cells to PLK1 inhibition.

**EXPERIMENTAL PROCEDURES**

**Materials**—A human NPM1 cDNA was a generous gift from Dr. Stephen Peiper (Thomas Jefferson University, Philadelphia, PA). Monoclonal anti-NPM1 and propidium iodide were purchased from Enzo Life Sciences (Farmington, NY), whereas anti-GRK4–6 was from Millipore (Billerica, MA). 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI), anti-poly(ADP-ribose) polymerase (PARP), anti-Na+/K+ ATPase antibodies, purified PLK1, and 5-fluoro-2'-deoxyuridine (FUDR) were from Sigma. All media were purchased from Mediatech, Inc. (Manassas, VA). BI 2536 and GSK461364 were purchased from Selleckchem (Houston, TX), dissolved in water, aliquoted, and stored at −20 °C until use. Anti-phospho-Ser-4 and anti-phospho-Thr-199 NPM1 antibodies were from Cell Signaling Technologies (Danvers, MA).

**Identification of Nuclear GRK Substrates**—A HeLa cell nuclear extract prepared from ten 15-cm dishes of confluent cells was diluted to 20 ml with 20 mM Tris-HCl, pH 8, and loaded on a 3-mL Q-Sepharose (Amersham Biosciences) column equilibrated with buffer A (20 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, 1 mM dithiothreitol) containing 50 mM NaCl. The column was washed with the same buffer and then eluted with a 30-ml linear gradient from 50 to 700 mM NaCl in buffer A. All purification steps were performed at 4 °C. For phosphorylation reactions, 10 μl of each fraction from the Q-Sepharose elution was incubated with or without 200 nM purified GRK2 or GRK5 in 20 μl of buffer B (20 mM Tris-HCl, pH 8.0, 4 mM MgCl2) containing 0.1 mM ATP and 1–2 μCi of [γ-32P]ATP. Reactions were incubated for 30 min at 30 °C, stopped with SDS sample buffer, and electrophoresed on a 10% SDS-polyacrylamide gel. Proteins were visualized by Coomassie Blue staining and autoradiography. This analysis identified a GRK5 substrate of ~40 kDa (p40) that eluted at ~600 mM NaCl. To identify p40, an aliquot of the peak fraction was electroeluted on a 10% SDS-polyacrylamide gel and stained by Coomassie Blue, and the ~40-kDa protein was excised, proteolyzed with trypsin, and analyzed by mass spectrometry. A subsequent database search identified the 40-kDa protein as nucleophosmin.

**Cell Culture**—HeLa cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10 mM HEPES and 10% fetal bovine serum (FBS) (Invitrogen). MDA-MB-231 cells were from American Tissue Culture Collection (ATCC). BT549 cells were from Dr. Stephen Peiper (Thomas Jefferson University, Philadelphia, PA). BT549 cells were grown in RPMI 1640 containing 10 mM HEPES, 10% FBS, whereas SKBR3 were grown in RPMI 1640 supplemented with 10 mM HEPES, 10% FBS, and 2% puromycin. The GRK5 shRNA sequence was 5'-ACGAGATGATAGAACATC-3'. For transient transfections, four GRK5 siRNAs were pooled together, and 60 pmol of the pool was used per transfection: 5'-CCCAACGGUCUUGCUAGAAA-3', 5'-GGGGAGAACCAUUCACAGGGA-3', 5'-CAAACCAUGUCAGCUAGAAA-3', and 5'-GAUUAUUGGCGCAUUGAGG-3'. Control siRNA scrambled sequences were purchased from Dharmacon. To determine cell death, trypan blue (Mediatech) exclusion was used to count non-viable cells with percent cell death expressed as (# stained cells/# total cells) × 100.

**Purification of NPM1**—NPM1 from HeLa cell nucleoli was purified following a previously published method (35). For purification of His-tagged NPM1, a NPM1 cDNA was subcloned in-frame within the cloning site of pTrcHis A (Invitrogen) using BamH1 and HindIII. NPM1 was expressed in Escherichia coli M16 cells (Qiagen, Valencia, CA). Cells were grown in terrific broth to A600 = 0.6, induced with 0.1 mM isopropyl β-D-1-thiogalactopyranoside, grown overnight at 30 °C, and then collected by centrifugation and frozen at −80 °C. Frozen cells were thawed, resuspended in buffer C (20 mM Tris-HCl, 0.5 mM NaCl, 5% glycerol) supplemented with a protease inhibitor tablet without EDTA (Roche Applied Science), harnessed using a Polytron, sonicated, and centrifuged at 32,000 × g. The supernatant was then incubated with 10 ml of a 50% nickel resin slurry (Qiagen), washed with buffer C containing 50 mM imidazole, and eluted with buffer C containing 200 mM imidazole. Eluted His-tagged NPM1 was concentrated and then applied to a gel filtration column using Buffer B. Peak fractions were pooled, concentrated, and frozen at −80 °C. The purified protein was analyzed by SDS-PAGE and Coomassie Blue staining, and the concentration was determined by Bradford assay.

**Glutathione S-Transferase (GST) Pulldown Assays**—NPM1 cDNA was subcloned into pGEX vector 4T2 (GE Healthcare) using EcoRI and Xhol. Site-directed mutagenesis was performed using a two-step PCR mutagenesis method. For GST-GRK5-(1–200) and GST-GRK5-(489–562), DNA sequences were generated using PCR and inserted within the BamH1-SalI fragment in the vector pGEX-4T-2 as previously described (36). Purification of GST-NPM1 and GST-GRK5 was done following previous methods (16). HeLa cells expressing population as previously described (16). HeLa cells expressing GRK5 knockdown were grown in DMEM supplemented with 10 mM HEPES, 10% FBS, and 2 μg/ml puromycin. The GRK5 shRNA sequence was 5’-ACGAGATGATAGAACATC-3’. For transient transfections, four GRK5 siRNAs were pooled together, and 60 pmol of the pool was used per transfection: 5’-CCCAACGGUCUUGCUAGAAA-3’, 5’-GGGGAGAACCAUUCACAGGGA-3’, 5’-CAAACCAUGUCAGCUAGAAA-3’, and 5’-GAUUAUUGGCGCAUUGAGG-3’. Control siRNA scrambled sequences were purchased from Dharmacon. To determine cell death, trypan blue (Mediatech) exclusion was used to count non-viable cells with percent cell death expressed as (# stained cells/# total cells) × 100.

**Glutathione S-Transferase (GST) Pulldown Assays**—NPM1 cDNA was subcloned into pGEX vector 4T2 (GE Healthcare) using EcoRI and Xhol. Site-directed mutagenesis was performed using a two-step PCR mutagenesis method. For GST-GRK5-(1–200) and GST-GRK5-(489–562), DNA sequences were generated using PCR and inserted within the BamH1-SalI fragment in the vector pGEX-4T-2 as previously described (36). Purification of GST-NPM1 and GST-GRK5 was done following previous methods (37). For GST-NPM1, GST fusion proteins were expressed in E. coli BL21 (DE3) cells grown in Luria broth. The culture was induced with 100 μM isopropyl β-D-1-thiogalactopyranoside and grown at 30 °C overnight. Cells were resuspended and sonicated in lysis buffer (50 mM sodium phosphate, pH 7.0, 0.5% Triton X-100, 5 mM NaCl, protease inhibitor mixture, 1 mM phenylmethylsulfonyl fluoride, 1 mM β-mercaptoethanol) and centrifuged at 60,000 × g, and the GST fusion
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proteins were affinity-purified with glutathione-Sepharose 4B beads (GE Healthcare). Protein purity was assessed by Coomassie Blue staining of SDS-polyacrylamide gels, and protein concentration were determined using a Bradford assay. Pulldown experiments were performed similar to published protocols (38). GST-GRK5-(1–200) and GST-GRK5-(489–562) were expressed in JL210(DE3) cells, induced with 100 μM isopropyl β-D-1-thiogalactopyranoside, grown at 30 °C for 2 h, and purified in a similar fashion to GST-NPM1.

Fluorescence Microscopy—Cells were grown on poly-L-lysine-coated coverslips for 72 h in the presence or absence of 5 nM BI 2536, then fixed with 4% paraformaldehyde and permeabilized with 0.4% Triton X-100 in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.7 mM KH2PO4, pH 7.4). Cells were stained with DAPI, washed twice with PBS, mounted, and then examined on a Nikon Eclipse E800 fluorescence microscope using a Plan Fluor 60×/H9262/1.30 Achromat oil objective. Images were collected using QED Camera software and processed with Adobe Photoshop and Image Pro Plus. The number of fragmented and elongated nuclei was scored and compared between treatments.

Immunoblotting and Immunoprecipitation—Nuclear extracts were prepared following a previously published protocol (39). Briefly, cells were resuspended in a hypotonic buffer (10 mM Tris-HCl, pH 8.0, 5 mM MgCl2, 10 mM KCl, 0.1 mM EDTA, 300 mM sucrose, 0.5 mM TCEP, 0.5 mM DTT), incubated on ice for 10 min, then centrifuged at 1000 × g for 20 min. The supernatant (cytosol) and particulate (nuclear) fractions were collected. The particulate fraction was washed vigorously 6 × with PBS, then incubated with hypertonic buffer (20 mM Tris-HCl, pH 8.0, 5 mM MgCl2, 420 mM NaCl, 0.2 mM EDTA, 25% glycerol, 5 mM DTT, and protease inhibitors) on a shaker at 4 °C followed by centrifugation at 18,000 × g for 30 min. The supernatant was used as the nuclear fraction. For whole cell lysates, cells were lysed by incubation in Triton lysis buffer (20 mM HEPES, pH 7.4, 1% Triton X-100, 150 mM NaCl, 50 mM EDTA containing protease inhibitors and PhosSTOP phosphatase inhibitor tablets (Roche Applied Science)) for 20 min at 4 °C followed by centrifugation at 18,000 × g for 30 min. Protein concentrations were determined by Bradford assay.

For co-immunoprecipitations, ~100 μg of nuclear extract was immunoprecipitated by overnight incubation at 4 °C with 1 μg of anti-NPM1, anti-GRK5, or corresponding antibody isoforms, previously cross-linked to 20 μl of protein A/G beads using dimethyl pimelimidate. Beads were then washed 6 × with PBS and eluted by boiling in SDS sample buffer. For immunoblotting, samples were electrophoresed and separated by SDS-PAGE, transferred to nitrocellulose, blocked with 5% skim milk in TBS, 0.1% Tween 20, and blotted with the appropriate primary and secondary antibodies. Immunoblots were incubated with SuperSignal Enhanced Chemiluminescent (Thermo Scientific) substrate and developed.

In Vitro Kinase Assay—GRK2 and GRK5 were purified as described (40, 41), whereas purified PLK1 was from Sigma. Protein phosphorylation was detected using either [γ-32P]ATP labeling or phospho-specific antibodies, and assays were performed similar to previous protocols (6). Briefly, indicated amounts of purified nucleophosmin were incubated with or without indicated amounts of purified GRK2 or GRK5 in 20 μl of buffer B with 0.1 mM ATP and 1–2 μCi [γ-32P]ATP. Reactions were incubated for the indicated times at 30 °C, stopped with SDS sample buffer, and electrophoresed on a 10% SDS-polyacrylamide gel. Proteins were visualized by autoradiography. Radioactivity was quantified by excising Coomassie Blue-stained bands from the dried gels and counting in a scintillation counter.

Flow Cytometry—Cells were treated for 72 h with 0–10 nM BI 2536, collected, fixed with 70% ethanol, rehydrated with PBS, and then stained with propidium iodide solution (0.2 mg/ml DNase-free RNase, 1 mg/ml propidium iodide, and 0.1% Triton X-100 in PBS). An additional series of studies involved a double thymidine block followed by release of the cells into 60 nM BI 2536. In these studies, BI 2536 effectively retarded both control and GRK5 shRNA cells at 10 h, with ~90% of cells trapped at G2/M as determined by flow cytometry analysis (% cells at G2/M at 10 h: control, 87.5 ± 1.7%; GRK5 shRNA, 87.9 ± 1.3%, n = 4). All samples were analyzed on a Beckman Coulter Epics XL-MCL Flow Cytometer.

Statistical Analysis—All statistics were performed using a Student’s t test.

RESULTS

Since previous studies showed that GRK5 can localize in the nucleus (13, 30), we attempted to identify potential nuclear substrates for GRK5. Nuclear extracts were prepared from HeLa cells and initially analyzed for GRKs as well as various cellular markers. The nuclear extract was highly enriched in the nuclear marker nucleolin and largely devoid of cytoplasmic (Hsp90) and membrane (Na+/K+ ATPase) markers (Fig. 1A). The nuclear fraction also contained endogenous GRK2 and GRK5 (Fig. 1A). To identify potential GRK substrates, the nuclear extract was chromatographed on an anion exchange column (Fig. 1B), and fractions were phosphorylated with or without the addition of purified GRK2 or GRK5. Although little phosphorylation was observed in most fractions, fraction 16 contained an ~40-kDa protein that was phosphorylated by GRK5 (Fig. 1C, left panel) but not by GRK2 (Fig. 1C, right panel). The 40-kDa band was excised from the gel, proteolyzed with trypsin, and then analyzed by mass spectrometry. This analysis identified 21 unique peptides of which 12 were from nucleophosmin (NPM1), providing 18% overall sequence coverage (data not shown). Immunoblotting with an anti-NPM1 antibody confirmed that NPM1 is in fraction 16 as well as in the whole cell, cytosolic, and nuclear extracts (Fig. 1D). The nuclear extract also contained NPM1 oligomers of ~200 kDa that have been previously reported (35).

To verify that NPM1 is a substrate for GRK5, we purified NPM1 from HeLa cell nucleoli (Fig. 2A, left panel) and found that it was phosphorylated by GRK5 (Fig. 2A, center panel) but not by GRK2 (Fig. 2A, right panel). We also expressed and purified His-tagged NPM1 from E. coli (Fig. 2B) and found that it was phosphorylated by GRK5 to a stoichiometry of ~1 mol of P_i/mol of NPM1 (Fig. 2, C and D). In contrast, NPM1 was only weakly phosphorylated by GRK2 (Fig. 2D). Thus, NPM1 appears to be a specific substrate for GRK5.
To determine whether endogenous NPM1 and GRK5 associate in cells, co-immunoprecipitation studies were performed using control and GRK5 shRNA-transfected cells (Fig. 3A). Endogenous GRK5 was found to co-immunoprecipitate with endogenous NPM1 from HeLa cells (Fig. 3B). The co-immunoprecipitation was largely lost in the GRK5 shRNA cells, whereas no GRK5 or NPM1 immunoprecipitation was observed when an identical mouse isotype IgG was used in place of anti-NPM1 antibody.
Similarly, endogenous NPM1 co-immunoprecipitated with GRK5, whereas reduced co-immunoprecipitation was observed in GRK5 shRNA cells. This blot is representative of three-four experiments. C, shown is detection of NPM1 in anti-GRK5 or anti-IgG immunoprecipitates derived from either control or GRK5 shRNA cells. This blot is representative of 3–4 experiments. D, in vitro GST pulldown assays demonstrate the N terminus of NPM1 interacts with GRK5. Upper panel, NPM1 is divided into three domains: the N-terminal oligomerization domain (residues 1–119), the histone-binding domain (residues 119–190), and the DNA/RNA binding domain (residues 190–294). Lower panel, the GST-tagged NPM1 domain truncations were expressed in E. coli and purified using glutathione beads (Coomassie stain). 80 pmol of GST (25 kDa), GST-NPM1 (65 kDa), GST-NPM1(1–190) (45 kDa), GST-NPM1(1–102) (36 kDa), and GST-NPM1(1–15) (25 kDa) on glutathione beads were incubated with 10 pmol of purified GRK5 for 1 h at room temperature, washed extensively, and eluted with SDS sample buffer. The proteins were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with the GRK4–6 antibody. Blots presented are representative of four experiments. E, GRK5 is subdivided into three domains: the N-terminal (1–180), catalytic (180–507), and C-terminal domain (507–590). GST (25 kDa), GST-GRK5(1–200) (45 kDa), and GST-GRK5-(489–562) (30 kDa) were expressed in E. coli and purified using glutathione beads (Coomassie stain). Eighty pmol of GST or GST-GRK5 truncations on glutathione beads were incubated with 10 pmol of purified NPM1 for 1 h at room temperature, washed extensively, and eluted with SDS sample buffer. The proteins were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-NPM1 antibodies. Blots presented are representative of four experiments.

We next focused on identifying potential GRK5 phosphorylation sites on NPM1. Two well-characterized phosphorylation sites on NPM1, Ser-4 and Thr-199, were initially explored using phospho-specific antibodies to determine whether these were

FIGURE 3. NPM1 co-immunoprecipitates with GRK5 and their interaction is mediated by their respective N termini. A, shown is an immunoblot (IB) for GRK5 in HeLa cells stably transfected with control (C) or GRK5 (5) shRNA. B, detection of GRK5 in anti-NPM1 or anti-IgG2a immunoprecipitates (IP) derived from control or GRK5 shRNA-transfected cell lines is shown. This blot is representative of three-four experiments. C, shown is detection of NPM1 in anti-GRK5 or anti-IgG immunoprecipitates derived from either control or GRK5 shRNA cells. This blot is representative of 3–4 experiments. D, in vitro GST pull-down assays demonstrate the N terminus of NPM1 interacts with GRK5. Upper panel, NPM1 is divided into three domains: the N-terminal oligomerization domain (residues 1–119), the histone-binding domain (residues 119–190), and the DNA/RNA binding domain (residues 190–294). Lower panel, the GST-tagged NPM1 domain truncations were expressed in E. coli and purified using glutathione beads (Coomassie stain). 80 pmol of GST (25 kDa), GST-NPM1 (65 kDa), GST-NPM1(1–190) (45 kDa), GST-NPM1(1–102) (36 kDa), and GST-NPM1(1–15) (25 kDa) on glutathione beads were incubated with 10 pmol of purified GRK5 for 1 h at room temperature, washed extensively, and eluted with SDS sample buffer. The proteins were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with the GRK4–6 antibody. Blots presented are representative of four experiments. E, GRK5 is subdivided into three domains: the N-terminal (1–180), catalytic (180–507), and C-terminal domain (507–590). GST (25 kDa), GST-GRK5(1–200) (45 kDa), and GST-GRK5-(489–562) (30 kDa) were expressed in E. coli and purified using glutathione beads (Coomassie stain). Eighty pmol of GST or GST-GRK5 truncations on glutathione beads were incubated with 10 pmol of purified NPM1 for 1 h at room temperature, washed extensively, and eluted with SDS sample buffer. The proteins were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-NPM1 antibodies. Blots presented are representative of four experiments.
phosphorylation of NPM1 by GRK5, and this was completely lost when Ser-4 or Thr-199 was mutated to alanine (Fig. 4C). In contrast, no NPM1 phosphorylation was observed with GRK2 (Fig. 4, A and B). To assess whether Ser-4 and Thr-199 were major sites of phosphorylation in control and GRK5 shRNA HeLa cells in the presence or absence of PLK1 inhibition. Although GRK5 knockdown or PLK1 inhibition alone did not significantly affect cell viability, the combination of PLK1 inhibition and GRK5 knockdown caused a dramatic increase in cell death at the 8-, 9-, and 10-h time points post-double thymidine release (Fig. 5C). Taken together, these results demonstrate that GRK5 contributes to regulating the phosphorylation of Ser-4 in NPM1 in HeLa cells.

The phosphorylation of NPM1 on Ser-4 by PLK1 is important for proper mitotic assembly as cells transfected with NPM1-S4A display elongated and fragmented nuclei (43), similar to what is seen with PLK1 inhibition (45). Because PLK1 inhibition ultimately results in cell apoptosis (44, 45), we next tested whether GRK5 knockdown had any effect on cell death in the presence or absence of PLK1 inhibition. Although GRK5 knockdown or PLK1 inhibition alone did not significantly affect cell viability, the combination of PLK1 inhibition and GRK5 knockdown caused a dramatic increase in cell death at the 8-, 9-, and 10-h time points post-double thymidine release (Fig. 5D).

To determine whether GRK5 knockdown altered the sensitivity of cells to the effects of PLK1 inhibition, we measured cell death in control and GRK5 shRNA cells after a 72-h treatment with 0–120 nM BI 2536. In HeLa cells lacking GRK5, there was an ~4.5-fold decrease in the EC50 of BI 2536-mediated cell death (control shRNA, 18.9 ± 5.2 nM; GRK5 shRNA, 4.1 ± 1.1 nM, n = 6, p < 0.05) (Fig. 6A). Similar results were observed in GRK5 siRNA-transfected cells (data not shown). To determine whether these effects were PLK1-specific, cells were incubated for 72 h with 0–120 nM GSK461364, a more selective PLK1
inhibitor (Fig. 6B). Similar to BI 2536, cells lacking GRK5 were ~4.5-fold more sensitive to GSK461364 compared with control cells ($EC_{50}$ for control shRNA cells, 17.0 ± 4.4 nm; $EC_{50}$ for GRK5-shRNA cells, 3.8 ± 0.7 nm, $n = 4$, $p < 0.05$). To determine whether these results might involve a general effect attributed to GRK5 knockdown, we measured the sensitivity of cells to FUDR, which kills cells by inhibiting thymidylate synthase. No differences were observed in the $EC_{50}$ values associated with FUDR in control versus GRK5 shRNA lines (Fig. 6C).

To further characterize the role of GRK5 in PLK1 inhibitor-induced cell death, we performed rescue experiments in the GRK5 shRNA HeLa cells by expressing RNAi-resistant mutants of wild type and catalytically inactive GRK5. These experiments demonstrate that reintroduction of wild type GRK5 into the GRK5 shRNA HeLa cells could partially rescue cell viability. Moreover, this rescue required the catalytic activity of GRK5 as no rescue was observed with the GRK5-K215R mutant (Fig. 6D). To determine the time course of cell death attributed to PLK1 inhibition, control and GRK5 shRNA cells were treated with either 10 or 30 nm BI 2536, and cell viability was quantified over a 120-h period. A significant increase in cell death was observed in the GRK5 shRNA cells at all time points examined with either 5 or 10 nm BI 2536 (Fig. 7A). PLK1-inhibited cells were also stained with propidium iodide, and their sub-G1 DNA content was examined by flow cytometry (Fig. 7, C and D). Increased sub-G1 DNA content suggests increased DNA fragmentation, which is another indicator for apoptosis. GRK5-shRNA cells had a significant increase in sub-G1 DNA content after a 72-h treatment with either 5 or 10 nm BI 2536 (Fig. 7C).

Because GRK5 levels may control the sensitivity of cells to PLK1 inhibition, we also tested if cell lines with higher GRK5 levels were more resistant to cell death associated with PLK1 inhibition. For these studies we chose a number of breast cancer cell lines. MDA-MB-231 and BT549 cells had higher GRK5 levels compared with T47D, SKBR3, and MCF7 cells with an ~5-fold range in expression (Fig. 8A, lower panel). The sensitivity of these cells to BI 2536-mediated cell death mirrored the GRK5 expression level with MDA-MB-231 and BT549 being least sensitive and T47D, MCF7, and SKBR3 being most sensitive (Fig. 8A, upper panel). To further test if this effect was associated with GRK5 levels in MDA-MB-231 cells, GRK5 expression was decreased by siRNA transfection, and the sensitivity to BI 2536 was tested (Fig. 8B). GRK5 knockdown increased both the efficacy and potency of BI 2536 (control siRNA = 11.1 ± 3.3 nm; GRK5 siRNA = 4.2 ± 1.7 nm, $n = 6$), thus providing additional evidence that GRK5 modulates the sensitivity of cells to PLK1 inhibition.
DISCUSSION

In addition to their role in mediating the phosphorylation, desensitization, and trafficking of GPCRs, GRKs also phosphorylate and regulate the function of various non-receptor proteins (3). In this report we provide evidence that GRK5 phosphorylates the nuclear protein, NPM1. GRK5 directly interacts with NPM1, forming a complex through their N-terminal domains. GRK5 phosphorylates NPM1 at Ser-4 and Thr-199 in vitro, with Ser-4 serving as the major phosphorylation site. In cells, GRK5 phosphorylates NPM1 on Ser-4, a site that is also phosphorylated by PLK1 (43). Interestingly, GRK5 phosphorylation of NPM1 may confer resistance to cell death mediated by PLK1 inhibition. Cell lines with higher GRK5 levels show increased resistance to PLK1 inhibition, whereas cells with lower GRK5 levels are more sensitive to undergoing cell death.

FIGURE 6. Cells with decreased GRK5 expression are more sensitive to PLK1 inhibition. A, shown is cell death in control or GRK5-shRNA stably transfected cells after 72 h of treatment with 0–120 nM BI 2536 as determined by trypan blue exclusion (n = 6). B, shown is cell death in control or GRK5-shRNA stably transfected cells after 72 h treatment with 0–120 nM GSK461364, as determined by trypan blue exclusion (n = 4). C, shown is dose-response cell death in control or GRK5-shRNA stably transfected cells after 72 h of treatment with 0–5 μM FUDR as determined by trypan blue exclusion (n = 3). EC50 for control shRNA cells, 0.24 ± 0.1 μM; EC50 for GRK5-shRNA cells, 0.27 ± 0.08 μM, n = 3, p > 0.05. D, cell viability of GRK5-shRNA cells transfected with either pcDNA3 wild type GRK5 or catalytically inactive GRK5 (GRK5-K215R) and treated with 10 nM BI 2536, t = 72 h (n = 5), was determined by trypan blue exclusion. *** denotes p < 0.05 compared with vector-transfected control (n = 4). Inset, immunoblotting for GRK5 by GRK4–6 antibody in control or GRK5-shRNA stably transfected cell lines transfected with vector, wild type GRK5, or GRK5-K215R. The cell viability of control shRNA cells was ∼50–55% after the 72 h treatment with 10 nM BI 2536 (see Fig. 6A). E and F, cell death of control or GRK5 shRNA stably transfected cells treated with 30 nM (E) or 10 nM (F) BI 2536 (n = 3) for 0–120 h.

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NPM1 is a multifunctional protein involved in a wide variety of different cellular activities with many of them controlled by phosphorylation (33, 34). Phosphorylation of NPM1 at Ser-4 by PLK1 is involved in mitotic spindle formation (43), and overexpression of a NPM1-S4A mutant leads to cytokinetic defects (43) perhaps leading to cell death (45). Phosphorylation at Thr-199 by cyclin-dependent kinase 1 is involved in regulating the centrosome (33), whereas the phosphorylation of both Ser-4 and Thr-199 controls centrosome duplication (33, 46). Interestingly, GRK5 is also localized in the centrosome, although it does not appear to regulate centrosomal duplication (16). Nevertheless, knockdown of GRK5 leads to increased apoptosis associated with PLK1 inhibition, suggesting interplay between GRK5 and PLK1 in regulating the phosphorylation of NPM1 and controlling normal cell function.

Our results demonstrate that GRK5 phosphorylates NPM1 on Ser-4 both in vitro and in cells and likely coordinates with PLK1 in this process. Considering that PLK1 inhibition leads to cell death (44, 45), GRK5 might function to maintain particular PLK1-linked cell division processes associated with NPM1 function. During cell division, NPM1 is involved in spindle and centrosome formation as well as kinetochore-microtubule attachments (47). As a result, a certain level of active NPM1...
could prevent spindle collapse associated with PLK1 inhibition (48) and thus help maintain cell viability. Decreasing GRK5 levels increased BI 2536-mediated cell death most likely because GRK5 no longer compensates for loss of PLK1 activity in phosphorylating NPM1, leading to increased nuclear elongation and fragmentation. These defects ultimately lead to increased apoptosis.

The ability of GRK5 to compensate for PLK1 activity is an interesting example of protein kinases that have overlapping substrate specificities. The function of GRK5 in phosphorylating NPM1 may be important as PLK1 is a therapeutic target in the treatment of a number of cancers, including esophageal cancer (49), neuroblastomas (50), and others (51). Reducing the GRK5 level and/or activity in cancer cells, because of its potential ability to compensate for PLK1 activity at NPM1, could enhance the potency of PLK1 inhibitors in mediating cancer cell death. This has been demonstrated here with decreasing GRK5 levels in MDA-MB-231 cells. With lowered GRK5 levels, lower levels of PLK1 inhibitor could be administered to treat certain cancers, potentially avoiding off-target effects such as neutropenia, which has limited the effectiveness of these drugs in some clinical trials (51). Furthermore, PLK1 inhibitors might prove more effective in treating tumors that have low GRK5 expression, thereby enabling the use of lower inhibitor concentrations and avoiding off-target effects.

Interestingly, GRK5 has recently been implicated in the development of prostate cancer (25). GRK5 also appears to have a role in cardiovascular disease as a GRK5-Q41L polymorphism that is prevalent in African Americans is protective in the development of heart failure (52). Moreover, GRK5 attenuates atherosclerosis in mice through multiple mechanisms including reduced NF-κB activity and desensitization of various GPCRs and growth factor receptors (53). Additional mouse knock-out and transgenic studies reveal that GRK5 has many additional physiological roles (54). These include roles in regulating muscarinic receptor function (55), neuronal morphogenesis (56), and possibly the development of Alzheimer disease (26). GRK5 also phosphorylates and regulates histone deacetylase 5 in the nucleus (13) and appears to regulate apoptosis (15) and cell cycle progression (16) through p53 phosphorylation. Although we do not know if GRK5 regulation of NPM1 contributes to any of these observed phenotypes in mice or humans, it is evident that GRK5 has many important physiological and pathophysiological roles.

GRK5-NPM1 complexes could suggest novel means by which GPCRs may regulate processes associated with NPM1. NPM1 itself has been demonstrated to interact and affect the activity of CXCR4 (37). This receptor could be an important modulator of NPM1 function as it has been reported to be localized in perinuclear regions in metastatic renal carcinoma (58). NPM1 has also been reported to interact with the GPCR adaptor proteins, arrestins (59). Because GRKs are activated by GPCR binding (57), GPCR activation might be able to regulate the phosphorylation state of NPM1.

In summary, we demonstrate NPM1 as a substrate for GRK5. GRK5-mediated phosphorylation of NPM1-Ser-4 leads to an increased resistance to PLK1 inhibitors, which could potentially reduce the effectiveness of these inhibitors to mediate cancer cell death. Therefore, this study both illustrates the increasing complexity of the GRK kinome and suggests that the protein composition of cancer cells needs to be considered as levels of other proteins acting at similar sites of action could affect chemotherapeutic outcomes.

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