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Geranylgeranyltransferase I Inhibitors Target RalB To Inhibit Anchorage-Dependent Growth and Induce Apoptosis and RalA To Inhibit Anchorage-Independent Growth▽

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Geranylgeranyltransferase I inhibitors (GGTIs) are presently undergoing advanced preclinical studies and have been shown to disrupt oncogenic and tumor survival pathways, to inhibit anchorage-dependent and -independent growth, and to induce apoptosis. However, the geranylgeranylated proteins that are targeted by GGTIs to induce these effects are not known. Here we provide evidence that the Ras-like small GTPases RalA and RalB are exclusively geranylgeranylated and that inhibition of their geranylgeranylation mediates, at least in part, the effects of GGTIs on anchorage-dependent and -independent growth and tumor apoptosis. To this end, we have created the corresponding carboxyl-terminal mutants that are exclusively farnesylated and verified that they retain the subcellular localization and signaling activities of the wild-type geranylgeranylated proteins and that Ral GTPases do not undergo alternative prenylation in response to GGTI treatment. By expressing farnesylated, GGTI-resistant RalA and RalB in Cos7 cells and human pancreatic MiaPaCa2 cancer cells followed by GGTI-2417 treatment, we demonstrated that farnesylated RalB, but not RalA, confers resistance to the proapoptotic and anti-anchorage-dependent growth effects of GGTI-2417. Conversely, farnesylated RalA but not RalB expression renders MiaPaCa2 cells less sensitive to inhibition of anchorage-independent growth. Furthermore, farnesylated RalB, but not RalA, inhibits the ability of GGTI-2417 to suppress survivin and induce $p27^{Kip1}$ protein levels. We conclude that RalA and RalB are important, functionally distinct targets for GGTI-mediated tumor apoptosis and growth inhibition.

Members of the Ras and Rho branches of the Ras superfamily of small GTPases are critically involved in the regulation of many biological events critical to the regulation of cellular homeostasis, such as cell cycle control, cell survival, death, differentiation, development, and growth (9, 53). The aberrant activation or inactivation of Ras family proteins is believed to be important in the induction of oncogenesis. In addition to the three Ras proteins (H-, N-, and K-Ras), other Ras family proteins with validated roles in oncogenesis include R-Ras, Ral, Rheb, Di-Ras, and Noey2/ARHI small GTPases. Rho family GTPases (e.g., RhoB, RhoC, Rac1b, and DBC2) are also implicated in oncogenesis (41).

The oncogenic functions of the Ras and Rho proteins require posttranslational processing by prenyltransferase enzymes (20, 23, 56). The two enzymes responsible for prenylation of Ras family proteins are farnesyltransferase (FTase) and geranylgeranyltransferase I (GGTase I) (3–5, 29, 42, 43, 55, 56), which covalently attach the 15-carbon farnesyl and 20-carbon geranylgeranyl lipids, respectively, to the cysteine of proteins with the carboxy-terminal tetrapeptide consensus sequence CAAX (C is cysteine, A is any aliphatic amino acid, and X is any carboxyl-terminal amino acid). In general, FTase farnesylates proteins in which X is methionine or serine (37), whereas GGTase I geranylgeranylates proteins in which X is leucine or isoleucine (12). Ras proteins that are mutationally rendered unprenylatable lose their oncogenic activity and fail to properly localize within the cell (20, 23). Similarly, prenylation of other proteins in the Ras and Rho families is essential to their activities (1, 20, 25).

The fact that prenylation is required for the oncogenic activity of small GTPases prompted us and others to design FTase and GGTase I inhibitors (FTIs and GGTIs) as potential anticancer drugs (14, 26, 35, 57). While numerous studies have shown that FTIs suppress oncogenic and tumor survival pathways, the actual mechanism by which FTIs inhibit tumor growth is not known (34, 44). Thus, while designed originally as anti-Ras inhibitors, the Ras isoforms most commonly mutated in human cancers (N- and K-Ras) escape FTI inhibition by undergoing alternative prenylation by GGTase I (40, 51, 54). Therefore, the critical farnesylated proteins that FTIs target to induce these effects are not known (34, 44). Similarly, the GGTase I substrates important for the antitumor activity of GGTIs are not yet clearly understood. While we have implicated Rac1 and Rac3 Rho family proteins as candidate targets for GGTIs (22), other important targets remain to be identified. Clues to what these targets may be are suggested by our previous studies demonstrating that GGTIs inhibit the activation of the Akt serine/threonine kinase and expression of survivin (10, 49). Furthermore, GGTIs also induce $p21^{waf}$, inhibit

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cyclin-dependent kinase activity and phosphorylation of Rb, and lead to G_0/G_1 cell cycle accumulation (30, 35, 49–52). In animal models, GGTIs both inhibit tumor growth in nude mouse xenografts and induce tumor regression in transgenic mice (48, 49). The GGTase I substrates targeted by GGTIs to induce their antineoplastic effects are not known. Logical candidates include other Ras and Rho family proteins with roles in oncogenesis.

Recently, the *Ra*s-*l*ike RalA and RalB small GTPases have been shown to play critical roles in Ras-mediated growth transformation of human cells (16, 27). Ral GTPases are activated by Ral guanine nucleotide exchange factors (RalGEFs; e.g., RalGDS), and RalGEFs function as key downstream effectors of activated Ras (38). Critical evidence for the important role of the RalGEF-Ral effector pathway in Ras-mediated oncogenesis is provided by studies in cell culture and mouse model systems. We showed previously that this effector pathway, and not the Raf-MEK-ERK mitogen-activated protein kinase effector pathway, is sufficient and necessary to promote Ras-mediated tumorigenic growth transformation (16). Similar observations were made by Weinberg and colleagues (36), who found cell type differences in the importance of RalGEF-Ral signaling in Ras transformation. Marshall and colleagues found that mice deficient in one RalGEF (RalGDS) were developmentally normal but showed impaired skin tumor growth caused by carcinogen-induced Ras activation (15). An unexpected outcome of the study of the role of Ral GTPases in oncogenesis has been the distinct functions of the highly related RalA and RalB isoforms. Although RalA and RalB share strong sequence similarities (85% identity), White and colleagues found that RalA is important for tumor cell anchorage-independent proliferation, whereas RalB promotes tumor cell survival (8). We recently determined that RalA, but not RalB, is critical for anchorage-independent and tumorigenic growth of pancreatic carcinoma cells, whereas RalB and to a lesser degree RalA are critical for pancreatic carcinoma invasion and metastasis (28). The distinct functions of RalA and RalB may be due, in part, to distinct downstream effector utilization (7, 45).

The increasing evidence for Ral GTPases in oncogenesis prompted our interest in evaluating Ral GTPases as important targets for GGTI antitumor activity. Both RalA and RalB C termini contain a CAAX sequence that predicts prenylation, and RalA has been shown to be geranylgeranylated (24). Furthermore, RalA and RalB are involved in many oncogenic steps that are inhibited by GGTIs. Therefore, in this paper we investigated whether some of the antineoplastic effects of GGTIs are mediated by inhibition of the geranylgeranylation and function of RalA and/or RalB. To this end, we have demonstrated that RalA and RalB are exclusively geranylgeranylated, generated farnesylated variants of Ral, and verified that they are GGTI insensitive, and we used these variants to rescue cancer cells from the antineoplastic effects of GGTIs. Our data suggest that inhibition of RalB mediates the effects of GGTIs on survivin, $p27^{Kip1}$, apoptosis, and anchorage-dependent growth, whereas inhibition of RalA mediates, at least in part, the effects of GGTIs on anchorage-independent growth.

MATERIALS AND METHODS

Synthesis of CAAX peptidomimetics. The GGTase I-specific peptidomimetics GGTI-2417 and GGTI-2418 were synthesized as described previously (33). The FTase-specific peptidomimetics FTI-2148 and FTI-2153 were synthesized as described previously (48).

Cloning of RalA and RalB mutants. We used our previously described RalA and RalB pBABE expression constructs (16) as template DNA for site-directed mutagenesis PCR driven by Platinum *Taq* polymerase (Invitrogen, Carlsbad, CA) according to the manufacturer's (QIAGEN, Valencia, CA) instructions with the following primers: FLAG-RalA-CCIL was generated using F, 5′-GCCGGATCCATGGATTACAAGGATGACGACGATAAGATCGTCGACTACCTAGCAAATAAGCCC-3′, and R, 5′-GCCGGATCCTTATAAAATGCAGCATCTTTCTCTGATTC-3′); FLAG-RalA-CCIS was generated using F, 5′-GCCGGATCCATGGATTACAAGGATGACGACGATAAGATCGTCGACTACCTAGCAAATAAGCCC-3′, and R, 5′-GCCGGATCCTTATGAAATGCAGCATCTTTCTCTGATTC-3′); FLAG-RalA-SCIL was generated using F, 5′-GCCGGATCCATGGATTACAAGGATGACGACGATAAGATCGTCGACTACCTAGCAAATAAGCCC-3′, and R, 5′-GCCGGATCCTTATAAAATGCAGGATCTTTCTCTGATTC-3′); FLAG-RalB-CCLL was generated using F, 5′-GCCGGATCCATGGATTACAAGGATGACGACGATAAGATCGCTGCCAACAAGAGTAAGGGCCAG-3′, and R, 5′-GCCGGATCCTCATAGTAAGCAACATCTTTCTTTAAAACT-3′); FLAG-RalB-CCLS was generated using F, 5′-GCCGGATCCATGGATTACAAGGATGACGACGATAAGATCGCTGCCAACAAGAGTAAGGGCCAG-3′, and R, 5′-GCCGGATCCTCATGATAAGCAACATCTTTCTTTAAAACT-3′); FLAG-RalB-SCLL was generated using F, 5′-GCCGGATCCATGGATTACAAGGATGACGACGATAAGATCGCTGCCAACAAGAGTAAGGGCCAG-3′, and R, 5′-GCCGGATCCTCATAGTAAGCAAGATCTTTCTTTAAAACT-3′). These PCR fragments were subcloned into pBABE Moloney murine retroviral plasmid by single enzyme digest with BamHI (New England Biolabs, Ipswich, MA) under standard restriction enzyme conditions. Plasmid sequences were verified by a standard Sanger sequencing reaction.

In vitro transcription-translation-prenylation assay. Plasmid DNA was amplified in a PCR using AccuPrime *Taq* polymerase (Invitrogen, Carlsbad, CA) and 1μg plasmid DNA by forward primer T7-FLAG (RalA, 5′-GCCGGATCCTAATACGACTCACTATAGGGTCGACTACCTAGCAAATAAGCCC-3′; RalB, 5′-GCCGGATCCTAATACGACTCACTATAGGGGCTGCCAACAAGAGTAAGGGCCAG-3′) and gene-specific reverse primers) (the same primers as mentioned previously in the cloning protocol). Subsequent cDNA was isolated using a QIAquick PCR purification column (QIAGEN, Valencia, CA), and 500 ng was used for T7 in vitro transcription-translation (TnT-coupled rabbit reticulocyte lysate system; Promega, Madison, WI). Briefly, reaction components were assembled on ice according to the manufacturer's protocol along with either 5 μCi [^{3}H]farnesyl pyrophosphate, 5 μCi [^{3}H]geranylgeranyl pyrophosphate, or 10 μCi [^{35}S]methionine in the presence or absence of GGTI-2418 or FTI-2148. The reaction mixture was incubated at 30°C for 120 min, and the reaction was stopped by addition of an equal volume of 2× sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. The samples were then loaded onto 12% acrylamide SDS-PAGE gels and separated at 50 V. Gels were then fixed in methanol-acetic acid (50% methanol, 10% glacial acetic acid, 40% double-distilled H_2O) for 30 min while gently shaking. Gels were then rinsed and incubated for 30 min with Amplify reagent (Amersham Biosciences, Piscataway, NJ) for 30 min while gently shaking. Gels were then transferred to Whatman paper at 80°C for 2 h on a Bio-Rad model 583 gel drier using a Bio-Rad HydroTech vacuum pump (Bio-Rad, Hercules, CA). Gels were visualized by autoradiography using Kodak BioMax FX film (Kodak, Rochester, NY) at −80°C.

Cells and culture. The human tumor cell line MiaPaCa2 (pancreatic carcinoma), monkey embryonic kidney Cos7 cells, and murine NIH 3T3 fibroblasts were purchased from ATCC (Manassas, VA) and grown in Dulbecco's modified minimal essential medium (DMEM; Invitrogen, Carlsbad, CA) at 37°C in a humidified incubator at 5% CO_2.

Transfection procedure. Cells were grown to 50 to 70% confluence and transfected with Transit-LT1 (Mirus, Madison, WI) according to the manufacturer's instructions. Briefly, 3 μl of Transit-LT1 reagent was suspended per 1 ml of OPTI-MEM medium (Invitrogen, Carlsbad, CA) and allowed to equilibrate at 24 to 27°C for 15 min. Approximately 1 μg of plasmid per ml of medium was suspended and allowed to complex with the liposomes for 15 min at 24 to 27°C. Cells were briefly washed with OPTI-MEM, and 2 ml containing 2 μg medium and 6 μl Transit-LT1 was plated on top of the cells and incubated at 37°C for 6 h. Two ml of DMEM containing 10% fetal bovine serum, without penicillin-streptomycin, was added, and the cells were further incubated at 37°C overnight.

Immunofluorescence. Cos7 cells were seeded at a 50% cell density in six-well plates containing sterilized glass coverslips and allowed to attach overnight. Cells were transfected overnight using Transit-LT1 in Opti-MEM medium following the manufacturer's instructions. Medium was replaced with complete growth medium containing either dimethyl sulfoxide (DMSO), 25 μM FTI-2153, or 25 μM GGTI-2417 and incubated at 37°C, 5% CO_2 for 48 h. Cells were then aspirated and washed twice with sterile phosphate-buffered saline (PBS; pH 7.4) and fixed in 4% paraformaldehyde. Cells were then rinsed twice with sterile PBS (pH 7.4) and permeabilized, on ice, with 0.1% Triton X-100. Cells were then blocked for 1 hour with 1% bovine serum albumin, rinsed twice, and incubated overnight with 1:100 anti-FLAG M2 monoclonal antibody (Sigma-Aldrich, St. Louis, MO) or 1:100 antihemagglutinin (anti-HA) monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Cells were rinsed three times in sterile PBS (pH 7.4) and then incubated for 1 hour with secondary fluorescein isothiocyanate-conjugated rabbit anti-mouse antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Cells were then washed twice in sterile PBS, mounted using Vectashield mounting reagent containing 4′,6′-diamidino-2-phenylindole (DAPI) for nuclear visualization, and analyzed using a Leica DMIL fluorescence microscope (Leitz, Wetzlar, Germany) at 525 nm (fluorescein isothiocyanate) and 365/420 nm (DAPI).

Luciferase assay. NIH 3T3 cells were plated (10^6 cells per well) in six-well plates and transfected using a standard calcium phosphate-mediated transfection with 4 μg of either pBABE, HA-H-Ras12V, FLAG-RalA72L-F, FLAG-RalA72L-GG, FLAG-RalA72L-S, FLAG-RalB72L-F, FLAGRalB72L-GG, or FLAG-RalB72L-S plus 1 μg of NF-κB-pTAL firefly luciferase plasmid (BD Biosciences, Franklin Lakes, NJ) and 0.2 μg *Renilla* luciferase plasmid for 5 h (both from BD Biosciences, Franklin Lakes, NJ). Cells were then incubated in full growth medium overnight followed by serum starvation for 24 h in DMEM supplemented with 0.5% fetal calf serum. Cells were then lysed, and luciferase activity was determined using the Promega dual luciferase assay system according to the manufacturer's instructions.

Western blotting. Cells were treated with GGTI-2417 for 48 h, harvested, and lysed in HEPES lysis buffer as described previously (51). Proteins were then resolved by 12.5% SDS-PAGE and immunoblotted with antibodies against unprenylated Rap1A/Krev-1 (121), $p21^{WAF1}$ (C-190), Akt 1-2 (N19), and RhoB (119), RhoA (26C4), anti-HA monoclonal antibody, and survivin (FL142) (all from Santa Cruz Biotechnology, Santa Cruz, CA), $p27^{KIP1}$ (G173-524; Pharmingen, San Diego, CA), phospho-serine 473 Akt (Cell Signaling, Danvers, MA), and β-actin (AC15) and anti-FLAG M2 monoclonal antibody (both from Sigma-Aldrich, St. Louis, MO). The enhanced chemiluminescence blotting system (NEN Life Science Products, Boston, MA) was used for detection of positive antibody reactions.

Trypan blue dye exclusion assay. Adherent cells were harvested using trypsinization and pooled with suspension cells from medium supernatant by pelleting at 300 × *g* for 5 min at 4°C. The cells were then aspirated and resuspended in an appropriate volume of medium by pipetting gently up and down. Two 20-μl aliquots were removed and combined with an equal volume of trypan blue and allowed to mix for 2 min. A 10-μl volume was loaded onto a hemacytometer, and cells were scored as live or dead based on trypan blue dye exclusion.

TUNEL analysis. Cells were seeded into 60-mm-diameter dishes and grown in DMEM supplemented with 5% fetal bovine serum for 24 h and then treated with GGTI-2417 for 48 h, which we determined to be the optimal time point for induction of apoptosis. Apoptosis was determined by terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling (TUNEL) by using an in situ cell death detection kit (Roche, Indianapolis, IN). The cells were trypsinized, and cytospin preparations were obtained by centrifugation at 1,500 × *g* (using a Cytospin-3 centrifuge; Therma-Shandon). Cells were fixed with freshly prepared paraformaldehyde (4% in PBS, pH 7.4). Slides were rinsed with PBS, incubated in permeabilization solution, and cross-reacted with TUNEL reaction mixture for 60 min at 37°C in a humidified chamber. The slides were rinsed, mounted, and analyzed under a light microscope.

Creation of retrovirus. Retrovirus was created by transient transfection of HEK-293T human embryonic kidney cells with pVPACK-Ampho, pVPACK-gag-pol, and pBABE retroviral plasmids according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). Briefly, 293T cells were seeded at 2.5×10^6 cells in a 60-mm^2 dish. Three μg of each plasmid was combined and brought to a volume of 225 μl in RNase/DNase-free water, following which 25 μl 2.5 M $CaCl_2$ was added dropwise while gently vortexing. This solution was then added to 250 μl 2× HEPES-buffered saline dropwise and incubated at room temperature for 5 min. The total volume of 500 μl was then added to the 293T cells and incubated for 8 h at 37°C. The medium was then removed, and cells were incubated for 48 h. The supernatant was then passed through a 0.45-μm nylon low-protein-binding filter (Fisherbrand, Houston, TX) to remove cells and cell debris. Retroviral titers were determined by serial dilution and infection of NIH 3T3 cells followed by selection and colony formation in puromycin.

Cellular fractionation. Membrane and cytosolic fractions were isolated using the MEM-PER membrane extraction kit (Promega, Madison, WI). Briefly, 1×10^6 Cos7 cells were transfected with the appropriate plasmid and collected 72 hours later. Cells were lysed, and membrane and cytosolic fractions were isolated according to the manufacturer's protocol. Membrane and cytosolic fractions were diluted fivefold and diluted further in 2× Laemmli sample buffer (Bio-Rad, Carlsbad, CA). Proteins were separated by SDS-PAGE and visualized by Western blotting.

Creation of stable cell lines. MiaPaCa2 cells were seeded into six-well plates at a 40% confluence and incubated with 8 μg/ml Polybrene (Millipore/Specialty Media, Billerica, MA) and 5×10^5 viral particles for 8 h. The medium was changed to complete growth medium, and cells were incubated at 37°C, 5% CO_2 for 24 h. The medium was then replaced with complete growth medium containing 4 μg/ml puromycin and incubated until colonies formed. All colonies were pooled and taken as a single polyclonal population and cultured in complete medium containing 2 μg/ml puromycin.

MTT metabolism assay. Cells were seeded in a 96-well plate at a density of 1,500 cells per well and allowed to attach overnight. Cells were then incubated for 96 h with various concentrations of GGTI-2417 or appropriate DMSO control. Medium was aspirated after 96 h, the optimal time for study of proliferation using this assay, and replaced with complete medium containing 1 mg/ml 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) and incubated for 3 h at 37°C in 5% CO_2 humidified incubator. Medium was then aspirated, and DMSO was added. Cells were incubated for 5 min at room temperature with shaking, following which absorbance was determined at 495 nm.

Soft agar clonogenicity assay. For soft agar growth assays, the cell lines were seeded at a cell density of 1,500/well in triplicate in 12-well culture dishes in 0.3% agar over a 0.6% bottom agar layer. Various concentrations of GGTI-2417 or vehicle (DMSO) were included in the 0.3% agar layer of cells. Cultures were fed and treated with drug or vehicle once weekly until colonies grew to a suitable size for observation (colony growth rates were 10 to 14 days for RalA-stable MiaPaCa2 cells and 3 weeks for RalB-stable MiaPaCa2 cells). Colonies were photographed after overnight incubation with 1 mg/ml MTT in the respective cell growth medium. The growth of colonies in the presence of inhibitor was compared with the control colonies treated with vehicle.

Statistical analysis. Statistical analysis was performed using a standard Student's *t* test via either Microsoft Excel (Microsoft, Redmond, WA) or GraphPad Software (San Diego, CA) statistical analysis tools.

RESULTS

Generation of CAAX box mutants that are farnesylated or unprenylated versions of both RalA and RalB. It was demonstrated previously that RalA is geranylgeranylated and that both RalA and RalB require the prenyl-accepting cysteine for proper localization (18, 24). However, direct evidence that RalA and RalB are exclusively geranylgeranylated, and thus validated targets of GGTIs, is lacking. Furthermore, whether the nature of the prenyl group influences the subcellular location and function of Ral proteins is not known. To this end, we used site-directed mutagenesis to generate two CAAX box mutant forms of wild-type or GTPase-deficient/constitutively activated (Q72L) RalA and RalB proteins. The first missense mutation replaced the carboxyl-terminal leucine with a serine residue to switch the prenyltransferase specificity from GGTase I (RalA-CCIL and RalB-CCLL) to a site preferred by the related enzyme FTase (RalA-CCIS and RalB-CCLS). The second mutation replaced the prenylated cysteine with a serine residue to prevent prenylation (RalA-SCIL and RalB-SCLL). Both GTP-locked (RalA72L, RalB72L) and wild-type RalA and RalB were used to generate the CAAX box mutants. Plasmid constructs containing these Ral cDNA sequences were then used in in vitro transcription-translation-prenylation assays in rabbit reticulocyte lysates with radiolabeled

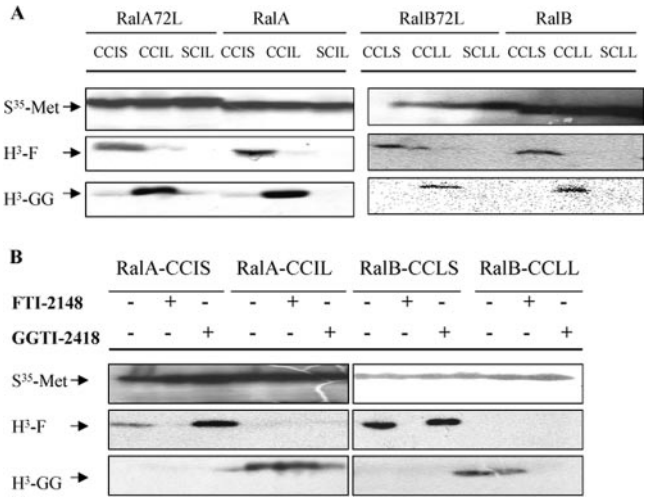

FIG. 1. RalA-CCIL and RalB-CCLL are geranylgeranylated, whereas the mutants RalA-CCIS and RalB-CCLS are farnesylated. (A) Ral DNAs were used in transcription-translation-prenylation assays using either radiolabeled [^{35}S]methionine, [^{3}H]FPP, or [^{3}H]GGPP and then run on an SDS-PAGE and visualized by autoradiography as described in Materials and Methods. Results are representative of two independent experiments. (B) RalA-CCIS, RalA-CCIL, RalB- CCLS, and RalB-CCLL DNAs were transcribed, translated, and prenylated using either [^{35}S]methionine, [^{3}H]FPP, or [^{3}H]GGPP in the presence of either vehicle (DMSO), 250 nM FTI-2148, or 250 nM GGTI-2418 and then run on an SDS-PAGE and visualized by autoradiography. Results are representative of two independent experiments.

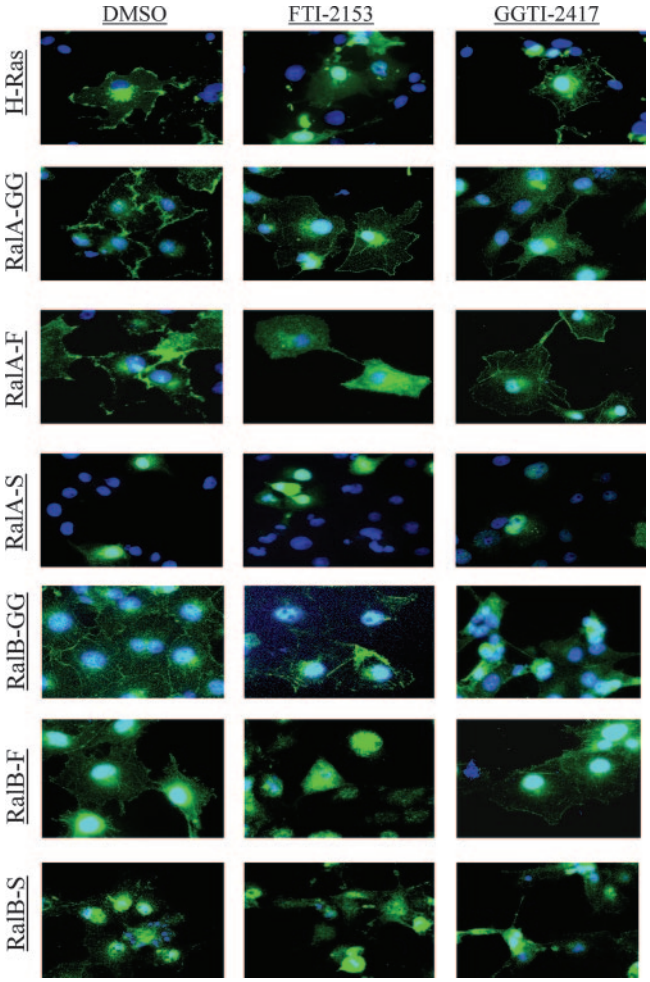

FIG. 2. Geranylgeranylated (GG) and farnesylated (F) RalA and RalB localize similarly and require prenylation for correct localization. Cos7 cells grown on coverslips were transiently transfected with plasmids expressing the indicated proteins and treated with the indicated inhibitors (25 μM) and then analyzed for FLAG-Ral or HA-H-Ras distribution using immunoflorescence as described in Materials and Methods. Nuclei were visualized using DAPI stain. Results are representative of three independent experiments.

[^{35}S]methionine, [^{3}H]farnesyl pyrophosphate (FPP), and [^{3}H]geranylgeranyl pyrophosphate (GGPP) to determine the relative strength of translation, farnesylation, and geranylgeranylation. Figure 1A shows that RalA-CCIL and RalB-CCLL were geranylgeranylated, while replacement of the carboxyl-terminal amino acid with a serine resulted in incorporation of a farnesyl instead of a geranylgeranyl moiety. Replacement of the prenylation site cysteine with a serine residue (CCIL and CCLL to SCIL and SCLL) rendered both RalA and RalB unprenylated (Fig. 1A).

Geranylgeranylated and farnesylated RalA and RalB are not alternatively prenylated in the presence of GGTIs and FTIs, respectively. It has been well documented that certain Ras family members are capable of being alternatively prenylated when FTase is inhibited (40, 51, 54). We reasoned that it is possible that RalA and/or RalB (geranylgeranylated or farnesylated forms) could be alternatively prenylated when GGTase I or FTase is inhibited. In order to determine whether the wild-type RalA-CCIL and RalB-CCLL were indeed targets only of GGTase I and could not be prenylated by FTase upon GGTase I inhibition, we added GGTI-2418, a potent and selective competitive inhibitor of GGTase I (33), to the reticulocyte lysate transcription-translation-prenylation mixture. Wild-type RalA-CCIL and RalB-CCLL were geranylgeranylated and were not alternatively farnesylated when GGTase I was inhibited by GGTI-2418, and their geranylgeranylation was little affected by inhibition of FTase by our previously characterized FTase-specific inhibitor, FTI-2148 (48). Furthermore, RalA-CCIS and RalB-CCLS remained farnesylated when GGTase I was inhibited and did not become geranylgeranylated when FTase I was inhibited (Fig. 1B). Minor variations in the apparent incorporation efficiencies of the radiolabeled prenyl groups in the absence or presence of inhibitors was seen regularly but without consistency. These results suggest that RalA-CCIL and RalB-CCLL are targets of GGTIs but not FTIs, and conversely, that the RalA-CCIS and RalB-CCLS CAAX box mutants are targets of FTIs but not GGTIs. Furthermore, neither the geranylgeranylated or farnesylated Ral proteins are alternatively prenylated under the pressure of GGTI or FTI treatment, respectively. Therefore, for the rest of the manuscript we will refer to RalA-CCIL and RalB-CCLL as RalA-GG and RalB-GG, respectively. Similarly, we will refer to RalA-CCIS and RalB-CCLS as RalA-F and RalB-F, respectively.

Prenylation is required for proper subcellular localization of RalA and RalB, and both RalA and RalB are mislocalized in response to prenyltransferase inhibition. The results of Fig. 1

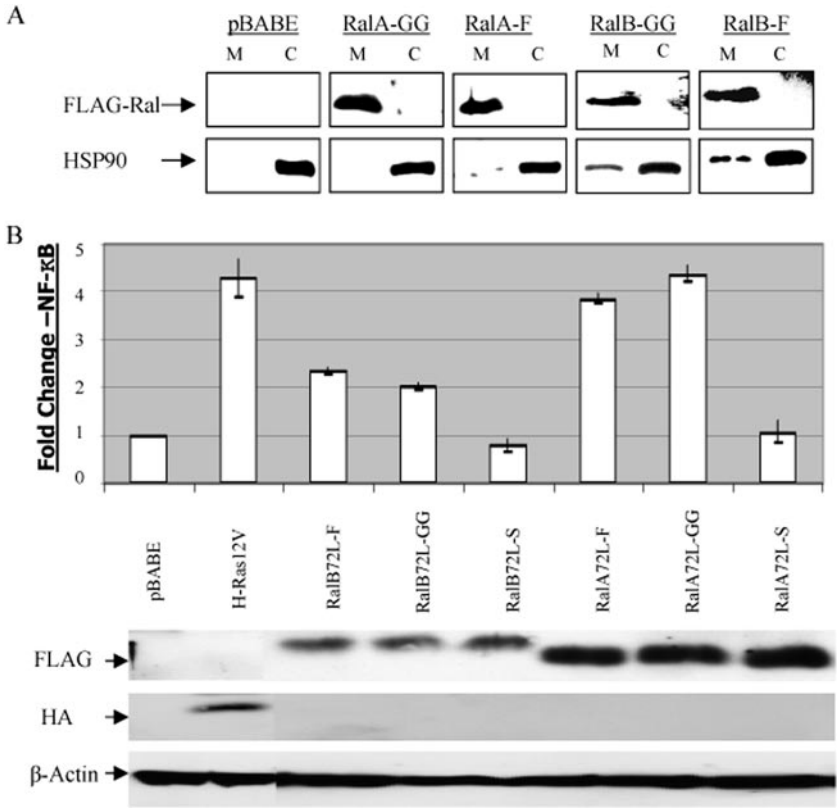

FIG. 3. Farnesylated and geranylgeranylated RalA and RalB are equivalent in mediating activation of NF-κB promoter activity. (A) Cos7 cells were transiently transfected with the indicated plasmids, and membrane (M) and cytosolic (C) fractions were isolated and probed by Western blotting. HSP90 was used as a cytosolic fraction marker. Results are representative of two independent experiments. (B) NIH 3T3 cells were serum starved and transiently cotransfected with plasmid expressing the indicated proteins and an NF-κB reporter plasmid. NF-κB promoter activity was detected following transfection as described in Materials and Methods. Expression was analyzed by Western blot analysis. Results are representative of three independent experiments.

demonstrated, in a reconstituted cell reticulocyte system, that the wild-type RalA-GG, RalB-GG, and CAAX box mutant RalA-F and RalB-F variants are not alternatively prenylated in vitro. We next determined whether prenylation is required for proper localization in intact cells and whether GGTI-2417 treatment is sufficient to disrupt localization of wild-type RalA-GG and RalB-GG. We also wanted to determine if the RalA-F and RalB-F mutants display a localization similar to RalA-GG and RalB-GG. To this end, we ectopically expressed FLAG epitope-tagged RalA-GG, RalA-F, RalA-S, RalB-GG, RalB-F, and RalB-S in Cos7 cells followed by treatment with the indicated prenyltransferase inhibitors. We found that both the geranylgeranylated and farnesylated forms of RalA and RalB demonstrated a similar localization to the plasma membrane (Fig. 2). This was confirmed by membrane/cytosol cellular fractionations where RalA-GG and RalB-GG as well as RalA-F and RalB-F localized to the membrane fractions (Fig. 3A). The fact that RalA and RalB localize similarly is interesting but different than results by Feig et al. (45). The reason for this difference at present is not known. The unprenylated versions of RalA (RalA-S) and RalB (RalB-S) were diffused in the cytoplasmic and the perinuclear regions, indicating that prenylation is required for proper plasma membrane localization of RalA and RalB (Fig. 2). Furthermore, in response to GGTase I inhibition, RalA-GG and RalB-GG were unable to localize to the plasma membrane, while no change in membrane localization was observed when FTase activity was inhibited. As expected, H-Ras was predominantly localized to the plasma membrane, and this was only affected by FTI but not GGTI treatment. Similarly, in response to FTase inhibition, RalA-F and RalB-F were unable to localize to the plasma membrane, while no change in membrane association was observed with GGTase I inhibition (Fig. 2). These data demonstrate that RalA-GG and RalB-GG are targets of GGTase I inhibitors and that the farnesylated Ral variants will be useful reagents to determine if inhibition of Ral GTPases contribute to the biological effects of GGTI-2417 treatment.

Farnesylated and geranylgeranylated RalA and RalB are equivalent in ability to activate NF-κB-responsive promoter elements. We next wanted to verify that Ral function was retained when modified by farnesylation. It had been reported previously that the constitutively activated RalB72L mutant protein is capable of activating NF-κB-dependent transcription (17). We therefore determined if farnesylated Ral and geranylgeranylated of Ral are equivalent in their ability to mediate activation of an NF-κB-dependent promoter. In order to assess the contribution of the prenyl moiety to NF-κB-driven transcription we transiently transfected NIH 3T3 cells with either the pBabe-puro empty vector or one encoding constitutively activated H-Ras (H-

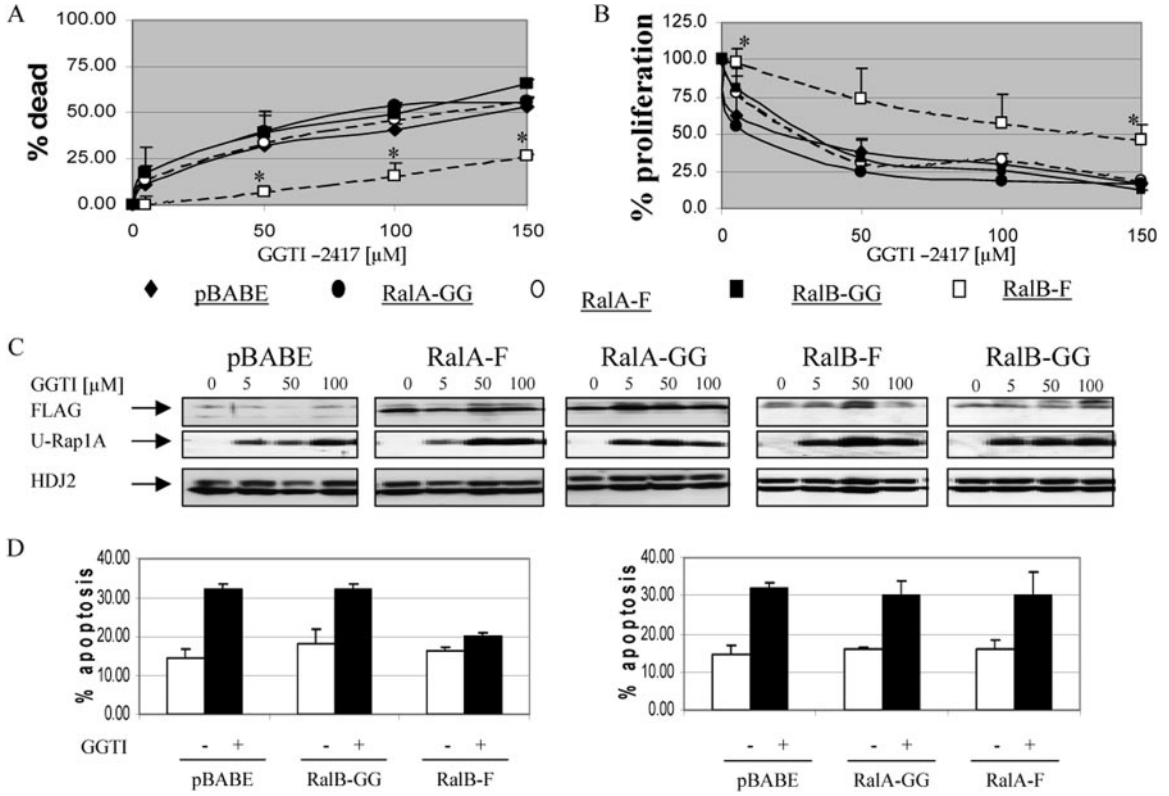

FIG. 4. Ectopic expression of farnesylated RalB, but not RalA, renders cells less sensitive to GGTI-2417 inhibition of survival and proliferation and induction of apoptosis in Cos7 cells. (A and B) Inhibition of RalB prenylation is required for GGTI induction of cell death and inhibition of proliferation. Cos7 cells were transfected with the indicated plasmids and then treated with GGTI-2417 or vehicle (DMSO) control. Cell viability was determined by the trypan blue dye exclusion assay. Data shown are the averages of three independent experiments. (C) Cell lysates from panels A and B were analyzed for expression by Western blot analysis as described in Materials and Methods. (D) Inhibition of RalB prenylation is required for GGTI induction of apoptosis. Cos7 cells were transiently transfected with plasmids expressing the indicated proteins and then treated for 72 h with 50 μM GGTI-2417 or DMSO control, collected, and analyzed for apoptosis via a TUNEL assay as described in Materials and Methods. Data shown are the averages of three independent experiments. *, $P < 0.05$.

Ras12V), RalB72L-GG, RalB72L-F, RalB72L-S, RalA72L-GG, RalA72L-F, or RalA72L-S. As expected, H-Ras12V stimulated an approximately fourfold increase in NF-κB luciferase activity (Fig. 3). As previously reported, RalB72L-GG activated NF-κB-dependent promoter activity. Importantly, RalB72L-F activated NF-κB luciferase activity to a similar extent as the geranylgeranylated version (approximately twofold). Similar to the results with RalB, ectopic expression of RalA72L-GG and RalA72L-F both stimulated fourfold activation of NF-κB. Importantly, neither nonprenylated RalB-S nor RalA-S stimulated activation of NF-κB (Fig. 3). These data demonstrate that prenylation is required for RalA and RalB activation of NF-κB and that farnesyl and geranylgeranyl moieties are equivalent in supporting Ral activation of NF-κB.

Ectopic expression of RalB-F renders Cos7 cells resistant to inhibition of proliferation, induction of cell death, and apoptosis by GGTI-2417. Since the farnesylated and geranylgeranylated forms of Ral proteins displayed similar subcellular localization and signaling properties, we reasoned that, if Ral GTPases were important functional targets of GGTIs, ectopic expression of farnesylated, but not geranylgeranylated, RalA and/or RalB would rescue cells from the effects of GGTase I inhibition. Figure 4A shows that ectopic expression of farnesylated RalB, but not RalA, rendered cells less sensitive to the induction of cell death by GGTI-2417. There were no statistically significant differences between any transfected groups (pBabe empty vector control, RalA-F, RalA-GG, and RalB-GG) in cell death except in the RalB-F-expressing Cos7 cells ($P < 0.05$ at 50, 100, and 150 μM). For example, GGTI-2417 induced 39% cell death at 50 μM in RalB-GG-transfected Cos7 cells, but when RalB-F was expressed GGTI-2417 induced only 7% cell death (Fig. 4A). Similarly, RalB-F-expressing Cos7 cells were 4.51-fold more resistant to inhibition of proliferation by GGTI-2417 than RalB-GG-expressing cells (50% inhibitory concentration [IC_{50}] of 131 μM and 29 μM, respectively) (Fig. 4B).

We next determined whether RalA-F and/or RalB-F could rescue cells from GGTI-2417 treatment-induced programmed cell death (apoptosis) by TUNEL assay. Figure 4D shows that only ectopic expression of farnesylated RalB, but not farnesylated RalA, demonstrated a significantly protective effect from GGTI-2417-induced apoptosis. GGTI-2417 increased the fraction of apoptotic cells from about 18 to 32% in RalB-GG, but only from 17 to 20% in RalB-F (Fig. 4D). In contrast, GGTI-2417 potently induced apoptosis in Cos7 cells expressing either RalA-GG or RalA-F, with GGTI-2417 treatment increasing the fraction of apoptotic cells from approximately 15% to 30% in both cell populations (Fig. 4D). These results sug-

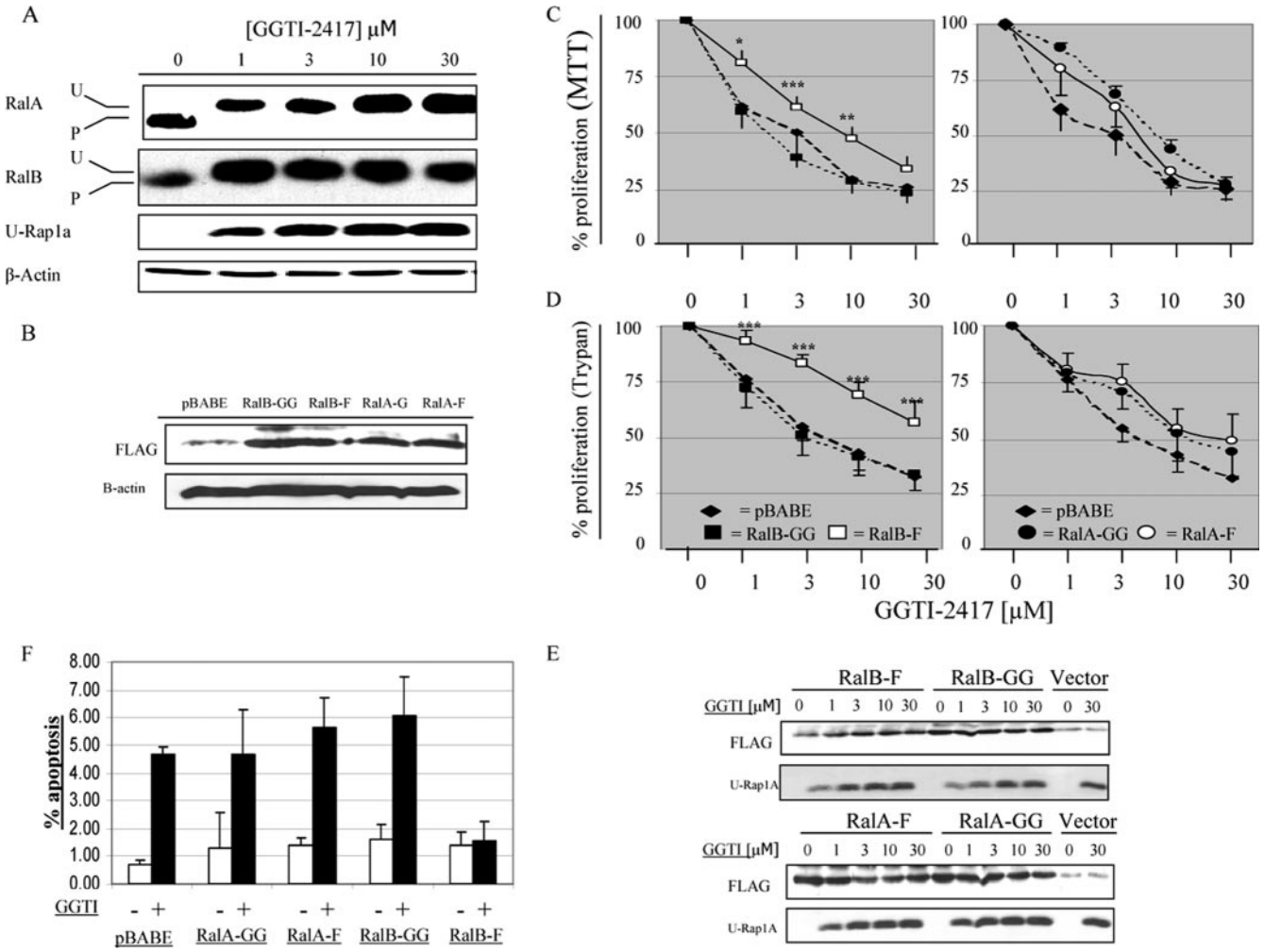

FIG. 5. Stable expression of farnesylated RalB, but not RalA, promotes resistance to the antiproliferative and proapoptotic effects of GGTI-2417 in MiaPaCa2 cells. (A) Dose-response results to determine the efficacy of GGTI-2417 in inhibiting RalA and RalB prenylation in MiaPaCa2 cells. MiaPaCa2 cells were treated with various concentrations of GGTI-2417 for 48 h and processed for Western blotting as described in Materials and Methods. (B) Stable MiaPaCa2 cell lines were created using retroviral infection of a puromycin resistance marker along with the indicated transgenes as described in Materials and Methods. Cells were lysed, and expression was assessed by Western blot analysis. (C) Stably expressing MiaPaCa2 cells were treated with the indicated concentrations of GGTI-2417 for 96 h. Proliferation was measured by the MTT viability assay as described in Materials and Methods. Data shown are the averages of at least three independent experiments. (D) MiaPaCa2 cells stably expressing the indicated Ral proteins were treated with the indicated concentrations of GGTI-2417 for 72 h. Proliferation was assessed by trypan blue dye exclusion assay as described in Materials and Methods. Data shown are the averages of at least three independent experiments. (E) MiaPaCa2 cells stably expressing the indicated Ral proteins were treated with GGTI-2417 (30 μM) and lysed, and expression was assessed by Western blot analysis. Data shown are representative of three independent experiments. (F) MiaPaCa2 cells stably expressing the indicated Ral proteins were treated with GGTI-2417 for 48 h, and apoptosis was assessed by a TUNEL assay as described in Materials and Methods. Data shown are the averages of three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

gest that inhibition of RalB and not RalA geranylgeranylation is an important target for GGTI-mediated apoptosis. All cell lines displayed similar accumulation of nongeranylgeranylated Rap1A (U-Rap1A) following GGTI-2417 treatment (Fig. 4C). No changes were observed in the gel mobility of the human homologue of DNAJ-2 (HDJ2), an exclusively farnesylated protein, indicating that FTase activity was not blocked by this treatment (Fig. 4C).

MiaPaCa2 human pancreatic cancer cells stably expressing farnesylated RalB are resistant to the antiproliferative and proapoptotic effects of GGTI-2417 compared to geranylgeranylated RalB-expressing cells. Since Cos7 are immortalized nontransformed cells of nonhuman primate origin, we endeavored to create a more relevant cell system to characterize the growth inhibitory and proapoptotic effects of GGTI-2417. We first determined by Western blotting that both RalA and RalB are efficiently targeted by GGTI-2417 in MiaPaCa2 cells. Figure 5A shows that GGTI-2417 concentrations as low as 1 μM inhibited RalA and RalB geranylgeranylation. We next established populations of MiaPaCa2 cells stably expressing RalA and RalB prenyl isoforms through retroviral transduction and selection (Fig. 5B). We then used these cells to examine the effects of ectopic wild-type or farnesylated RalA and RalB expression on GGTI-2417-mediated inhibition of proliferation

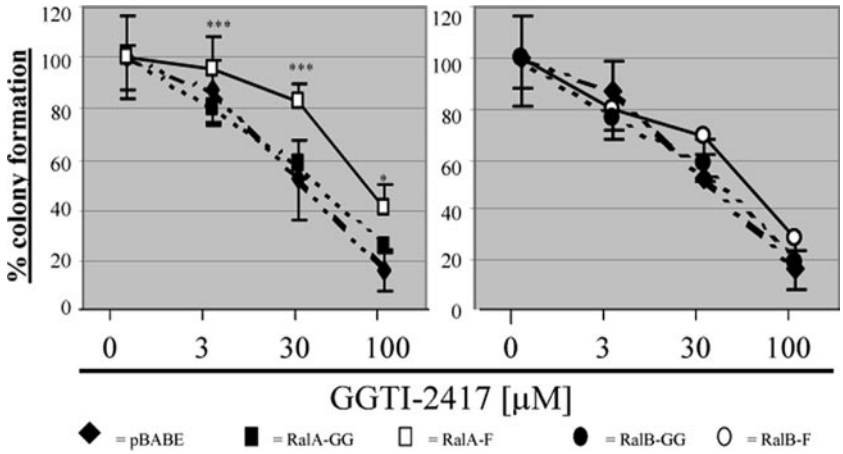

FIG. 6. Stable expression of farnesylated RalA, but not RalB, induces resistance to inhibition of anchorage-independent growth by GGTI-2417 in MiaPaCa2 cells. MiaPaCa2 cells stably expressing the indicated Ral proteins were seeded into 12-well plates in 0.3% soft agar and treated with the indicated concentrations of GGTI-2417 for 10 days as described in Materials and Methods. Data shown are the averages of two independent experiments repeated in triplicate. *, $P < 0.05$; ***, $P < 0.001$.

of MiaPaCa2 cells by both MTT metabolism viability (Fig. 5C) and trypan blue dye exclusion assays (Fig. 5D). Similar to our observations in Cos7 cells, MiaPaCa2 cells stably expressing farnesylated as opposed to geranylgeranylated RalB were less sensitive to the anchorage-dependent antiproliferative effects of GGTI-2417. Specifically, using the MTT assay RalB-GG-expressing cells were inhibited by 50% at 1.6 ± 0.3 μM (mean ± standard error) GGTI-2417, whereas RalB-F-expressing cells (IC_{50} of 8.3 ± 1.4 μM; $P = 0.0015$) were more than fivefold resistant. There were no statistically significant differences between RalB-GG and empty vector cells. Furthermore, no statistically significant differences in proliferation inhibition sensitivity were observed for empty vector-, RalA-F-, or RalA-GG-expressing cells (Fig. 5C). Similar results were obtained using total cell counting by trypan blue dye exclusion assay, demonstrating no difference in sensitivity between RalA-F- and RalA-GG-expressing cells and a greater-than-sixfold statistically significant difference ($P < 0.0001$) in IC_{50} values between RalB-GG- and RalB-F-expressing cells (IC_{50} of 4.8 ± 1.6 μM and greater than 30 μM, respectively) (Fig. 5D). There were no statistically significant differences between empty vector (5.6 ± 1.7 μM) and RalB-GG (4.8 ± 1.6 μM) cells. These data both confirmed and extended our observations in Cos7 cells and further indicated that inhibition of RalB, not RalA, prenylation is an important target for GGTI inhibition of anchorage-dependent cell proliferation.

To further examine the role of Ral proteins in GGTI-2417 induction of apoptosis, we determined if ectopic expression of RalA-GG, RalA-F, RalB-GG, or RalB-F altered MiaPaCa2 sensitivity. Specifically, we observed that GGTI-2417 treatment induced a greater-than-threefold increase in apoptosis in all cell lines except those expressing RalB-F (Fig. 5F). Of primary importance, GGTI-2417 induced a 3.6-fold induction of apoptosis (1.6 to 6.07%) in RalB-GG-expressing cells compared to no statistically significant induction of cell death in RalB-F-expressing cells (1.42 to 1.56%). This indicates that, consistent with the Cos7 results, RalB but not RalA is a critical target for the apoptotic cell death caused by GGTI-2417 treatment of MiaPaCa2 cells.

Stable expression of farnesylated RalA, but not RalB, induces partial resistance to inhibition of anchorage-independent growth by GGTI-2417 in MiaPaCa2 cells. We next determined whether GGTI-2417 inhibition of growth in soft agar might be due, in part, to inhibition of RalA and/or RalB function. To this end, we used a soft agar clonogenicity assay to measure the effects of GGTI-2417 on MiaPaCa2 cells stably expressing geranylgeranylated or farnesylated RalA or RalB. We found that MiaPaCa2 cells stably expressing either RalB-GG or RalB-F did not differ in sensitivity to GGTI-2417 treatment. However, MiaPaCa2 cells stably expressing RalA-F were less sensitive to inhibition of soft agar growth by GGTI-2417 than those cells stably expressing ectopic RalA-GG (Fig. 6). For example, at 30 μM, GGTI-2417 inhibited colony formation by 41.3% ± 5.9% and 17.8% ± 8.0% ($P < 0.001$) in RalA-GG- and RalA-F-expressing cells, respectively. Taken as a whole, these data implicate RalA, but not RalB, inhibition of prenylation as a potential mechanism for GGTI reversion of the transformed growth phenotype of MiaPaca2 cells as measured by anchorage-independent soft agar growth.

Stable expression of farnesylated RalB but not RalA in MiaPaCa2 cells inhibits the ability of GGTI-2417 to increase $p27^{Kip1}$ and decrease survivin protein levels. Recent data from our lab showed that GGTI treatment affects several signal transduction pathways critical to cell cycle division and tumor cell survival (30, 35, 49–52; A. Kazi, A. Carie, M. A. Blaskovich, C. Bucher, V. Thai, S. Moulder, H. Peng, D. Carrico, E. Pusateri, A. D. Hamilton, and S. M. Sebti, submitted for publication). In order to examine the contribution of inhibition of the geranylgeranylation of RalA and/or RalB to the effects of GGTI-2417 on signaling pathways, we treated MiaPaCa2 cells stably expressing RalA-GG, RalA-F, RalB-GG, and RalB-F with GGTI-2417. In control, empty vector-transfected MiaPaCa2 cells, GGTI-2417 treatment resulted in inhibition of RalA and Rap1 geranylgeranylation, increased the protein levels of $p27^{Kip1}$, RhoA, and RhoB, and decreased the levels of two antiapoptotic proteins, activated phosphorylated Akt (P-Akt) and survivin (Fig. 7). Expression of RalA-F, RalA-GG, or RalB-GG did not affect the ability of GGTI-2417 to induce its

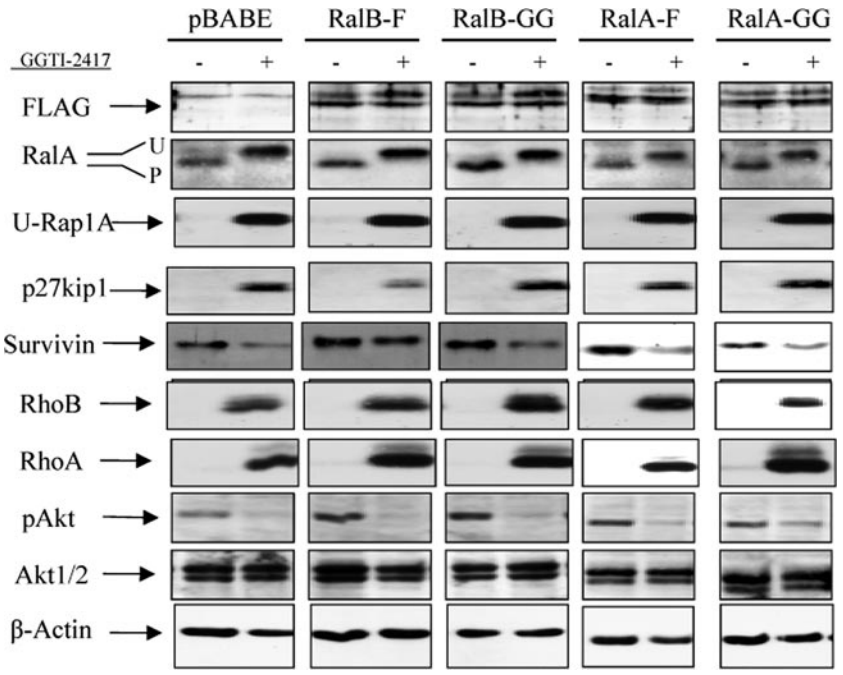

FIG. 7. RalB-F, but not RalA-F, inhibits the ability of GGTI-2417 to increase $p27^{Kip1}$ and decrease survivin protein levels. Stably expressing MiaPaCa2 cells were seeded into six-well plates and treated with GGTI-2417 (30 μM) for 48 h. Cells were lysed, and expression was assessed by Western blot analysis. Data shown are representative of three independent experiments.

effects on these signaling molecules. However, expression of RalB-F inhibited the ability of GGTI-2417 to increase $p27^{Kip1}$ and to decrease survivin protein levels. For example, while $p27^{Kip1}$ protein levels were increased in cells expressing RalB-GG (3.12-fold ± 0.88-fold), cells expressing RalB-F showed a 50% (1.63-fold ± 0.64-fold) attenuated increase of $p27^{Kip1}$ protein levels ($P = 0.0073$). Furthermore, GGTI-2417 treatment reduced survivin levels by 46 ± 11% in empty vector- and RalB-GG-expressing cells. However, MiaPaCa2 cells expressing RalB-F were less sensitive to depletion of survivin levels, with only a 12 ± 3% reduction of survivin levels following GGT-2417 treatment ($P = 0.0006$). By contrast, expression of either RalA-F or RalA-GG had no effect on GGTI reduction of survivin or induction of $p27^{Kip1}$. To determine if knockdown of RalA or RalB affected the levels of survivin or $p27^{Kip1}$, we used small interfering RNA (siRNA) to specifically inhibit RalA and RalB expression in parental MiaPaCa2 cells. We found that knockdown of RalB, but not RalA, inhibited the survivin protein levels by 70.2%, whereas neither RalA nor RalB knockdown affected the levels of $p27^{Kip1}$ (data not shown).

DISCUSSION

Inhibition of protein geranylgeranylation strongly attenuates multiple tumorigenic pathways, such as anchorage-dependent and -independent tumor growth and protection from apoptosis (30, 35, 49–52). However, many proteins are substrates for GGTase I-mediated protein geranylgeranylation, and it is not known which of these are critical targets for the antineoplastic effects of GGTIs (56). Since recent studies demonstrated the important roles of the RalA and RalB small GTPases in human oncogenesis (7, 8, 15, 16, 28, 36, 38, 45), we evaluated the possibility that these GGTase I substrates are important targets for GGTIs. In this study we have demonstrated that the GTPases RalA and RalB are geranylgeranylated by GGTase I, require prenylation for proper localization, and are downstream targets of pharmacological inhibitors of GGTase I. We designed farnesylated, GGTI-insensitive variants of Ral GTPases and found that farnesylated RalB, but not RalA, confers resistance to the proapoptotic and anti-anchorage-dependent growth effects of GGTI-2417 on Cos7 and MiaPaCa2 pancreatic carcinoma cells. Conversely, farnesylated RalA, but not RalB, expression renders MiaPaCa2 cells less sensitive to inhibition of anchorage-independent growth. Finally, we determined that farnesylated RalB, but not RalA, inhibited the ability of GGTI-2417 to suppress survivin and induce $p27^{Kip1}$. We conclude that RalA and RalB are important, functionally distinct targets for GGTI-mediated antineoplastic effects. Additionally, our studies extend recent observations showing that the highly related RalA and RalB proteins serve distinct functions in oncogenesis.

The determination of the GGTase I substrates that are critical for the antitumor activity of GGTIs is complicated by the fact that over 60 CAAX-terminating proteins may be substrates for this prenyltransferase (56). We previously established and validated a substitutive chemical biology approach to define the contribution of inhibition of specific GGTase I substrates to GGTI inhibition of cell survival, proliferation, transformation, and cell signaling (22). In this study, we applied this approach and we generated farnesylated mutants of both RalA and RalB and showed that they localized similarly to authentic geranylgeranylated wild-type versions and similarly activated NF-κB and were resistant to inhibition by GGTI

treatment. Thus, this substitutive chemical biology approach is a valid means of uncoupling RalA and RalB from GGTase I dependency while preserving their wild-type subcellular localization and signaling activity.

Our results demonstrate a partial requirement for inhibition of RalA prenylation in the inhibition of anchorage-independent growth by GGTI-2417. Since expression of farnesylated RalB did not attenuate the ability of GGTI-2417 to inhibit anchorage-independent growth, we reason that the ability of RalA to regulate clonogenicity is a divergent function from that of the 85% identical protein RalB. The ability of farnesylated RalA but not RalB to regulate anchorage-independent growth is similar to results reported by Chien et al. (15), who showed that depletion of RalA but not RalB by siRNA inhibited anchorage-independent growth in HeLa and SW480 cancer cell lines. However, other processes commonly associated with anchorage-independent proliferation, such as cell migration and chemotaxis, bear a requirement for RalB as opposed to RalA; such results were first reported by Oxford et al. (32), who showed depletion of RalB but not RalA by siRNA inhibited cell migration in a panel of renal carcinoma cell lines. In particular, we found that RNA interference-mediated suppression of RalA, but not RalB, greatly impaired the soft agar growth of MiaPaCa2 and other pancreatic carcinoma cells (27, 28).

Interestingly, we found further divergent functions for the Ral family of small GTPases. Specifically, inhibition of RalB, but not RalA, prenylation was required for GGTI-mediated inhibition of proliferation and induction of apoptosis. In both the Cos7 and the MiaPaCa2 cell lines, expression of RalB-F but not RalA-F rendered cells less sensitive to GGTI-mediated inhibition of proliferation and induction of apoptosis. In MiaPaCa2 cells this rescue was concurrent with abrogation of GGTI effects on increasing $p27^{Kip1}$ and decreasing survivin protein levels, but not with inhibiting Akt activation levels and inducing RhoA and RhoB levels, both of which we have shown previously to be induced by GGTIs (11). These results suggest that, at least in the MiaPaCa2 pancreatic carcinoma cells, the ability of RalB-F to abrogate the GGTI antiproliferative and proapoptotic mechanism of action is associated with abrogation of $p27^{Kip1}$ induction and suppression of survivin levels. In further support of this, we used siRNA to specifically inhibit RalA and RalB expression and found that knockdown of RalB, but not RalA, strongly attenuated survivin expression but did not affect $p27^{Kip1}$ levels. These results concur with previous data that some tumor cells have at least a partial requirement for RalB in suppressing apoptosis (8) and suggest that RalB maintenance of survivin expression could be an important mechanism by which RalB promotes tumor cell survival. These results agree with our previous finding that ectopic expression of survivin partially abrogates GGTI induction of programmed cell death (10). Taken as a whole the results of this study raise interesting questions about the mechanism of GGTI-mediated antineoplastic activity. Both RalA and RalB are important regulators of many cellular processes that have not previously been implicated in the GGTI mechanism of action. One of the most critical of these in oncogenesis is cellular trafficking both at the receptor level through endocytosis and through formation and delivery of the exocyst complex (2, 6, 13, 21, 47), a process reported to be required for the transforming ability of RalA and RalB (27). While both RalA and RalB utilize the exocyst complex, RalA seems to primarily utilize the exocyst for transformation, whereas RalB preferentially utilizes components of the exocyst for exocyst-independent functions in regulating cell mobility (7, 39). In fact, RalA and RalB are the only known binding partners for two competitive regulatory elements of the exocyst: Exo84 and Sec5. Therefore, the inhibition of RalA and RalB by GGTI-2417 represents a novel mechanism to inhibit exocyst function in transformed cells.

Beyond the exocytic and endocytic trafficking pathways, RalA and RalB may regulate important interactions with the actin cytoskeleton through their association with RalBP1, the Rho GTPase-activating protein, and filamin, an actin-binding partner (31). Indeed, while many small GTPases, such as Cdc42, Rac, and Rho, bind filamin, only RalA and RalB bind filamin in a GTP-dependent manner. Therefore, the effects of GGTIs on cytoskeletal organization may be mediated by inhibition of RalA and/or RalB geranylgeranylation. In the course of our study we observed that RalB-F, but not RalA-F, inhibited GGTI-induced cell rounding in MiaPaCa2 cells concurrent with inhibition of actin fiber formation (data not shown). This is indicative of a disruption in the cell-cell junctions and the underlying actin-driven membrane formations. This raises the provocative hypothesis that at least part of the GGTI effects could be due to inhibition of normal cell-cell patterning. This is consistent with the fact that RalA is required for basolateral sorting and delivery of E-cadherin (45), an important component of cell junctions and sheet patterning. Furthermore, it has been previously reported that cadherins mediate growth suppression by potently inducing $p27^{Kip1}$ levels in a variety of cell lines (46). It is thus an intriguing possibility that aberrant activation of Ral proteins could mediate further aberrations in vesicle sorting of cadherins and contribute to suppression of $p27^{Kip1}$ levels. Disruption of Ral-mediated vesicle sorting of cadherins by GGTIs could constitute a mechanism for the antineoplastic activity of GGTase I inhibition. Further, cadherin clustering and expression levels can regulate survivin levels (19). This suggests that sorting of basolateral and apical membrane proteins, such as cadherins, could be a Ral-dependent pathway that is required for tumor cell proliferation and survival and is sensitive to perturbation by GGTIs.

The antineoplastic activity of GGTIs is likely to be a consequence of inhibiting the function of multiple, functionally distinct GGTase I substrates. However, our chemical biology approach has clearly delineated nonoverlapping roles for RalA and RalB in anchorage-independent and -dependent growth and demonstrates that inhibition of RalA and RalB geranylgeranylation is an important step in the mechanism of action of GGTIs. Specifically, we have identified three novel pathways that are associated with the GGTI response: first, a RalB-dependent induction of the $p27^{Kip1}$ pathway; second, a RalB maintenance of the survivin pathway; third, a RalA anchorage-independent proliferation pathway. The first two pathways are associated with the ability of GGTI to inhibit anchorage-dependent proliferation and survival. Further characterizing these pathways should prove to be an interesting and important contribution to the study of Ral biology. We feel these results should prompt a thorough examination of the GGTI mechanism of action with particular attention focused

on the Ral family in both future clinical trials and in preclinical models.

ACKNOWLEDGMENTS

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