Regulator of G Protein Signaling 6 (RGS6) Induces Apoptosis via a Mitochondrial-dependent Pathway Not Involving Its GTPase-activating Protein Activity*

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Regulator of G protein signaling 6 (RGS6) is a member of a family of proteins called RGS proteins, which function as GTPase-activating proteins (GAPs) for Gα subunits. The role of RGS6 as a G protein GAP, the link between G protein activation and cancer, and a reduction of cancer risk in humans expressing a RGS6 SNP leading to its increased translation, we hypothesized that RGS6 might function to inhibit growth of cancer cells. Here, we show a marked down-regulation of RGS6 in human mammary ductal epithelial cells that correlates with the progression of their transformation. RGS6 exhibited impressive anti-proliferative actions in breast cancer cells, including inhibition of cell growth and colony formation and induction of cell cycle arrest and apoptosis by mechanisms independent of p53. RGS6 activated the intrinsic pathway of apoptosis involving regulation of Bax/Bcl-2, mitochondrial outer membrane permeabilization (MOMP), cytochrome c release, activation of caspases-3 and -9, and poly(ADP-ribose) polymerase cleavage. RGS6 promoted loss of mitochondrial membrane potential (ΔΨm) and increases in reactive oxygen species (ROS). RGS6-induced caspase activation and loss of ΔΨm was mediated by ROS, suggesting an amplification loop in which ROS provided a feed forward signal to induce MOMP, caspase activation, and cell death. Loss of RGS6 in mouse embryonic fibroblasts dramatically impaired doxorubicin-induced growth suppression and apoptosis. Surprisingly, RGS6-induced apoptosis in both breast cancer cells and mouse embryonic fibroblasts does not require its GAP activity toward G proteins. This work demonstrates a novel signaling action of RGS6 in cell death pathways and identifies it as a possible therapeutic target for treatment of breast cancer.

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2 The abbreviations used are: RGS, regulator of G protein signaling; GAP, GTPase-activating protein; DCF, dichlorofluorescein; MEF, mouse embryonic fibroblast; MOMP, mitochondrial outer membrane permeabilization; ROS, reactive oxygen species; Rb, retinoblastoma tumor suppressor protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Z, benzylxoyacarbonyl; fmk, fluoromethyl ketone; EGFP, enhanced green fluorescent protein; RGS6L, Long N-terminal form of RGS6.
Here, we discovered that RGS6 possesses powerful apoptotic actions in breast cancer cells. RGS6 apoptotic mechanisms are mediated by mitochondria and ROS and are independent of p53 as well as the GAP activity of RGS6. This work provides the first insight into how an RGS protein promotes apoptosis, thus adding to the diversity of known RGS protein functions beyond their ability to inactivate G proteins.

**EXPERIMENTAL PROCEDURES**

**Materials**—PMSF, aprotinin, leupeptin, sodium orthovanadate, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), doxorubicin, crystal violet, N-acetyl cysteine, 2’,7’-dichlorodihydrofluorescein diacetate, PEG-sugar oxide dismutase, PEG-catalase, JC-1, carbonyl cyanide 3-chlorophenylhydrazone, L-N6-nitroarginine methyl ester hydrochloride, dimethylthiourea, Z-VAD-FMK, Z-LEHD-FMK, Z-DEVD-FMK, annexin V/Propidium Iodide apoptosis detection kit and antibodies for β-actin were obtained from Sigma. Cytochrome c and Bcl-2 antibodies from BD Pharmingen (San Diego, CA) and Bax antibody from BD Biosciences Corp. (Brisbane, CA). DMEM and FBS were from Invitrogen, and phospho-Rb (Ser795), cyclin D1, and cyclin E antibodies were from Cell Signaling Technology, Inc. (Danvers, MA). Kp 7–6 was from Calbiochem. The Cell Death Detection kit was from Roche Applied Science, Supersignal® West Pico chemiluminescent substrate was from Thermo Scientific, and nitrocellulose membrane was from Bio-Rad.

**RGS6 cDNAs**—Full-length cDNAs encoding various splice forms of RGS6 were amplified and cloned as described previously (14). We used RGS6 cDNAs cloned into EGFP and mCherry vectors (Clontech).

**Cell Culture and Transfection**—Human breast cancer cell lines T47D, MCF-7, and MDA-MB-231 were cultured in DMEM supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified 5% CO2 atmosphere. These cells were transiently transfected with vectors containing RGS6 or other cDNAs using a Bio-Rad Gene Pulser exactly as described previously (19). MEFs were isolated from embryonic day 14.5 RGS6+/+ and RGS6−/− mouse embryos using standard protocols. MEFs were transiently transfected with vectors containing RGS6L and RGS6LΔN401V using Lipofectamine 2000 (Invitrogen) by following the manufacturer’s protocol.

**Cell Viability**—Cell viability was measured by both MTT reduction (20) and trypan blue exclusion assays. For MTT assays, cells were transfected with EGFP control or RGS6L-EGFP cDNAs. At various times following transfection, MTT solution was added following medium removal, and cells were incubated for 6 h at 37 °C. Formazan crystals in the viable cells were solubilized with dimethyl sulfoxide, and the absorbance at 550 nm was determined. For trypan blue exclusion assays, both floating and attached cells were harvested from dishes and collected by centrifugation at 800 × g for 5 min at 4 °C. Cell pellets were resuspended in PBS and mixed with trypan blue (0.2%), and viable (unstained) and dead (stained) cells were counted using a hemocytometer.

**Apoptosis Assay**—We used the Roche cell death detection kit to quantify the extent of apoptosis in cells treated with etoposide (positive control) or transfected with EGFP or various EGFP-tagged forms of RGS6. This ELISA kit quantifies formation of cytoplasmic histone-associated DNA fragments (mono- and oligosomes) after apoptotic cell death. Results are expressed as a fold increase in enrichment factor (cytoplasmic nucleosomes).

**Annexin V Assay**—Phosphatidylserine exposure in the outer membrane, an early event in apoptosis, was measured using an annexin V/Propidium Iodide kit (Sigma) according to the manufacturer’s instructions. Annexin positive cells
were counted using a fluorescent microscope and expressed relative to the number of total cells (% annexin-positive).

**Clonogenic Survival Assay**—Colony-forming assays were performed in MCF-7 and MDA-MB-231 cells following transfection with EGFP or RGS6L-EGFP. Twenty-four h following transfection, cells were trypsinized, plated at a density of 100 cells/well, and incubated for 10 days at 37 °C with growth medium being replaced every 3 days. Colonies were fixed with methanol and stained with 0.5% crystal violet (in methanol: water, 1:1). Results are expressed relative to the number of colonies present in EGFP transfectants.

**Cell Cycle Analysis**—Thirty-six h following transfection of MCF-7 and MDA-MB-231 cells with EGFP or RGS6L-EGFP, cells were harvested, fixed in 70% ethanol, and stored at −20 °C. Cells then were washed twice with ice-cold PBS, incubated with RNase (5 µg/ml) and the DNA intercalating dye propidium iodide. Cell cycle analysis was performed by flow cytometry using a Becton Dickinson FACScan flow cytometer. Cellular debris was excluded from analysis by raising the forward scatter threshold, and the DNA content of the nuclei was registered on a logarithmic scale.

**Measurement of Intracellular ROS Levels**—Intracellular ROS levels were estimated using the cell-permeable oxidation-sensitive probe, CM-H$_2$DCFDA (21). MCF-7 cells were transfected with mCherry or RGS6L-mCherry and grown for 36 h. Cells were then scraped from dishes, collected by centrifugation as above, and washed three times with ice-cold PBS prior to resuspension in PBS and incubation with CM-H$_2$DCFDA (5 µM) at 37 °C for 20 min. After incubation, cells were washed two times with cold PBS and lysed in PBS containing 1% Tween 20. The ROS level in lysates was determined by measurement of fluorescence of dichlorofluorescein (DCF) (excitation at 480 nm and emission at 530 nm). Intracellular hydrogen peroxide levels in MCF-7 cells were measured 36 h after transfection with EGFP or RGS6L-EGFP according to the manufacturer’s protocol.

**Measurement of Mitochondrial Membrane Potential**—Mitochondrial membrane potential ($\Delta$Ψ$_{m}$) was measured by the incorporation of cationic fluorescent dye JC-1 (5,5’$,6,6’$-tetra-chloro-1,1’$,3,3’$-tetraethyl benzimidazolylcarbo cyanine iodide) into the mitochondria as described (22). MCF-7 cells were transfected with mCherry or RGS6L-mCherry and
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grown for 36 h. Cells were harvested as above for ROS studies and incubated with shaking for 30 min at 37 °C in PBS containing IC-1 (2.5 μg/ml). Cells were then washed twice with ice-cold PBS and resuspended in PBS, and mitochondrial membrane potential was quantified by measuring the ratio of fluorescence at 590/530. Carbonyl cyanide 3-chlorophenylhydrazone was used as a mitochondrial uncoupler.

Measurement of Cytochrome c Release—To determine effects of RGS6L on cytochrome c release, cytosolic extracts were prepared as described by Shishodia et al. (23) to assure removal of mitochondria and other insoluble fragments prior to immunoblotting.

Caspase Assays and Immunoblotting—Activities of caspases-3, -8, and -9 in cellular lysates were assayed using Biovision’s caspase activity kits according to the manufacturer’s protocol. At various times following transfection with EGFP or RGS6L-EGFP, MCF-7 cells were incubated in the presence and absence of inhibitor peptides (40 μM) specific to different caspases (caspase 9, Z-LEHD-FMK; caspase 8, Z-IETD-FMK; and caspase-3, Z-DEVD-FMK) for 1 h before cell lysis. For immunoblotting, cells were lysed in lysis buffer (20 mM Tris (pH 7.4), 250 mM NaCl, 2 mM EDTA, 0.1% Triton X-100, 1 mg/ml aprotinin, 1 mg/ml leupeptin, 0.4 mM PMSF, and 4 mM NaN3VO4). Insoluble material was removed by centrifugation at 20,000 × g for 10 min at 4 °C. Immunoblotting was performed as described previously (24).

Immunohistochemistry—Formalin-fixed, paraffin-embedded breast tumor sections were obtained from the University of Iowa Department of Pathology and processed to examine expression of RGS6. Briefly, sections were dewaxed in xylene, treated with a graded series of alcohol, immersed in 3% hydrogen peroxide to block endogenous peroxide activity, blocked with 5% BSA, and then incubated overnight at 4 °C with anti-RGS6 (developed in our laboratory). Following washing (four times) in PBS for 10 min each, sections were incubated for 1 h at room temperature with peroxidase-conjugated secondary antibody. A positive reaction was detected by exposure to stable DAB for 3 min. The sections were finally counterstained in Harris hematoxylin and observed under microscope. A histo-score (H-score) was calculated by multiplying the percentage of positive cells by the average intensity. Average H-scores from 10 randomly chosen fields were used to grade RGS6 protein levels in each sample.

Statistical Analysis—Data were analyzed by paired t test and one-way analysis of variance. Results were considered significantly different at p < 0.05. Values are expressed as means ± S.E.

RESULTS

Loss of RGS6 Expression in Human Breast Carcinomas—Because of the role of G protein activation in tumorigenesis, including breast cancer, and the known activity of RGS6 as a G protein GAP, we investigated whether RGS6 expression is dysregulated in human breast cancer. Fig. 1A shows the results of immunohistochemical staining for RGS6 in normal and cancerous human breast specimens. As shown, RGS6 was expressed exclusively in ductal epithelial cells in normal mammary tissue. Expression of RGS6 in these cells was significantly lower in noninvasive carcinomas (ductal carcinoma in situ) and was dramatically lower in invasive tumors, with loss correlating to tumor grade, as shown in Fig. 1B. Thus, a reduction of RGS6 expression in mammary ductal epithelial cells apparently occurs with breast cancer disease progression.

RGS6 Inhibits Growth of Breast Cancer Cells—In view of these findings, we considered the possibility that RGS6 might normally function to suppress breast cancer cell growth or survival. We first examined effects of expression of RGS6 on the survival of a variety of human breast cancer cell lines. Expression of enhanced green fluorescent protein (EGFP)-
tagged RGS6 protein (RGS6L-EGFP) dramatically inhibited the survival of MCF-7, MDA-MB-231, and T47D cells in a time-dependent manner (Fig. 2A), whereas expression of EGFP did not reduce survival (data not shown, see Fig. 2B). This cytotoxic action of RGS6L was independent of p53 because its expression produced comparable cell killing in cells with wild-type p53 (MCF-7) and with inactive mutant p53 (MDA-MB-231, T47D). We also evaluated the ability of RGS6L to promote cell death by measurement of trypan blue exclusion. As shown in Fig. 2B, RGS6L expression promoted a time-dependent cell death in MCF-7 and MDA-MB-231 cells with ~50% cell death occurring within 48 h. We next assessed the effect of RGS6 expression on the colony-forming ability of breast cancer cells, another means of measuring reproductive cell death. Expression of RGS6L-EGFP inhibited colony formation of both MCF-7 and MDA-MB-231 cells by ~80% compared with control EGFP-transfected cells (Fig. 2, C and D). Thus, RGS6 exhibited a powerful p53-independent cytotoxic activity in human breast cancer cells.

RGS6 Induces Breast Cancer Cell Apoptosis—We next undertook experiments to delineate the mechanisms underlying the cell-killing activity of RGS6 in breast cancer cells. We first examined whether the cytotoxic actions of RGS6 were a result of the induction of apoptosis. Fig. 3A shows that expression of RGS6L-EGFP induced apoptosis in a time-dependent manner in both MCF-7 and MDA-MB-231 cells, as measured by increased cytoplasmic histone-associated DNA fragments compared with EGFP-transfected control cells. Remarkably, expression of RGS6 was equally (MDA-MB-231 cells) or more (MCF-7 cells) effective in inducing apoptosis than that promoted by treatment of cells with etoposide (25 μM), a well-known apoptosis-inducing DNA-damaging agent. We also determined effects of RGS6 on early apoptotic events, namely the translocation of phosphatidylserine to the outer cell surface that can be detected by annexin V staining. Fig. 3B shows that expression of RGS6L-mCherry produced a time-dependent increase in annexin-positive MDA-MB-231 and MCF-7 cells, which was significantly higher than that observed in mCherry control cells. Thus, as assessed by both early and late apoptosis markers, RGS6 strongly induces apoptosis in both noninvasive p53 wild-type MCF-7 cells and invasive p53 mutant MDA-MB-231 cells.

RGS6 Induces Cell Cycle Arrest in Breast Cancer Cells—To further characterize the growth regulatory actions of RGS6 on breast cancer cells, we performed cell cycle analysis by flow cytometry. For these studies, we examined the cell cycle profile of cells at a time point (36 h) when the growth inhibitory actions of RGS6 were becoming apparent (Fig. 2A). RGS6L expression induced a G1/S arrest of both MCF-7 and MDA-MB-231 cells as shown by the increased proportion of cells in the G1 phase and associated decreased proportion of cells in S phase compared with EGFP-transfected control cells (Fig. 4A). In keeping with these findings, expression of RGS6L in MCF-7 cells produced time-dependent decreases in phospho-Rb (Ser795) and cyclins D1 and E (Fig. 4B), cell cycle regu-
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G1/S phase arrest. These results show that the cyto-

toxic effects of RGS6L are initially associated with a cell cycle

arrest preventing their passage into the S phase of the cell

cycle.

RGS6 Induces Apoptosis by Intrinsic Apoptosis Pathways

Involving Altered Mitochondrial Function—Focusing on

MCF-7 cells, we undertook a variety of studies to elucidate

the specific mechanism by which RGS6 induced apoptosis

in breast cancer cells. We first determined that RGS6L

does not activate a Fas-dependent (extrinsic) death path-

way by showing that induction of apoptosis by RGS6L was

insensitive to treatment with Fas/Fas ligand antagonist (Kp 7–6) (Fig. 5A). This conclusion was supported by fur-

ther studies demonstrating that RGS6L does not pro-

mote activation of the extrinsic pathway-regulated caspase-8 (Fig. 5D) (25).

The intrinsic pathway of apoptosis proceeds through a

signaling cascade involving Bax activation and its targeting

to mitochondria, which results in MOMP and a release of
cytochrome c that activates the initiator caspase-9.

Caspase-9 then activates the executioner caspases-3 and -7, which induce apoptosis by cleavage of an essential complex

I subunit (NDUSF1), which causes the loss of mito-

chondrial transmembrane potential (ΔΨm) and ATP synthesis

and the generation of ROS (25). ROS can also induce

MOMP as a feed-forward mechanism in apoptosis (26).

Fig. 5B shows that expression of RGS6L-EGFP resulted in

increased activation of Bax, as shown by the increase ratio

of Bax to Bcl-2, and release of cytochrome c (Cyto

C) from MCF-7 cells. No time-dependent changes were observed in EGFP transfectants so only one time point is shown (48 h). β-Actin is shown as a loading control. Results are representative of three experiments. C, a pan-caspase inhibitor inhibits RGS6L cytotoxicity. MCF-7 cell transfection and survival was measured as in A. Cells were treated with Z-VAD-FMK (1 h) 12 h after transfection. Cell survival is expressed as percent of EGFP control cells. Results are means ± S.E. of three experiments (*, p < 0.001 compared with EGFP control).

D, RGS6L promotes activation of caspases-3 and -9 in MCF-7 cells. Cells were treated with inhibitors specific to each caspase (40 μM for 1 h) 12 h after transfection with EGFP or RGS6L-EGFP, and activity measurements were made for each caspase as described under “Experimental Procedures.” Results are means ± S.E. of three experiments (*, p < 0.05; **, p < 0.001 compared with EGFP control). E, effects of inhibitors to caspase-9 (LEHD), -3 (DEVD), and -8 (IETD) on RGS6L-induced cytotoxicity. Inhibitors were added as in D, and survival was measured as in C. Results are means ± S.E. of three experiments (*, p < 0.01 compared with untreated cells). F, RGS6L promotes poly(ADP-ribose) polymerase (PARP) cleavage. No time-dependent changes were observed in EGFP transfectants, so only one time point is shown (48 h). Results are representative of three experiments.
caspase-9 and the executioner caspase-3 in the intrinsic pathway of apoptosis but not of caspase-8 (Fig. 5D), a caspase activated by Fas-dependent death pathways. As expected, RGS6L-induced activation of caspases-9 and -3 were significantly reduced by inhibitors specific to each of these caspases. Brief treatment (1 h) of MCF-7 cells with either caspase-3 or caspase-9 but not caspase-8 inhibitors, significantly improved survival in RGS6L-expressing MCF-7 cells (Fig. 5E). A hallmark of apoptosis is DNA damage and the ability of poly(ADP-ribose) polymerase to

**FIGURE 6.** ROS generation contributes to RGS6L-induced apoptosis in MCF-7 cells. A, RGS6L promotes generation of ROS in MCF-7 cells, which is blocked by scavengers of superoxide and hydrogen peroxide. MCF-7 cells were transfected with mCherry or RGS6L-mCherry and were incubated with and without different ROS scavengers (1 h) 12 h after transfection. ROS was measured by CM-H$_2$DCFDA fluorescence 24 h later as described under “Experimental Procedures.” Results are means ± S.E. of three experiments (†, p < 0.001 compared with mCherry control; *, p < 0.05; **, p < 0.01; ***, p < 0.001 compared with RGS6L-mCherry). B, RGS6L promotes production of hydrogen peroxide in MCF-7 cells. Measurements of hydrogen peroxide were made 36 h following transfection with EGFP or RGS6L-EGFP as described under “Experimental Procedures.” Results are means ± S.E. of eight measurements for EGFP and RGS6L-EGFP transfectants (*, p < 0.001 versus EGFP-transfected control cells). C, effects of ROS scavengers on RGS6L-induced apoptosis in MCF-7 cells. ROS scavenger treatments were as in A, and formation of cytoplasmic histone-associated DNA fragments was measured 48 h following transfection. Results are means ± S.E. of three experiments (#, p < 0.001 compared with mCherry transfected cells; *, p < 0.05; **, p < 0.01; ***, p < 0.001 compared with RGS6L-mCherry transfected cells. D, effects of ROS scavengers on RGS6L-induced cytotoxicity in MCF-7 cells. ROS scavenger treatments were as in A, and cell survival was measured 48 h following transfection by MTT assay. Results are means ± S.E. of three experiments (*, p < 0.05; **, p < 0.01; ***, p < 0.001 compared with RGS6L-mCherry transfected cells. E, effects of ROS scavengers on RGS6L-induced activation of caspase-9 and -3 in MCF-7 cells. Treatment with N-acetyl cysteine was as in A, and caspase activity was measured 48 h following transfection with EGFP or RGS6L-EGFP. F, effects of ROS scavengers on RGS6L-induced loss of mitochondrial membrane potential (Δψ$_{m}$) in MCF-7 cells. ROS scavenger treatments were as in A in RGS6L-mCherry transfectants. Untransfected cells were treated with carbonyl cyanide 3-chlorophenylhydrazone (CCCP, 1 μM for 1 h) at the same time point. Cells were harvested 36 h following transfection, and mitochondrial membrane potential was measured as described under “Experimental Procedures.” Results are means ± S.E. of three experiments (*, p < 0.01; ***, p < 0.001 versus mCherry control; †, p < 0.001 versus RGS6L-mCherry).
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mediate such repair is prevented by its cleavage by caspase-3 (27). Fig. 5F shows that expression of RGS6L in MCF-7 cells markedly increased poly(ADP-ribose) polymerase cleavage, confirming a robust activation of caspase-3 in response to RGS6L expression. Together, these experiments demonstrate that RGS6L-induced cytotoxicity is mediated by a mechanism involving activation of Bax, MOMP, and cytochrome c-mediated activation of initiator caspase-9 resulting in activation of the executioner caspase-3.

ROS Generation Contributes to RGS6L-induced Apoptosis—Because the intrinsic pathway of apoptosis ultimately leads to mitochondrial dysfunction and ROS production, which can induce further mitochondrial dysfunction and apoptosis, we assessed the role of ROS in RGS6L-induced apoptosis. Fig. 6A shows that RGS6L induced significant increases in ROS, as measured using a ROS-sensitive fluorophore. RGS6L-induced increases in ROS were markedly reduced by treatment of cells with a general ROS scavenger N-acetyl cysteine (10 mM) and an enzymatic scavenger for hydrogen peroxide (PEG-catalase, 1000 units/ml), slightly reduced by the superoxide scavenger (PEG-SOD, 200 units/ml), and unaffected by scavengers for peroxynitrite (L-NAME, 1 mM) or hydroxyl radicals (DMTU, 1 mM). These findings are consistent with an RGS6-induced mitochondrial dysfunction because mitochondria-generated superoxide is rapidly converted to hydrogen peroxide in cells. Fig. 6B confirms these findings by demonstrating that RGS6L-induced significant increases in cellular hydrogen peroxide levels. To determine the role of RGS6L-induced ROS in its cytotoxic actions, we examined the effects of these ROS scavengers on MCF-7 cell survival and apoptosis. Indeed, scavengers of superoxide and hydrogen peroxide significantly attenuate RGS6L-induced apoptosis (Fig. 6C) and increase cell survival (Fig. 6D). Moreover, RGS6L-induced activation of caspase-9 and -3 were significantly attenuated by cellular incubation with N-acetyl cysteine (Fig. 6E). Lastly, we determined whether RGS6-induced ROS functions in a feed-forward mechanism of mitochondrial dysfunction that has been described (26). Fig. 6F shows that RGS6L-induced loss of mitochondrial membrane potential, a hallmark of MOMP, was blocked by treatment of cells with either N-acetyl cysteine or PEG-catalase. Together these results suggest a mechanism whereby RGS6L-induced alterations in mitochondrial function, likely from MOMP resulting from alterations in Bax activity, lead to ROS generation, which contributes to feed-forward mechanism of mitochondrial dysfunction and caspase-mediated apoptosis.

RGS6L-induced Apoptosis Does Not Require Its GAP Activity—We next assessed whether the ability of RGS6L to induce apoptosis was dependent upon its GAP activity toward G proteins. We assessed the effect of mutating a critical Asn in RGS6L (Asn401), required for G protein interaction with and GAP activity toward Ga subunits (28), on RGS6L-induced apoptosis. We showed previously that RGS6L\textsuperscript{N401V} is incapable of interacting with Ga\textsubscript{11} (29). We found that RGS6L\textsuperscript{N401V} was as effective as RGS6L in both increasing cytoplasmic histone-associated DNA fragments (Fig. 7A) and activating caspases-9 and -3 (Fig. 7B). These results demonstrate that the ability of RGS6 to induce apoptosis does not require its GAP activity toward G proteins.

Loss of RGS6 Impairs Growth Suppression and Apoptosis—To further prove the role of RGS6 in regulating cellular growth and apoptosis, we used MEFs isolated from RGS6\textsuperscript{+/−} and RGS6\textsuperscript{−/−} mice. When these cells were treated with doxorubicin, a well known chemotherapeutic agent that induces DNA damage and apoptosis, RGS6\textsuperscript{+/−} MEFs showed resistance to doxorubicin-induced growth suppression (Fig. 8A) and apoptosis (Fig. 8B) as compared with RGS6\textsuperscript{−/−} MEFs. Consistently, expression of wild-type RGS6 (RGS6L-EGFP) and GAP-deficient mutant (RGS6L\textsuperscript{N401V}) rendered growth suppression (Fig. 8C) as well as apoptosis (Fig. 8D) in RGS6\textsuperscript{−/−} MEFs. These findings demonstrate the antiproliferative property of RGS6.

DISCUSSION

These results demonstrate an entirely novel signaling action of RGS6 in growth suppression and apoptosis induction in human breast cancer cells. RGS6 activated intrinsic apoptosis pathways in which ROS generation play a key role in the mitochondrial permeability transition and caspase activation. Importantly, the ability of RGS6 to promote apoptosis is independent of its GAP activity toward G proteins, defining a noncanonical role of RGS6 in cell death pathways. This study also provides new evidence that loss of RGS6 expression correlates with progression of human breast cancer. Together, these observations identify RGS6 as a possible therapeutic target for treatment of...
breast cancer in humans, which remains a leading cause of death in women (30).

The possibility that RGS6 might possess antiproliferative actions was first suggested by the finding that a SNP in RGS6 that increased its translation 3-fold was associated with significant reduction in the risk of bladder cancer in humans (18). This observation provided the first link between RGS proteins and cancer. It is surprising, given the evidence that G protein-coupled receptors and G proteins are crucial players in tumorigenesis (15), that there has been limited study of the effects of RGS proteins on cell proliferation and survival. In fact, we are aware of only two reports in which RGS proteins have been linked to changes in cell proliferation, i.e. RGS17 was found to stimulate growth of lung cancer cells (31) and RGS16 was found to inhibit epidermal growth factor receptor-stimulated growth of breast cancer cells by binding to a subunit of PI3K (32), an activity of RGS16 not involving G proteins. Likewise, a single report by Dulin et al. (33) showed that expression of RGS3T in CHO cells increased the number of TUNEL-positive cells observed in response to serum withdrawal. This effect was ascribed to the unique N terminus of RGS3T and not to its G protein-regulating RGS domain.

Here, RGS6, a member of the R7 family of RGS proteins, was found to possess an impressive ability to induce death of breast cancer cells, apparently via its ability to induce cell cycle arrest and apoptosis. It is not surprising that we have found that RGS6 expression is low or absent in rapidly dividing and malignant cells as would be expected from its powerful antiproliferative activities described here. Indeed, we have been unable to establish stable cell lines expressing RGS6, which is consistent with our present finding that expression of RGS6 almost completely blocked colony formation by breast cancer cells. RGS6 induces growth arrest by mechanisms that appear to be p53-independent as it had indistinguishable effects on cell survival, cell cycle arrest and apoptosis in MCF-7 and MDA-MB-231 cells, which express wild-type and an inactive mutant of p53, respectively. Our results show that RGS6 induces G1/S phase arrest, a finding consistent with the observed decreases in phosphorylated Rb and expression of cyclins D1 and E, all of which are required for transition from the

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G₁ to the S phase of the cell cycle. Thus, cyclin D-Cdk4/6 promotes mid-G₁ phase progression, cyclin E-Cdk2 promotes the G₁/S phase transition, and Cdk4/6-mediated phosphorylation of Rb prevents its association and inhibitory effects on E2F transcription factors that activate genes for cell cycle regulatory proteins (e.g. cyclin E) and proteins needed for DNA replication in S phase (34–39).

Our findings provide the first mechanistic insight into how a member of the RGS protein family induces cell death. We have shown that RGS6 activates the intrinsic pathway of apoptosis involving regulation of Bax/Bcl-2, MOMP, cytochrome c release, and activation of caspases-9 and -3. We showed that RGS6 promoted loss of mitochondrial transmembrane potential, known to be mediated by caspase activation, and increases in ROS. Using agents to sequester various ROS, we found that RGS6-mediated apoptosis was mediated by increases in superoxide anion and its metabolite hydrogen peroxide, which was shown to be increased by RGS6. In addition, we found that the ability of RGS6 to promote loss of mitochondrial transmembrane potential and to activate caspases-9 and -3 was mediated by these ROS. These results are entirely consistent with an amplification loop in which ROS provide a feed-forward signal to induce mitochondrial membrane potential changes leading to MOMP, cytochrome c release, and caspase activation as recently reviewed (26). The mechanisms underlying activation of the proapoptotic Bax/Bak proteins that are essential for MOMP are complex and involve conformational changes, mitochondrial translocation and possible interactions with BH-3-only proteins (25), which themselves are regulated by phosphorylation and transcription. Although the precise mechanisms by which Bax activation occurs are not fully known, our results are consistent with RGS6-induced changes in Bax/Bcl-2 as a triggering event in its apoptotic actions.

Our results show that RGS6 induces cell death by a non-canonical mechanism that is independent of its ability to inactivate G proteins. We found that loss of RGS6 impairs doxorubicin-induced growth suppression and apoptosis in MEFs and that RGS6L\textsuperscript{2401V}, a mutant of RGS6 that does not interact with or function as a GAP for G proteins, was equally as active as wild-type RGS6 in its induction of apoptosis and caspase activation. Thus, although we hypothesized that RGS6 might possess antiproliferative activity by virtue of its ability to suppress G protein activation, RGS6 promoted apoptosis by a mechanism that does not depend upon its ability to inhibit G proteins. Because some members of the RGS protein family can modulate G protein signaling indirectly by forming G protein-coupled receptor signaling complexes (40) or by inhibiting the G protein effectors phospholipase Cβ and adenyl cyclase (7, 41), our results do not exclude an effect of RGS6 on G protein signaling per se. On the other hand, RGS6 may activate cell death pathways by mechanisms beyond its ability to perturb G protein signaling. Indeed, we previously showed that RGS6 interacts directly via its GGL domain with neuronal differentiation factor SCG10 (29) and transcriptional repressor DMAP1 (17) to induce neuronal differentiation and to inhibit DMAP1 transcriptional repression, respectively. Future work will focus on resolving this question as well as identifying the structural elements of RGS6 that are required for its cytoxic activity.

In summary, this work identifies a novel signaling action of RGS6 in mitochondrial and ROS-mediated cell death pathways as well as in cell cycle arrest of breast cancer cells that occurs by p53-independent mechanisms. Our findings broaden our understanding of the functions of RGS proteins to include powerful proapoptotic actions mediated by mechanisms beyond their canonical roles as GAPs for G proteins. These findings identify RGS6 as a possible therapeutic target for treatment of breast cancer, where we found dramatic down-regulation of RGS6 expression.

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