Identification of a Protein, G0S2, That Lacks Bcl-2 Homology Domains and Interacts with and Antagonizes Bcl-2

Christian Welch,1 Manas K. Santra,1 Wissal El-Assaad,2 Xiaochun Zhu,1 Wade E. Huber,1 Richard A. Keys,1 Jose G. Teodoro,2 and Michael R. Green1

1Howard Hughes Medical Institute, Programs in Gene Function and Expression and Molecular Medicine, University of Massachusetts Medical School, Worcester, Massachusetts and Goodman Cancer Centre and Department of Biochemistry, McGill University, Montreal, Quebec, Canada

Abstract

The Bcl-2 family of proteins consists of both antiapoptotic and proapoptotic factors, which share sequence homology within conserved regions known as Bcl-2 homology domains. Interactions between Bcl-2 family members, as well as with other proteins, regulate apoptosis through control of mitochondrial membrane permeability and release of cytochrome c. Here we identify a novel regulator of apoptosis that lacks Bcl-2 homology domains but acts by binding Bcl-2 and modulating its antiapoptotic activity. To identify regulators of apoptosis, we performed expression profiling in human primary fibroblasts treated with tumor necrosis factor-α (TNF-α), a potent inflammatory cytokine that can regulate apoptosis and functions, at least in part, by inducing expression of specific genes through NF-κB. We found that the gene undergoing maximal transcriptional induction following TNF-α treatment was G0S2, switch gene 2 (G0S2), the activation of which also required NF-κB. We show that G0S2 encodes a mitochondrial protein that specifically interacts with Bcl-2 and promotes apoptosis by preventing the formation of protective Bcl-2/Bax heterodimers. We further show that ectopic expression of G0S2 induces apoptosis in diverse human cancer cell lines in which endogenous G0S2 is normally epigenetically silenced. Our results reveal a novel proapoptotic factor that is induced by TNF-α through NF-κB and that interacts with and antagonizes Bcl-2. [Cancer Res 2009;69(17):6782–9]

Introduction

Apoptosis has been implicated in a variety of biological processes including normal development, tissue homeostasis, and defense against pathogens (reviewed in ref. 1). Over the past decade, interest in apoptotic mechanisms has been greatly stimulated by the discovery that deregulation of cell death contributes to several human pathologies including cancer, autoimmune disorders, and degenerative diseases (reviewed in refs. 2, 3).

A key component of the apoptotic machinery is a proteolytic system involving a family of cysteine proteases called caspases. Two pathways leading to caspase activation have been characterized: the extrinsic pathway, which involves the so-called "death receptors," and the intrinsic pathway, which involves the release of proapoptotic proteins from the mitochondria. Commitment to apoptosis is governed by proteins of the Bcl-2 family, which are defined by the inclusion of one or more Bcl-2 homology domains (reviewed in refs. 2, 4). The BH3 domain, for example, is a potent "death domain" that is critical for heterodimerization with other Bcl-2 family proteins. The Bcl-2 family includes both antiapoptotic members, such as Bcl-2 and Bcl-xL, and proapoptotic members that either resemble Bcl-2 (e.g., Bak and Bad) or bear only the BH3 interaction domain (e.g., Bax, Bid, and Noxa). Although the precise mechanism by which Bcl-2 proteins function is under investigation, it is clear that they regulate the release of proteins from the mitochondria that induce apoptosis.

A well-studied example of the extrinsic pathway is that induced by the inflammatory cytokine, tumor necrosis factor-α (TNF-α; reviewed in ref. 5). TNF-α promotes apoptosis through binding to its cell surface receptor, TNFR1, leading to the activation of downstream signal transduction pathways. Signaling through TNFRI elicits rapid activation of two major heterodimeric transcription factors, activator protein-1 (also called c-Jun) and NF-κB. NF-κB can either inhibit or induce apoptosis depending on cell type, extent of NF-κB activation, and nature of the apoptotic signal (reviewed in ref. 6). For example, NF-κB has been shown to antagonize TNF-α–induced apoptosis by activating survival factors such as cellular FLICE inhibitory protein, cellular inhibitors of apoptosis cIAP1 and cIAP2, and TNFR-associated factors TRAF1 and TRAF2. NF-κB also up-regulates several antiapoptotic Bcl-2 family members and inhibits the intrinsic mitochondrial-dependent apoptosis pathway following DNA damage. By contrast, NF-κB can promote cell death, for example, when cells are cultured under conditions of serum withdrawal or treated with UV irradiation. Consistent with the ability of NF-κB to exhibit both proapoptotic and antiapoptotic activities, NF-κB does not always function as an oncogene and in some cases can act as a tumor suppressor (6).

Based on these considerations, we hypothesized the existence of novel, NF-κB–dependent, TNF-α–inducible modulators of apoptosis. Here we perform expression profiling in human primary fibroblasts to identify new TNF-α target genes that encode regulators of apoptosis.

Materials and Methods

Expression profiling. Early (<10)–passage primary foreskin fibroblasts (PFF; American Type Culture Collection) were treated with 25 ng/mL TNF-α or PBS for 16 h, and poly(A)+ mRNA was isolated using an Oligotex Direct mRNA Midi Kit (Qiagen). mRNA (2 μg) was used to generate cDNA using an oligo(dT) T7 primer and Superscript cDNA Synthesis Kit (Invitrogen).

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Requests for reprints: Jose G. Teodoro, Goodman Cancer Centre, McGill University, 364 Plantation Street, Worcester, MA 01605. Phone: 514-398-3273; Fax: 514-398-6769; E-mail: jose.teodoro@mcgill.ca or Michael R. Green, Molecular Medicine, University of Massachusetts Medical School, 1160 Pine Avenue, Room 616, Montreal, Quebec, Canada H3A 1A3. Phone: 514-988-7699; Fax: 514-988-6769; E-mail: michael.green@umassmed.edu.

©2009 American Association for Cancer Research. doi:10.1158/0008-5472.CAN-09-0128

Cancer Res 2009; 69: (17). September 1, 2009 6782 www.aacrjournals.org

Downloaded from cancerres.aacrjournals.org on April 13, 2017. © 2009 American Association for Cancer Research.
The cDNA was in vitro transcribed using the T7 Megascript Kit (Ambion). The resulting cRNA was fragmented by heating at 94°C for 30 min and hybridized against Affymetrix Hu6800 and Hu35K microarrays, which were scanned and analyzed using Affymetrix Microarray Suite software. Raw data have been submitted to the Array Express repository.1

**G0S2 expression analysis.** For Northern blots, 1 μg poly(A) mRNA was loaded per lane and hybridized with a random primer-labeled probe for G2G1 switch gene 2 (G0S2) or glyceroldehyde-3-phosphate dehydrogenase (GAPDH). For immunoblotting, PFFs were treated with TNF-α, and 16 h later, whole-cell extracts were made by lysing cells in 1× Laemmli buffer. Blots were probed with a G0S2 rabbit polyclonal antibody (generated by Covance Laboratories by immunizing rabbits with purified, recombinant His-tagged G0S2 produced in Escherichia coli) or tubulin monoclonal antibody (Sigma) followed by the appropriate horseradish peroxidase–conjugated α-Ig secondary antibody (Amersham Biosciences).

Reverse transcription PCR analysis. PFFs were infected with Ad-LacZ or Ad-I-B-SR and treated with TNF-α (25 ng/mL) or PBS. Twenty-four hours later, total RNA was isolated using Trizol reagent (Invitrogen) and used to perform reverse transcription PCR (RT-PCR). For the demethylation experiments, cells were treated with 5-aza-2′-deoxycytidine and trichostatin A as described previously (7).

Plasmid and adenovirus construction. G0S2 was cloned by RT-PCR amplification using poly(A) mRNA isolated from TNF-α–treated PFFs. The PCR product, including the sequence for the hemagglutinin (HA) epitope, was subcloned into pcDNA3-HA (Invitrogen) to generate pcDNA-G0S2-HA. Plasmids expressing enhanced green fluorescent protein (EGFP)-G0S2 and G0S2-enhanced yellow fluorescent protein (EYFP) were derived by subcloning the G0S2 sequence from pcDNA3-G0S2-HA into pEGFP-C1 (Clontech) or pEYFP (Clontech), respectively. To generate GFP-tagged G0S2 deletion mutants, truncated G0S2 sequences were PCR amplified and cloned into pEGFP-C1. Point mutations in G0S2 were generated using the QuikChange Mutagenesis Kit (Stratagene) using pcDNA3-G0S2-HA as the template. All clones were confirmed by restriction digest analysis and DNA sequencing.

To construct plasmids expressing NH2-terminal FLAG-tagged Bcl-2 family members, the corresponding cDNAs (obtained from Stanley Korsmeyer) were PCR amplified and cloned into pBFLAG-myc-CMV-26 (Sigma). To construct the plasmid expressing Bax-V5, Bax cDNA was PCR amplified and cloned into pcDNA4-V5/V5HisA (Invitrogen). Plasmids expressing Bcl-2-enhanced cyan fluorescence protein (ECPF) and Bad-ECFP were generated by cloning PCR-amplified cDNAs into pECFP-C1. Adenovirus vectors expressing wild-type G0S2 (Ad-G0S2) or the G0S2(R57A,D58A) mutant, both containing a single HA epitope at the COOH terminus, were generated using the AdEasy XL Adenoviral Vector System (Stratagene). Adenovirus vectors Ad-LacZ (8) and Ad-I-B-SR (9) have been described previously. Target cells were infected with adenovirus vectors at ~80% confluence at a multiplicity of infection of 35 plaque-forming units/cell.

**Immunofluorescence and fluorescence microscopy.** H1299 cells (American Type Culture Collection) were transfected using FuGENE 6 transfection reagent (Roche). Thirty-six hours later, cells were fixed in 4% paraformaldehyde (in PBS), permeabilized in 0.5% Triton X-100 (in PBS), and stained with an α-HA monoclonal antibody (Sigma) followed by α-mouse Ig Texas red–conjugated secondary antibody (Jackson Laboratories) or Mitotracker (Molecular Probes/Invitrogen). Cells were visualized with a Zeiss Axioshot2 fluorescence microscope using Axiosvision 3.1 software.

**Biochemical fractionation.** Mitochondria were isolated from TNF-α–treated PFFs using the ApoAlert Cell Fractionation kit (Clontech). Immunoblotting was done using antibodies to G0S2, Aurora A (Novus Biologicals), and cyclooxygenase IV (BD Clontech).

**Coimmunoprecipitations.** For cotransfection experiments, ~2 × 107 cells were cotransfected with appropriate plasmids, and 24 h later, cells were harvested and lysed in 1% CHAPS as described previously (10). Following centrifugation, supernatants were incubated with 20 μL equilibrated EZview Red α-HA beads or α-FLAG M2 affinity beads (Sigma) for 4 h at 4°C. Beads were washed three times in 1% CHAPS and bound

Figure 1. G0S2 is a NF-κB–dependent downstream target of TNF-α that encodes a mitochondrial protein. A, G0S2 expression was analyzed by Northern blot (left) or immunoblotting (right) in TNF-α–treated PFFs. GAPDH and tubulin were monitored as loading controls. B, RT-PCR analysis monitoring G0S2 expression in TNF-α–treated PFFs infected with Ad-LacZ or Ad-I-B-SR. C, top, immunofluorescence of G0S2-HA–expressing H1299 cells stained with an α-HA antibody (left) or Mitotracker (middle). Right, merged images. Bottom, immunofluorescence of H1299 cells expressing EGFP-G0S2 or empty vector (EGFP-C1). Cells were visualized for GFP (left) or Mitotracker (middle). D, biochemical fractionation. Whole-cell extracts prepared from TNF-α–treated PFF cells were separated into mitochondrial (Mito) and cytoplasmic (Cyto) fractions and immunoblotted for G0S2, Aurora A (a cytoplasmic protein), or cyclooxygenase IV (COX IV; a mitochondria marker).
proteins were eluted. Immunoprecipitated material and whole-cell extracts were blotted and probed with α-FLAG M2 (Sigma), α-HA (Sigma), α-EGFP (Clontech), or α-V5 (Invitrogen) monoclonal antibodies. For adenovirus experiments, HeLa cells (American Type Culture Collection) were infected, and 24 h later, mitochondria were isolated as described above and lysed in 1% CHAPS. G0S2 was immunoprecipitated using EZview Red α-HA beads, and immunoblotting was done using α-Bcl-2 (BD Pharmingen) and α-HA monoclonal antibodies.

GST pull-down assays. In vitro translation of G0S2 was done using the T7 TnT Quick-Coupled In vitro Translation System (Invitrogen) using the plasmid pcDNA3-G0S2-HA. The GST-Bcl-2 fusion protein, in which the NH2-terminal domain (amino acids 1-20) of Bcl-2 was deleted, was expressed in E. coli from pGEX-4T-1 (Amersham) as described previously (11). GST or GST-Bcl-2 (1 μg) immobilized on 10 μL glutathione-Sepharose was incubated with 10 μL in vitro translated G0S2 for 4 h at 4°C in GST lysis buffer. Pull-downs were washed four times with lysis buffer [50 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, 0.5 mmol/L DTT, 0.05% Triton X-100], analyzed by SDS-PAGE, and visualized using a Fujifilm FLA-5000 PhosphorImager system.

Fluorescence resonance energy transfer. Twenty-four hours after cotransfection, HCT116 cells (American Type Culture Collection) were transfected to poly-lysine–coated glass slides and examined using a Leica confocal laser scanning microscope system (TCS SP2 AOBS; Leica Microsystems). The 458 nm laser-line was used to excite the focused cells, and the images were captured with a liquid-cooled CCD camera. Digitized microscope images were analyzed to select a region of interest in a single cell to optimize the signal-to-noise ratio. Spectra were recorded from three cells and the spectra were averaged and plotted. To record ECFP emission spectra, the ECFP donor in the selected region of interest of a single cell was excited with the 405 nm laser-line to avoid leak-through of ECFP emission. Spectra, the ECFP donor in the selected region of interest of a single cell was excited with the 405 nm laser-line to avoid leak-through of ECFP emission. Spectra were recorded from three cells and the spectra were averaged and plotted. To record EYPF emission spectra, the EYPF fluorophore was excited by the 488 nm laser-line.

Figure 2. G0S2 directly interacts with the antiapoptotic protein Bcl-2. A, HEK293 cells were cotransfected with plasmids expressing G0S2-HA and a FLAG-tagged Bcl-2 protein. G0S2 was immunoprecipitated using an α-HA antibody, and the coimmunoprecipitate (co-IP) was analyzed by immunoblotting with an α-FLAG antibody. The presence of G0S2 and Bcl-2 member was also monitored in the whole-cell extract (WCE). B, G0S2 was immunoprecipitated from the mitochondrial fraction of Ad-G0S2– or Ad-LacZ–infected HeLa cells using an α-HA antibody, and the immunoprecipitate was analyzed using an α-Bcl-2 antibody. C, GST pull-down assays using purified GST-tagged Bcl-2 and 35S]methionine-labeled G0S2. D, fluorescence resonance energy transfer (FRET) analysis. Fluorescence emission spectra in HCT116 cells coexpressing G0S2-EYPF and either Bcl-2-ECFP or Bad-ECFP. Arrow, peak of fluorescence resonance emission at 525 nm (fluorescence resonance energy transfer signal).

Apoptosis assays. Cells were stained using the Annexin V-PE Apoptosis Detection Kit-I (BD Pharmingen) and analyzed using a Guava Personal flow cytometer (Guava Technologies). UV treatment of HeLa/Bcl-2 and HeLa/Neo cells (generated by stable transfection with plasmids expressing FLAG-Bcl-2 or empty vector [pXFLAG-myc-CMV-26; Sigma]) was done by irradiating cells for 30 s in a UV Stratalinker (Stratagene). For luciferase-based assays, H1299 cells were cotransfected with pcDNA3-luciferase (12) and appropriate plasmids. For experiments in primary cells, PFs were treated with camptothecin in the presence or absence of TNF-α (25 ng/mL). After 72 h, cells were trypsinized, washed with cold PBS three times, stained, and analyzed as described above.

RNA interference. PFF cells were stably transfected with a G0S2 short hairpin RNA (source location V2LHS-114719) or a nonsilencing short hairpin RNA (5′-TCTCGCTTGCCGAGAATG-3′) obtained from Open Biosystems.

Analysis of G0S2 expression in tumor samples. Human samples of normal lung, adenocarcinoma, and squamous cell carcinoma were obtained from the University of Massachusetts Cancer Center Tissue Bank. Gene expression levels were analyzed by quantitative RT-PCR using Platinum SYBR Green qPCR SuperMix-UDG with Rox (Invitrogen) and the following primers: G0S2 (forward 5′-GCGGCTGAGGACTGTTG-3′ and reverse 5′-CTTGGTCCTCGAGGACCTG-3′) and GADD42 (forward 5′-GGTGACCACTGTTAGCCG-3′ and reverse 5′-GCAGTGACCTGTTGTCATGAG-3′). For each sample, GADD42 provided an internal normalization control. The average G0S2 expression level in the 10 normal samples was assigned as 1.

Results

Identification of G0S2 as a NF-κB–dependent downstream target of the TNF-α pathway. To identify transcriptional targets downstream of TNF-α, we used high-density cDNA microarrays to compare the expression profiles of human PFs treated in the
presence or absence of TNF-α. Of the 33,000 genes represented on the microarray, 138 showed increased expression and 27 showed decreased expression by $\geq 4$-fold on TNF-α treatment (Supplementary Table S1). As expected, this approach identified numerous genes known to be induced by TNF-α including TNFAIP2, TNFAIP3, and TNFAIP6 (13) as well as VCAM1 (14) and PTSG2 (also known as COX2; ref. 15). The gene most highly induced following TNF-α treatment was G0S2, which encodes a protein of unknown function. Northern blot analysis indicated that G0S2 is widely expressed in normal human tissues and is present at particularly high levels in peripheral blood, skeletal muscle, and heart (Supplementary Fig. S1).

To verify the results of the microarray experiment, we analyzed G0S2 mRNA and protein levels in PFFs following TNF-α treatment. The Northern blot analysis indicated that G0S2 is widely expressed in normal human tissues and is present at particularly high levels in peripheral blood, skeletal muscle, and heart (Supplementary Fig. S1).

Figure 3. G0S2 induces apoptosis in transformed cells dependent on interaction with Bcl-2. A, H1299 and HCT116 cells were infected with Ad-G0S2 or Ad-LacZ or mock-infected, and cell death was monitored by Annexin V-PE staining. B, top, schematic representations of GFP-tagged G0S2 deletion mutants; bottom, H1299 cells were cotransfected with plasmids expressing FLAG-Bcl-2 and GFP-tagged G0S2 deletion mutants. Bcl-2 was immunoprecipitated with an α-FLAG antibody and the immunoprecipitate was analyzed with an α-GFP antibody. Also shown are the levels of GFP-tagged G0S2 and FLAG-Bcl-2 in WCE. C, top, schematic diagram showing point mutations constructed in the central region of G0S2; bottom, H1299 cells were cotransfected with plasmids expressing FLAG-Bcl-2 and a HA-tagged G0S2 deletion mutant. G0S2 was immunoprecipitated with an α-HA antibody and the immunoprecipitate was analyzed with an α-FLAG antibody. D, apoptosis assays. H1299 cells were infected with Ad-LacZ, Ad-G0S2, or Ad-G0S2(R57A,D58A) and monitored for apoptosis. Inset, immunoblot showing the G0S2 mutant was expressed at levels comparable with the wild-type protein. Bars, SD.

G0S2 is a mitochondrial protein. As a first step toward elucidating the function of G0S2, we determined its subcellular localization. H1299 non–small cell lung cancer cells were transfected with a plasmid encoding a COOH-terminal HA-tagged version of G0S2 (G0S2-HA) and the protein was detected by indirect immunofluorescence using an α-HA antibody. Figure 1C (top left) shows that G0S2-HA displayed a staining pattern that was characteristic of the mitochondria, which was confirmed by colocalization with the mitochondrial dye Mitotracker (Fig. 1C, top middle and right). The localization pattern was not a consequence of the COOH-terminal epitope tag, as a NH2-terminal EGFP-tagged version of G0S2 also localized to the mitochondria (Fig. 1C, bottom).
Biochemical fractionation experiments and subsequent immunoblot analysis using an α-G0S2 antibody confirmed that endogenous G0S2 was present in the mitochondria (Fig. 1D).

**G0S2 directly interacts with the antiapoptotic protein Bcl-2.** The mitochondrial localization of G0S2, in conjunction with the well-established role of NF-κB in apoptosis, prompted us to test whether G0S2 interacts with one or more members of the Bcl-2 family. HEK293 embryonic kidney cells were cotransfected with plasmids expressing G0S2-HA and a FLAG-tagged Bcl-2 family member. G0S2-HA was immunoprecipitated using an α-HA antibody and the immunoprecipitate was analyzed for the Bcl-2 family member by immunoblotting with an α-FLAG antibody. Figure 2A shows that, of the proteins tested, only Bcl-2 was readily detectable in the G0S2 immunoprecipitate. Significantly weaker interactions were also detected between G0S2 and the antiapoptotic proteins Bcl-xL and Mcl-1, which were evident only on overexposure of the autoradiogram (data not shown).

To confirm the association between G0S2 and Bcl-2, HeLa cells were infected with Ad-G0S2 (in which G0S2 was epitope tagged at the COOH terminus with HA), and G0S2 was immunoprecipitated from the mitochondrial fraction using an α-HA antibody and the immunoprecipitate was analyzed for endogenous Bcl-2 using an α-Bcl-2 antibody. Figure 2B shows that Bcl-2 was present in the immunoprecipitate from Ad-G0S2–infected cells but not from control cells infected with Ad-LacZ. *In vitro* GST pull-down assays using purified GST-tagged Bcl-2 and *in vitro* translated [35S]methionine-labeled G0S2 confirmed that the interaction between G0S2 and Bcl-2 was direct (Fig. 2C).

Finally, to determine whether G0S2 directly interacted with Bcl-2 *in vivo*, we used a fluorescence resonance energy transfer assay. Plasmids expressing ECFP fused to the COOH terminus of Bcl-2 or Bad (Bcl-2-ECFP or Bad-ECFP, respectively) and EYFP fused to the COOH terminus of G0S2 (G0S2-EYFP) were transiently transfected into HCT116 colorectal cells in pairwise combinations, and the cell lines were analyzed by fluorescence resonance energy transfer. The results of Fig. 2D show that a fluorescence resonance energy transfer signal was observed in cells expressing Bcl-2-ECFP and G0S2-EYFP, consistent with the results of Fig. 2A to C, but not in cells expressing Bad-ECFP and G0S2-EYFP. Collectively, the results of Fig. 2 indicate that G0S2 directly interacts with Bcl-2 both *in vitro* and *in vivo*.

**G0S2 induces apoptosis in transformed cells dependent on interaction with Bcl-2.** The above findings raised the possibility that G0S2 could have proapoptotic or antiapoptotic activity. We therefore tested whether ectopic expression of G0S2 could induce apoptosis in transformed cells. H1299 and HCT116 cells were infected with Ad-G0S2 or Ad-LacZ and 36 h later stained with Annexin V-PE. Figure 3A shows that, in both cell lines, expression of Ad-G0S2 induced pronounced apoptosis, whereas little or no cell death was observed in Ad-LacZ–infected or mock-infected cells. G0S2 expression and induction of apoptosis could be detected by 20 h post-infection and the level of apoptosis increased over 72 h (Supplementary Fig. S3).

To examine whether the ability of G0S2 to induce apoptosis and to interact with Bcl-2 were related activities, we sought to generate G0S2 mutants that were abrogated for their ability to interact with Bcl-2. We first delineated the region of G0S2 required for Bcl-2 interaction using a panel of G0S2 deletion mutants in which fragments of G0S2 were fused to the COOH terminus of GFP (Fig. 3B, top). The communoprecipitation experiments of Fig. 3B (bottom) show that only constructs harboring the central region of G0S2 (amino acids 33-67) interacted with Bcl-2.

We next constructed a series of point mutants in the central region by mutating pairs of amino acids to alanine (Fig. 3C, top). Figure 3C shows that mutations in amino acids 35 to 46 retained interaction with Bcl-2. Finally, we used a fluorescence resonance energy transfer assay (Fig. 3C, bottom) to confirm that Bcl-2 interacted with G0S2 mutants harboring mutations in amino acids 35 to 46.

**Figure 4.** G0S2 promotes apoptosis by preventing Bcl-2/Bax heterodimerization. A, HEK293 cells were cotransfected with plasmids expressing G0S2-HA, FLAG-Bcl-2, and/or V5-Bax. Bcl-2 was immunoprecipitated with an α-FLAG antibody and the immunoprecipitate was analyzed with an α-V5 antibody. B, left, HeLa cells stably expressing Bcl-2 or vector (Neo) were treated with UV; top right, HeLa/Bcl-2 and HeLa/Neo cells were infected with Ad-LacZ or Ad-G0S2 and monitored for apoptosis 48 h later; bottom right, immunoblot analysis monitoring expression of Bcl-2, G0S2, and tubulin. C, H1299 cells were cotransfected with plasmids expressing luciferase and Bcl-2, Bax, and/or G0S2. Luciferase activity was quantified and expressed relative to the levels observed in untransfected cells. Bars, SD.
G0S2 protein. However, mutations in amino acids 50 to 58 reduced interaction with Bcl-2; in particular, the G0S2 mutant derivative in which arginine 57 and aspartic acid 58 were mutated to alanine (R57A,D58A) was dramatically reduced for Bcl-2 interaction.

We then tested whether the G0S2(R57A,D58A) mutant was impaired for apoptotic activity. H1299 cells were infected with an adenovirus expressing wild-type G0S2 or the G0S2(R57A,D58A) mutant, and apoptosis was monitored by staining with Annexin V-PE. Figure 3D shows that the ability of G0S2(R57A,D58A) to induce apoptosis was significantly reduced compared with the wild-type protein. The reduced ability of G0S2(R57A,D58A) to interact with Bcl-2 and induce apoptosis was not a result of mislocalization of the protein, as the mutant protein retained mitochondrial localization (Supplementary Fig. S4). These results indicate that interaction with Bcl-2 is required for G0S2 to induce apoptosis.

**G0S2 promotes apoptosis by preventing Bcl-2/Bax heterodimerization.** We next investigated the mechanism by which G0S2 induces apoptosis. Bcl-2 mediates its survival function, at least in part, by heterodimerizing with Bcl-2 family members, such as Bax (20), and countering their proapoptotic activity. The coimmunoprecipitation experiment of Fig. 4A shows that addition of G0S2 inhibited the ability of Bcl-2 and Bax to coimmunoprecipitate, suggesting that G0S2 inhibits Bcl-2 function by preventing the formation of protective Bcl-2/Bax heterodimeric complexes.

A prediction of this model is that, in the presence of G0S2, Bcl-2 would be unable to carry out its antiapoptotic function. To test this idea, HeLa cells stably expressing either Bcl-2 (HeLa/Bcl-2) or, as a control, vector (HeLa/Neo) were infected with Ad-G0S2 or Ad-LacZ. Figure 4B shows, as expected, that overexpression of Bcl-2 protected HeLa cells from UV-induced apoptosis (left) but failed to protect HeLa cells from G0S2-mediated apoptosis (right).

As an alternative experimental approach, we used a cotransfection strategy. In this experiment, apoptosis was monitored using an assay in which luciferase activity serves as a proxy for cell death (12). H1299 cells were cotransfected with plasmids expressing luciferase and, as an apoptotic stimulus, Bax. Figure 4C shows, as expected, that expression of Bax alone induced cell death as evidenced by a dramatic reduction in luciferase activity compared with that observed in cells transfected with empty vector. Coexpression of Bcl-2 and Bax prevented the reduction in luciferase activity, consistent with previous studies showing that expression of Bcl-2 can inhibit Bax-induced apoptosis (20, 21). Significantly, expression of G0S2 together with Bax and Bcl-2 resulted in a level of luciferase activity comparable with that obtained on expression of Bax alone, indicating the antiapoptotic activity of Bcl-2 was inhibited. Collectively, the results of Fig. 4 indicate that G0S2 induces apoptosis by inhibiting the ability of Bcl-2 to form antiapoptotic Bcl-2/Bax heterodimers.

**G0S2 sensitizes primary cells to undergo apoptosis.** The finding that G0S2 could induce apoptosis in transformed cells prompted us to ask whether G0S2 also had a proapoptotic effect in primary cells. As has been observed in previous studies (see, for example, refs. 16, 22), we found that treatment of primary fibroblasts, such as PFFs, with TNF-α did not induce apoptosis (see below). However, we tested whether TNF-α treatment sensitized cells to apoptosis. Toward this end, PFFs were treated with a series of low concentrations of the DNA-damaging agent camptothecin, a known inducer of apoptosis, in the presence or absence of TNF-α. The results of Fig. 5A indicate that TNF-α treatment resulted in increased apoptosis, indicating that the net effect of TNF-α treatment is proapoptotic.

To determine whether G0S2 was responsible for the proapoptotic activity of TNF-α, we performed a RNA interference experiment. The results of Fig. 5B indicate that short hairpin RNA–mediated knockdown of G0S2 (Supplementary Fig. S5) abrogated the ability of TNF-α to increase the level of camptothecin-induced apoptosis. Thus, G0S2 is a TNF-α-inducible gene required for sensitizing PFFs to undergo apoptosis.
Finally, we asked whether ectopic expression of G0S2 was sufficient to sensitize PFFs to undergo apoptosis. PFFs were infected with Ad-G0S2 or Ad-LacZ and 24 h later challenged with various doses of camptothecin. The results of Fig. 5C show that ectopic expression of G0S2 resulted in a higher level of apoptosis, whereas little or no cell death was observed in control Ad-LacZ–infected cells. Thus, G0S2 is sufficient to sensitize primary cells to undergo apoptosis in response to DNA damage.

**G0S2 is epigenetically silenced in human cancer cell lines.** Many genes that negatively regulate cell survival are epigenetically silenced in human cancers through promoter hypermethylation (reviewed in ref. 23). Notably, the G0S2 promoter contains a CpG-rich island (24), suggesting that it may undergo epigenetic silencing. To test this hypothesis, we monitored G0S2 expression in a panel of human cancer cell lines before and after treatment with the DNA demethylating agent 5-aza-2'-deoxycytidine and the histone deacetylase inhibitor trichostatin A. The RT-PCR results of Fig. 6A show that G0S2 expression was induced by 5-aza-2'-deoxycytidine/trichostatin A treatment in all human cancer cell lines examined but not in normal PFFs. Cell lines in which G0S2 was highly induced were also analyzed for G0S2 protein by immunoblotting; as expected, in all cases, 5-aza-2'-deoxycytidine/trichostatin A treatment resulted in a substantial increase in G0S2 protein levels (Fig. 6B). These results indicate that the G0S2 gene is epigenetically silenced in a variety of human cancer cell lines.

Several of the cell lines in which G0S2 was epigenetically repressed were derived from non–small cell lung cancers. We therefore analyzed whether G0S2 expression was down-regulated in a panel of non–small cell lung tumors. The quantitative RT-PCR results of Fig. 6C indicate that G0S2 expression was down-regulated >5-fold in 70% of lung adenocarcinoma samples and 90% of lung squamous cell carcinoma samples analyzed. In addition, a search of the Oncomine cancer profiling database (25) revealed that G0S2 was frequently down-regulated in a variety of other solid tumors (Supplementary Fig. S6).

**Discussion**

Here we have shown that the G0S2 gene is induced in human primary cells by TNF-α treatment in a manner that depends on NF-κB. Moreover, we found that G0S2 induces apoptosis in human cancer cell lines and sensitizes primary cells to undergo apoptosis. G0S2 induces apoptosis by directly interacting with Bcl-2 and preventing its ability to heterodimerize with Bax.

We found that the G0S2 gene is epigenetically silenced in several human cancer cell lines and is down-regulated at high frequency in non–small cell lung cancers. Our results are consistent with those of a previous study, which found that the G0S2 promoter is hypermethylated in approximately one-third of primary head and neck squamous cell carcinomas (26). The proapoptotic activity of G0S2 and its epigenetic repression in tumors and human cancer cell lines raise the possibility that G0S2 is a tumor suppressor.
gene. Consistent with this idea, we found that short hairpin RNA–mediated knockdown of G0S2 promoted oncogene-induced transformation (Supplementary Fig. S7).

Our results fit in with several studies that have identified other TNF-α-inducible proapoptotic NF-κB targets such as TIEG and JEX-1 (27, 28). Interestingly, in some cell types, activation of NF-κB is required to mediate toxicity of cancer therapeutic agents. For example, activation of NF-κB is required for cell death induced by doxorubicin and its analogues (29, 30). Similarly, cellular resistance to vincristine has been shown to be mediated by inhibition of NF-κB activation (31). Taken together, these data suggest that in some cancer types NF-κB activation may enhance the activity of conventional chemotherapies. It may therefore be beneficial to determine if silencing of G0S2 correlates with low response of tumors to chemotherapy or poor patient outcome.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Received 1/13/09; revised 5/27/09; accepted 7/1/09; published OnlineFirst 8/25/09.

Grant support: Research in the laboratory of J.G. Teodoro is supported by Canadian Institute of Health Research grant MOP-86752. M.R. Green is an investigator of the Howard Hughes Medical Institute.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Identification of a Protein, G0S2, That Lacks Bcl-2 Homology Domains and Interacts with and Antagonizes Bcl-2


Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-09-0128

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2009/08/03/0008-5472.CAN-09-0128.DC1

Cited articles
This article cites 31 articles, 13 of which you can access for free at:
http://cancerres.aacrjournals.org/content/69/17/6782.full.html#ref-list-1

Citing articles
This article has been cited by 13 HighWire-hosted articles. Access the articles at:
/content/69/17/6782.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.