The Glucocorticoid-induced Gene tdag8 Encodes a Pro-apoptotic G Protein-coupled Receptor Whose Activation Promotes Glucocorticoid-induced Apoptosis*

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The apoptotic action of glucocorticoids on lymphocytes makes them effective therapeutics for many lymphoid malignancies. Although it is clear that glucocorticoid-induced apoptosis requires transcription, the gene products that induce apoptosis remain unknown. Using gene expression profiles of lymphoma cell lines and primary thymocytes treated with the synthetic glucocorticoid dexamethasone, we discovered that induction of tdag8 (T-cell death-associated gene 8) was a common event in each model system investigated. Activation of TDAG8 by its agonist psychosine markedly enhanced dexamethasone-induced apoptosis in a TDAG8-dependent manner. Expression of a TDAG8-GFP fusion protein was sufficient to induce apoptosis, and repression of endogenous TDAG8 using RNA interference partially inhibited dexamethasone-induced apoptosis. Together, these data suggest that TDAG8 is a regulator of glucocorticoid-induced apoptosis and that agonists of TDAG8 may be promising agents to improve the efficacy of glucocorticoids for the treatment of leukemia and lymphoma.

The fundamental mechanism of glucocorticoid-induced apoptosis presents a fascinating and important physiological question. The lytic action of adrenocorticotropic hormone (ACTH) and cortisol on lymphoid tissues and leukemia was first reported in the early 1940s and 1950s (1–3). In fact, glucocorticoid-induced apoptosis is one of the earliest described physiological models of apoptosis and is particularly important during thymocyte maturation and lymphocyte homeostasis (4–8). Despite their longstanding clinical use and physiological importance, a better understanding of the mechanism by which glucocorticoids induce apoptosis is necessary to enhance the efficacy and minimize the adverse effects of glucocorticoid therapy.

Unlike many death-promoting stimuli, glucocorticoids induce an active program of cellular suicide that requires transcriptional regulation. Inhibition of either transcription or translation blocks glucocorticoid-induced apoptosis of thymocytes, indicating that de novo RNA and protein synthesis are required (9, 10). The presence of a functional glucocorticoid receptor is also required. It was first observed that lymphocyte sensitivity to glucocorticoids was directly related to the ability of cells to selectively bind cortisol (11–13). Furthermore, glucocorticoid-resistant strains of childhood T-cell acute lymphocytic leukemia often result from diminished abundance, alternative splicing, or truncation of the glucocorticoid receptor (14–16).

Several transcriptionally regulated signaling pathways appear to be important in the initiation of glucocorticoid-induced apoptosis (reviewed in Refs. 17 and 18). These include activation of the proteasome (19, 20), generation of reactive oxygen species (21–23), and an increase in intracellular calcium (9, 24, 25). How these pathways signal the commitment of the lymphocyte to apoptosis is unclear. It is known that Bax and Bak, pro-apoptotic members of the Bel-2 family of apoptosis regulatory proteins, are critical for glucocorticoid-induced apoptosis, because thymocytes from mice lacking bax and bak are resistant to glucocorticoids (26). Once activated, Bax and Bak release cytochrome c from mitochondria, resulting in caspase activation and the subsequent degradation of cellular protein and chromatin (27, 28).

Although glucocorticoid-induced apoptosis requires transcriptional regulation by the glucocorticoid receptor and activation of Bax, the molecular events connecting these processes remain elusive. It is becoming increasingly clear that BH3-only members of the Bel-2 family play a critical role in activating Bax and Bak and often act as cellular sensors whose induction or activation are very early events in the execution phase of apoptosis (29). The BH3-only protein that is of particular interest in glucocorticoid-induced apoptosis is Bim. Bim is induced by glucocorticoids (30), is capable of activating Bax directly (29, 31), and the induction of Bim correlates with the onset of glucocorticoid-induced apoptosis (32).

Whereas glucocorticoids are capable of inducing an intrinsic form of apoptosis that does not require extracellular pro-apoptotic signals (such as Fas or tumor necrosis factor-α), environmental cues can influence the apoptotic response. Many cytokines, including interleukins 2 and 4 (33, 34), interleukin 7 (35), interferon-α (36), and activation of the T-cell receptor inhibit glucocorticoid-induced apoptosis in multiple model systems (37). Although inhibition of these signaling pathways may help overcome glucocorticoid resistance, identification of the receptors whose activation enhances glucocorticoid-induced apoptosis will open new opportunities for the enhancement of glucocorticoid therapy.

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1 The abbreviations used are: ACTH, adrenocorticotropic hormone; GFP, green fluorescent protein; RNAi, RNA interference; siRNA, small interfering RNA.

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TDAG8 Activation Enhances Glucocorticoid-induced Apoptosis

In an attempt to identify glucocorticoid-regulated genes that promote apoptosis, we have performed microarray expression analysis on three models of glucocorticoid-induced apoptosis (30, 38). We have found that tdag8 (T-cell death-associated gene 8), a G protein-coupled receptor selectively expressed in lymphoid tissues (primarily thymus, spleen, and peripheral blood monocytes), is rapidly and markedly induced by glucocorticoids in all three model systems. Activation of TDAG8 with the glycosphingolipid agonist psychosine markedly enhances glucocorticoid-induced apoptosis and the induction of Bim. This enhancement is only mediated by agonists of TDAG8 and is suppressed by a partial reduction of TDAG8 expression by RNA interference. Furthermore, expression of a TDAG8-GFP fusion protein is sufficient to induce apoptosis, and repression of endogenous TDAG8 partially protects against glucocorticoid-induced apoptosis. These studies define a role for TDAG8 as a regulator of glucocorticoid-induced apoptosis and suggest that agonists of TDAG8 may be promising adjuvants to enhance the efficacy of glucocorticoid therapy for the treatment of leukemia and lymphoma.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Unless otherwise noted, all of the chemicals were purchased from Sigma. All of the sphingolipids were obtained from Avanti Polar Lipids (Alabaster, AL), with the exception of N-acetylpsychosine, which was purchased from Calbiochem (San Diego, CA). Lyophilized sphingolipids were dissolved in dimethyl sulfoxide to a final concentration of 10 mM. Working solutions of sphingolipids were prepared immediately before use by diluting 10 mM stock solutions into a 10-fold excess solution of 0.1% (w/v) fatty acid-free bovine serum albumin in phosphate-buffered saline.

**Lymphoma Cell Culture**—WEHI7.2 and S49.A2 murine T-cell lymphoma cell lines were generous gifts of Diane Dowd (Case Western Reserve University) and Roger Miesfeld (University of Arizona), respectively. WEHI7.2 and S49.A2 cells were cultured in Dulbecco’s modified Eagle’s medium, supplemented with 2 mM l-glutamine (Invitrogen), 10% heat-inactivated bovine calf serum (HyClone), 12.5 units/ml penicillin, and 12.5 μg/ml streptomycin (Invitrogen). WEHI7.2 and S49.A2 cultures were seeded at a density of 1–2 × 10⁶ cells/ml and grown in a humidified 5% CO₂ incubator at 37 °C. WEHI7.2 cells were transfected with either pLacZ-GFP or pEmdGFP-cb5 and transferred to a 0.4-cm gap GeneDraft. TDAG8 was a generous gift of Kevin Lynch (University of Virginia). The controllable GFP chimera was produced chemically competent DH5α (New England Biolabs). Ligase reaction products were cloned into pSilencer2.1-U6 hygro using 400 units of T4 ligase (Promega). Sense and antisense oligonucleotides were synthesized by Qiagen. Sense and antisense oligonucleotides. Hybridization of the probe occurred at 68 °C for 16 h in QuikHyb solution (Stratagene). The membranes were subsequently washed at 65 °C twice for 20 min in 2× SSC, once for 30 min in 2× SSC with 0.1% SDS, and once for 30 min in 0.1× SSC with 0.1% SDS.

**Immunoblot Analysis**—The cell cultures were harvested by centrifugation, washed twice in phosphate-buffered saline, and lysed in radioimmune precipitation assay buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.1% sodium dodecyl sulfate, and 0.5% sodium deoxycholate) to which complete protease inhibitor tablets (Roche Applied Science) were added according to the manufacturer’s instructions. The protein concentration in cell lysates was quantified by the Bradford method. Sample volume and protein concentration in each of the samples were normalized by the addition of radioimmune precipitation assay buffer prior to loading. The lysates were mixed with equal volumes of 2× sample loading buffer (100 mM Tris-HCl, pH 6.8, 5% sodium dodecyl sulfate, 20% glycerol, 0.2% bromophenol blue) and then boiled for 10 min. The proteins were resolved by electrophoresis through 12.5% Tris-HCl SDS-PAGE gels with a 5% stacking gel and then immobilized by electrotransfer onto polyvinylidene fluoride membranes. Nonspecific protein binding was blocked with Tris-buffered saline containing 0.1% Tween 20 and 5% nonfat dry milk prior to incubation with primary antibodies. The anti-Bim polyclonal antibody was purchased from Sigma. Horseradish peroxidase-conjugated goat anti-rabbit IgG and enhanced chemiluminescence substrate (Amersham Biosciences) were used for antibody detection.

**Quantitative Apoptosis Assay: Cellular DNA Content**—Culture samples containing 1.5 × 10⁶ cells were collected by centrifugation at 200 × g for 5 min, washed once with phosphate-buffered saline, then suspended in 500 μl ice-cold methanol, and incubated for a minimum of 5 min at −20 °C. Methanol-fixed samples were then centrifuged at 2000 × g for 5 min and then boiled for 10 min. The DNA was extracted from the samples with chloroform:isoamyl alcohol (24:1) and then immersed at room temperature for 1 h in propidium iodide staining solution containing 50 μg/ml propidium iodide (Molecular Probes), 0.1% Nonidet P-40, 20 μg/ml RNase A, and 0.1% sodium azide in phosphate-buffered saline. Propidium iodide fluorescence was measured using a FACSScan XL flow cytometer (Coulter). Nonaggregated samples containing DNA content less than that of the G0 population were scored as apoptotic. Data analysis was performed using WinList 3D version 5.0 (Verity Software House).

**TDAG8-GFP Expression**—The phTDAG8-GFP expression vector, containing enhanced GFP fused to the carboxyl terminus of human TDAG8 was a generous gift of Kevin Lynch (University of Virginia). The construction and initial characterization of this vector has previously been described (40). The controlled plasmid, pEmdGFP-cb5, contains an emerald GFP that localizes to the membrane of the endoplasmic reticulum and remains within the cell following methanol fixation (41).

**Immunofluorescence**—For detection of TDAG8-GFP, cells were counted by flow cytometry, and the number of single GFP positive cells was enumerated. To achieve single-cell resolution, the W3/13 FACS profile was used. Data analysis was performed using WinList 3D version 5.0 (Verity Software House).

**Northern Blotting**—Total RNA was extracted from WEHI7.2 cells by lysis with TRIzol (Invitrogen) and then purified using the aqueous phase with an RNeasy mini-column kit. Total RNA samples were separated on a 1% agarose-formaldehyde gel and transferred to a GeneScreen Plus membrane (PerkinElmer Life Sciences) in 10× SSC by downward capillary transfer. The RNA was fixed to the membrane by cross-linking with ultraviolet light (245 nm, 30 s, 1200 μJ) using a Stratalinker UV oven (Stratagene). A full-length cDNA probe for TDAG8 was isolated by reverse transcriptase-PCR from WEHI7.2 cells, and labeled with [32P]dCTP using random priming.
TDAG8 RNAi hairpin oligonucleotides into the pSilencer vector was confirmed by sequencing (Cleveland Genomics). A pSilencer-GFP plasmid containing the RNAi hairpin oligonucleotide homologous to 21 bp of the coding region of enhanced GFP was used as a negative control. The pSilencer-TDAG8 or pSilencer-GFP plasmids were transfected into WEHI7.2 cells by electroporation, as described under “TDAG8-GFP Expression.” Electroporated cells were seeded into a T-175 flask containing 70 ml of fresh growth medium. Two hours following electroporation, hygromycin (Invitrogen) was added to the medium to make a final concentration of 1 mg/ml. Approximately 1 week later, dead cells were removed from the culture using Ficoll-Paque Plus (Amersham Biosciences). The remaining live cells were then counted, seeded at a density of 1.5–2.0 × 10^5 cells/ml and maintained in Dulbecco’s modified Eagle’s growth medium containing 1 mg/ml hygromycin. The ability of pSilencer-TDAG8 to repress TDAG8 message was confirmed by Northern blotting of stable clones treated with 1 μM dexamethasone for 16 h.

**RESULTS**

**Glucocorticoids Induce tdag8 in Multiple Models of Glucocorticoid-mediated Apoptosis**—Alteration of gene expression is a requisite step in glucocorticoid-induced apoptosis. To identify genes potentially involved in the apoptotic response to glucocorticoids, we employed microarray expression analysis to identify glucocorticoid regulated genes in three models of glucocorticoid-induced apoptosis. Transcript abundance for over 10,000 genes and expressed sequence tags was measured in the WEHI7.2 and S49.A2 murine lymphoma cell lines as well as in primary murine thymocytes treated with 1 μM dexamethasone over a range of treatment times between 6 and 24 h. From over 10,000 genes represented on the array, 207 genes, deemed significantly changed by the Affymetrix Microarray Analysis Suite v5.0 statistical algorithm, were similarly regulated by dexamethasone treatment in all three model systems (Fig. 1A).

Because small changes in the abundance of apoptosis regulatory proteins can have profound influences on cell fate, these 207 gene changes were not filtered by an arbitrary fold change parameter. Instead, the significance analysis of microarrays algorithm was used to assign a measure of significance to these 207 genes (39). The five most significant gene inductions resulting from this analysis are listed in Table 1. Among these genes are known regulators of apoptosis and nuclear receptor...
function. Interestingly, several of these genes appear to mediate glucocorticoid resistance. These include the stress response gene dig2, whose expression protects against glucocorticoid-induced apoptosis (38); FKBP51, a known glucocorticoid-regulated component of the glucocorticoid receptor chaperone complex that can mediate glucocorticoid resistance by inhibiting cortisol binding (43–45); and the ABC transporter 2, which has been identified as a mediator of multidrug resistance (46, 47).

Also included in this list is bim, a pro-apoptotic member of the bcl-2 family that acts at the mitochondria to activate Bac and release cytochrome c, promoting critical and irreversible steps in the execution phase of the apoptotic process (48, 49). The earliest and most pronounced of gene changes within this group of five was the induction of tdag8, a G protein-coupled receptor predominantly expressed within the immune system. Dexamethasone induced tdag8 in both cell lines and primary thymocytes at every time point analyzed (Fig. 1, A–D). Its expression was increased after as little as 2 h in thymocytes and was increased up to 11-fold by 24 h in WEHI7.2 cells. The induction of tdag8 was confirmed by Northern blotting. In the S49.A2 cell line, tdag8 was induced by 3 h and progressively accumulated throughout the 36-h time course (Fig. 1E).

To test the involvement of the glucocorticoid receptor in TDAG8 induction, WEHI7.2 cells were cultured for 16 h in the presence of 1 μM dexamethasone, after which the medium was replaced with fresh medium containing a 10-fold excess of the glucocorticoid-antagonist RU-486. The abundance of TDAG8 mRNA was determined by Northern blots of total RNA samples harvested at varying times following the removal of dexamethasone and the addition of RU-486. Within 2 h of incubation with RU-486, tdag8 message abundance was reduced to basal levels, suggesting that glucocorticoid receptor activation is required for the induction of tdag8 and that in the absence of dexamethasone, the elevated tdag8 message is rapidly degraded (Fig. 1F).

The TDAG8 Agonist Psychosine Markedly Enhances Glucocorticoid-induced Apoptosis—The work of Im et al. (40) has demonstrated that the glycosphingolipid psychosine is a selective agonist of TDAG8. The induction of TDAG8 in multiple models of glucocorticoid-induced apoptosis and the ability of TDAG8 to induce apoptosis suggested that agonists of TDAG8 ought to induce apoptosis in glucocorticoid-treated cells. To investigate this hypothesis, WEHI7.2 cells were treated with dexamethasone for 16 h to elevate TDAG8 expression. Psychosine was then added to these cultures and incubated for an additional eight hours, after which the cells were harvested to measure apoptosis. At doses consistent with the EC50 value for TDAG8 activation (40), psychosine markedly enhanced dexamethasone-induced apoptosis, as determined by DNA fragmentation and caspase activation (Fig. 2, A and B). Very little toxicity was observed when psychosine alone was added to the culture at concentrations of up to 10 μM. Thus, while being relatively nontoxic when administered alone, psychosine greatly enhanced apoptosis mediated by dexamethasone. This enhancement of glucocorticoid-induced apoptosis was also observed in the CEM-C7–14 cell line derived from human childhood T-cell acute lymphocytic leukemia. In this cell line, psychosine caused a 5-fold increase in apoptosis compared with that observed when cells were treated with dexamethasone alone. The structurally similar yet biologically inactive psychosine analog N-acetylpsychosine did not enhance glucocorticoid-induced apoptosis in CEM-C7–14 (Fig. 2C).

Previously, we have demonstrated that dexamethasone induces the expression of the pro-apoptotic protein Bim and that this induction is closely correlated with the onset of apoptosis (30). Immunoblot analysis of Bim demonstrated that psychosine amplified the dexamethasone-mediated induction of Bim (Fig. 2D). BimCterminal was nearly twice as abundant in cells treated with the combination of psychosine and dexamethasone as in cells treated with dexamethasone alone (Fig. 2E). Thus, the TDAG8 agonist psychosine enhanced glucocorticoid-induced apoptosis as measured by three hallmarks of glucocorticoid-induced apoptosis: DNA fragmentation, caspase activation, and the induction of Bim.

Only Agonists of TDAG8 Are Capable of Enhancing Glucocorticoid-induced Apoptosis—A glycosphingolipid, psychosine consists of a galactose linked to the amide head of sphingosine by an ether bond (Fig. 3A). Previous work has demonstrated that psychosine and glucosylpsychosine, in which glucose is substituted for the galactose in psychosine, are agonists of TDAG8, causing an accumulation of intracellular calcium and suppressing forskolin-induced cAMP generation (40). These glycosphinigolipids do not activate receptors homologous to TDAG8 such as OGR1, G2A, and GPR4 (50–52). Glucosylpsychosine was as capable as psychosine at enhancing glucocorticoid-induced apoptosis (compare Figs. 2A and 3B). N-Acetylpsychosine differs from psychosine only in the substitution of a methyl group for a proton on the primary amine, yet it is incapable of activating TDAG8. Consistent with its inability to activate TDAG8, N-acetylpsychosine had no appreciable effect on dexamethasone-induced apoptosis (Fig. 3C). Sphingosylphosphorylcholine and lysophosphatidylcholine are not agonists of TDAG8 but do activate receptors that share significant homology with TDAG8. Neither sphingosylphosphorylcholine nor lysophosphatidylcholine enhanced apoptosis in dexamethasone-treated cultures (Fig. 3, D and E). Furthermore, the endothelial differentiation gene family of Gαq coupled G protein-coupled receptors are expressed in a wide variety of tissues, including lymphocytes (53). Two ligands for endothelial differentiation gene receptors, sphingoines-1-phosphate and lysophosphatidic acid, did not enhance glucocorticoid-induced apoptosis as markedly as psychosine (Fig. 3, F and G). These data, summarized in Table II, indicate that only lipids that are known agonists of TDAG8 were able to enhance glucocorticoid-induced apoptosis.

Psychosine Enhances Glucocorticoid-induced Apoptosis through a TDAG8-dependent Mechanism—Although only

| Gene induced | Accession number | Fold change |
|--------------|------------------|-------------|
| TDAG8        | U39827           | 5.3 6.1 8.6 11.3 3.5 3.2 4.0 4.0 5.7 2.8 |
| DIG2/RTP801  | A1849939         | 3.7 5.7 2.3 3.0 4.6 3.2 3.2 5.7 5.7 4.6 |
| ABC transporter 2 (ATP-binding cassette) | U60901 | 3.0 2.6 3.3 2.5 1.7 2.6 2.8 2.5 1.6 0.7 |
| FK506-binding protein 5 (51 kDa) | U12959 | 2.3 2.6 3.3 2.6 1.3 1.3 3.0 3.0 5.7 3.5 |
| Bcl-2 interacting mediator of cell death (Bim) | AAT796690 | 1.4 2.0 2.1 2.3 2.3 1.7 2.1 2.3 2.6 1.4 |

TABLE I
Five most significant gene inductions

| Gene | Accession number | Fold change |
|------|------------------|-------------|
| WEHI7.2 |           | 6h 12h 18h 24h 6h 12h 18h 24h 2h 12h |
| S49.A2 |           | 6h 12h 18h 24h 6h 12h 18h 24h 2h 12h |
| Thymus |           | 6h 12h 18h 24h 6h 12h 18h 24h 2h 12h |
| TDAG8 | U39827 | 5.3 6.1 8.6 11.3 3.5 3.2 4.0 4.0 5.7 2.8 |
| DIG2/RTP801 | A1849939 | 3.7 5.7 2.3 3.0 4.6 3.2 3.2 5.7 5.7 4.6 |
| ABC transporter 2 (ATP-binding cassette) | U60901 | 3.0 2.6 3.3 2.5 1.7 2.6 2.8 2.5 1.6 0.7 |
| FK506-binding protein 5 (51 kDa) | U12959 | 2.3 2.6 3.3 2.6 1.3 1.3 3.0 3.0 5.7 3.5 |
| Bcl-2 interacting mediator of cell death (Bim) | AAT796690 | 1.4 2.0 2.1 2.3 2.3 1.7 2.1 2.3 2.6 1.4 |
TDAG8 agonists were capable of enhancing glucocorticoid-induced apoptosis, the possibility still remained that psychosine exerted its activity through a TDAG8-independent mechanism. To clearly demonstrate that psychosine enhanced glucocorticoid-induced apoptosis through a TDAG8-dependent process, the apoptotic sensitivity of cells to psychosine and dexamethasone was assessed in cells in which TDAG8 was repressed by RNA interference. Because of the poor transfection efficiency of lymphocytes, stable populations of WEHI7.2 cells expressing an antibiotic selectable RNA interference vector were used. Additionally, the time required for stable selection allows for the turnover of protein synthesized prior to the reduction of TDAG8 mRNA, optimizing the likelihood that the reduction of TDAG8 mRNA reflects a genuine decrease in TDAG8 protein. Because effective antibodies against TDAG8 are not currently available, use of this stable RNAi vector system is essential. In

**FIG. 2. Activation of endogenous TDAG8 enhances glucocorticoid-induced apoptosis.** WEHI7.2 cells were treated with 1 μM dexamethasone (black bars) or ethanol control (white bars) for 16 h. Psychosine was then added to the cultures at the concentrations indicated, and the cells were incubated for an additional 8 h. The cells were then fixed and apoptosis was measured by quantification of cells containing sub-G1 DNA (A) or active caspase-3 (B). The error bars represent the S.E. (n = 3). C, as above, human T-cell acute lymphocytic leukemia cells CEM-C7-14 were treated with 1 μM dexamethasone (black bars) or ethanol control (white bars) in the presence of vehicle (BSA), 10 μM psychosine, or 10 μM acetyl-psychosine, an inactive psychosine derivative. Apoptosis was quantified after 24 h. D, immunoblot analysis of Bim expression in WEHI7.2 cells treated with 1 μM dexamethasone, 10 μM psychosine, 1 μM dexamethasone with 10 μM psychosine, or vehicle control for 24 h. Three predominant isoforms, BimEL, BimL, and BimS, were observed. E, BimEL band intensity was quantified by densitometry in four independent experiments. Fold change in intensity relative to ethanol-treated vehicle control are indicated. The error bars represent the S.E. (n = 4).
this vector system, expression of hairpin small interfering RNA (siRNA) homologous to either the TDAG8 open reading frame or a control gene (GFP) were driven by the U6 RNA polymerase III promoter. The efficacy of t dag8 repression was measured by Northern blotting of total RNA from WEHI7.2 cells stably expressing either the TDAG8 or control siRNA vector that were treated with 1 μM dexamethasone for 16 h. When stably expressed in WEHI7.2 cells, the TDAG8 siRNA vector reduced expression of glucocorticoid-induced t dag8 by as much as 70% in stable clones (Fig. 4A). Furthermore, the TDAG8 siRNA effectively repressed expression of murine Myc-TDAG8 fusion protein driven by a cytomegalovirus promoter when co-transfected with the TDAG8 siRNA vector at equimolar concentrations of plasmid (data not shown). Following a 16-h incubation with 1 μM dexamethasone, psychosine was added in varying concentrations to WEHI7.2 cells stably expressing either the TDAG8 or control siRNA vectors. After incubating for a total of 36 h in the presence of dexamethasone, the cells were harvested, and the percentage of apoptotic cells was determined. The combination of dexamethasone and psychosine was 20% less toxic across multiple doses of psychosine in cells expressing the TDAG8 siRNA as compared with controls (Fig. 4B, p =

FIG. 3. Glucocorticoid-induced apoptosis is selectively enhanced by psychosine, not by agonists of other lipid-activated G protein-coupled receptors. A, molecular structure of psychosine. A galactose residue is joined to the primary amine of sphingosine through an ether bond. B–E, WEHI7.2 cells were treated with 1 μM dexamethasone for 16 h then treated with glucosylpsychosine (B), N-acetylpsychosine (C), sphingosylphosphorylcholine (D), lysosphatidylincholine (E), sphingosine-1-phosphate (F), or lysophosphatidic acid (G) for an additional 8 h in the continued presence of dexamethasone. Apoptosis was then quantified by measuring sub-G₁ DNA. The error bars represent the S.E. (n = 3).
Expression of TDAG8 Induces Apoptosis in WEHI7.2 Cells and Repression of Endogenous TDAG8 Reduces Sensitivity to Glucocorticoid-induced Apoptosis—Thus far, we have demonstrated that activation of TDAG8, with its agonist psychosine, markedly enhances glucocorticoid-induced apoptosis. We next sought to determine whether TDAG8 can mediate glucocorticoid-induced apoptosis in the absence of exogenously administered psychosine. To determine whether the induction of TDAG8 by dexamethasone is alone capable of inducing a death signal, the ability of a TDAG8-GFP fusion protein to induce apoptosis when transiently expressed in WEHI7.2 cells was assayed. The TDAG8-GFP fusion protein, in which enhanced GFP is fused to the carboxyl terminus of the human homolog of TDAG8, is known by the work of others to retain its activity, because it is capable of inducing receptor-dependent calcium transients and suppressing forskolin-induced cAMP generation (40). When expressed in WEHI7.2 cells, TDAG8-GFP localized predominantly to the plasma membrane (Fig. 5A). DNA content and TDAG8-GFP expression were measured 24 h after transfection with TDAG8-GFP. 30% of cells expressing TDAG8-GFP exhibited DNA fragmentation (sub-G1 DNA), whereas only 8% of the TDAG8 negative cells within the same transfected population were apoptotic (Fig. 5B). Cells transfected with untagged GFP were used to control for the potential toxicity of the transfection and of GFP expression. Apoptosis, as determined by nuclear fragmentation, was increased only in cells expressing TDAG8-GFP. Similar to the results obtained when measuring caspase activation, expression of TDAG8-GFP was alone sufficient to induce apoptosis, increasing the amount of fragmented nuclei in the culture from 7 to 35% (n = 4, p = 0.004, paired, two-tailed t test). Apoptosis was not increased in cells expressing untagged GFP (Fig. 5C).

Next, RNA interference was used to repress expression of endogenous TDAG8. Sensitivity to dexamethasone was then assayed in WEHI7.2 cells in which TDAG8 expression had been reduced. Cells expressing either the TDAG8 or control siRNA were treated with dexamethasone at a range of concentrations for 36 h. At each dose, the cells expressing the TDAG8 siRNA were more resistant to glucocorticoid-induced apoptosis than those expressing the control siRNA. At the highest dose of dexamethasone (1 μM), 45% of the control culture was apoptotic after 36 h, whereas only 33% of the TDAG8 siRNA expressing cells were apoptotic (Fig. 5D). Although this difference is small, it is highly significant across multiple doses of dexamethasone (p = 0.002, n = 3, two-tailed, paired t test).

**DISCUSSION**

The advent of microarray expression analysis has provided a powerful tool with which glucocorticoid-mediated changes in gene transcription can rapidly and accurately be identified. Microarray expression analysis drew our attention to the G protein-coupled receptor TDAG8, which is markedly induced by dexamethasone in three models of glucocorticoid-induced apoptosis. The data presented here identify TDAG8 and its agonists as regulators of glucocorticoid-induced apoptosis. The TDAG8 agonist psychosine markedly enhanced glucocorticoid-induced apoptosis. The percentage of cells that exhibited DNA fragmentation and caspase activation was greatly increased when treated with the combination of dexamethasone and psychosine in both murine and human models of T-cell leukemia. Psychosine also enhanced the glucocorticoid-mediated induction of Bim. This not only provides a third indicator that psychosine enhances glucocorticoid-induced apoptosis but also suggests that the apoptotic program enhanced by psychosine shares common distal events with glucocorticoid-induced apoptosis. Whether the induction of Bim is secondary to the induction of TDAG8 is an important question, because it suggests a mechanism by which TDAG8 may be exerting its pro-apoptotic effect. Suppression of TDAG8 by RNA interference did not noticeably reduce the dexamethasone-mediated induction of Bim (data not shown). However, given the subtle protective effect of the TDAG8 RNAi against glucocorticoid-induced apoptosis, its effect on Bim expression may be too subtle to detect. Unambiguous determination of whether the glucocorticoid-mediated Bim induction is secondary to TDAG8 induction must await the development of a TDAG8-null mouse.

The data presented here indicate that it is through the activation of TDAG8 that psychosine enhances glucocorticoid-induced apoptosis. Lysoosphospholipid agonists for receptors that share significant homology with TDAG8, but do not activate TDAG8, did not synergize with dexamethasone. Therefore, the enhancement of glucocorticoid-induced apoptosis is not the result of a nonselective action of lysoosphospholipids but
appears to be unique to agonists of TDAG8. Furthermore, the enhancement of glucocorticoid-induced apoptosis by psychosine was TDAG8-dependent, because repression of TDAG8 by RNAi reduced the ability of psychosine to enhance glucocorticoid-induced apoptosis.

Psychosine enhanced sensitivity to glucocorticoids at concentrations as low as 1 μM, consistent with concentrations of psychosine observed in vivo. Although no data are currently available to indicate the concentration of psychosine in the thymus or in serum, it is present at concentrations of ~0.2 μM in the
spleen and ~1 µm in the spinal cord in healthy mice (54). The abundance of psychosine in serum and in lymphoid organs, the mechanisms that regulate its synthesis and secretion, and whether psychosine participates in an autocrine or paracrine signaling loop among lymphocytes remain to be determined. It is interesting to note that, from 10,000 genes profiled by microarrays, we identified 207 that were regulated similarly in three models of glucocorticoid-induced apoptosis. Within the top five genes that were most significantly regulated by dexamethasone, we have identified genes that clearly participate in glucocorticoid signaling like FKBPS1 and genes that clearly enhance or suppress the induction of apoptosis: Bim (30), DIG2 (dexamethasone-induced gene 2) (38), and TDAG8. The role of the fifth gene, the ABC transporter 2, in glucocorticoid-induced apoptosis, is less clear. This particular transporter (ABC3) serves many functions, including peptide transport into the endoplasmic reticulum and transport of hydrophobic xenobiotics from the cytoplasm to the extracellular space (46, 47, 55). Interestingly, other members of this transporter family are known to transport glycosphingolipids from the Golgi, their site of synthesis, to the extracellular leaflet of the plasma membrane (56). This raises the intriguing possibility that, in addition to inducing TDAG8, dexamethasone also induces expression of a transporter that shuttles psychosine or a similar TDAG8 agonist to the plasma membrane, activating TDAG8 in an autocrine fashion. This would provide an additional step in the regulation of TDAG8 activity and the subsequent induction of apoptosis.

The ability of TDAG8 to induce apoptosis, coupled with the strong induction of TDAG8 by dexamethasone, suggests the hypothesis that the induction of TDAG8 by glucocorticosteroids may alone be a significant contributor to the induction of apoptosis. Two potential explanations exist for the observation that expression of TDAG8-GFP promoted an apoptotic program in the absence of exogenously applied agonist. First, as discussed above, an endogenous ligand may be present in the culture system that activates TDAG8 in either an autocrine or a paracrine fashion. Alternatively, vector-driven expression of TDAG8-GFP may result in such abundant receptor protein levels that, even if only 1% of the receptors are in their active conformation, they are in sufficient number to induce apoptosis. An effective means of quantifying receptor protein expression is required to determine whether the abundance of TDAG8 following dexamethasone treatment is similar to the amount of TDAG8-GFP that is sufficient to induce apoptosis.

Few other studies have investigated the function of TDAG8 in lymphoid tissues. The observation that enforced expression of TDAG8 was sufficient to induce apoptosis demonstrates that TDAG8 is capable of transducing a pro-apoptotic signal. TDAG8 is a member of the ovarian cancer G protein-coupled receptor 1 (OR1) family of genes (reviewed in Ref. 57). There are four receptors within this subfamily of the rhodopsin-Like G protein-coupled receptors: OR1, G2A, GPR4, and TDAG8. Together, these four receptors share 36–51% identity in amino acid sequence. All members of the OR1 family are activated by lysophospholipids and are coupled to Gαq or Gα11 heterotrimeric G proteins (57). It is interesting to note that several other members of the OR1 family, including OR1 and G2A, have been implicated as mediators of growth arrest and apoptosis (52, 58). Like TDAG8, other members of the OR1 family have only recently been paired with their ligands. Thus, determining the functions of these receptors in normal and pathologic physiology is a developing area of investigation.

During the course of this work, Tosa et al. (59) developed transgenic mice in which a tdag8 transgene was driven by an lck proximal promoter to generate immature thymocytes that overexpress TDAG8. In this model system, thymocytes from the transgenic mice were more sensitive to dexamethasone but not to apoptosis induced by T-cell receptor activation or ionizing radiation. In addition to identifying the potent enhancement of glucocorticoid-induced apoptosis observed upon activation of TDAG8, we repressed TDAG8 expression to assess the extent of its contribution as a mediator of glucocorticoid-induced apoptosis. Repression of TDAG8 by RNA interference offered a small but significant degree of protection against glucocorticoid-induced apoptosis. Given the likelihood that glucocorticoid-induced apoptosis is a multigenic process and the potency with which a few G protein-coupled receptors can transduce a strongly amplified signal, this subtle repression in apoptosis is a striking phenotype. However, because RNA interference only partially reduces TDAG8 mRNA expression and TDAG8 is still induced by dexamethasone in cells expressing the TDAG8 siRNA, it is difficult to determine to what extent TDAG8 mediates glucocorticoid-induced apoptosis. For now, the data are consistent with the conclusion that activation of TDAG8 markedly enhances glucocorticoid-induced apoptosis. To what extent it contributes to glucocorticoid-induced apoptosis in the absence of pharmacological concentrations of agonist will be more properly addressed in the future using a TDAG8-null mouse.

For nearly half a century, glucocorticoids have been used extensively to treat lymphoid malignancies without a clear understanding of the mechanism by which the apoptotic process is initiated. The practical goal of dissecting the mechanism of glucocorticoid-induced apoptosis is to identify novel means of improving glucocorticoid therapy. Here we have provided evidence that activation of the glucocorticoid-induced gene tdag8 markedly enhances glucocorticoid-induced apoptosis. Furthermore, we have shown that expression of TDAG8 is alone sufficient to induce apoptosis, and repression of TDAG8 offers subtle but significant protection against glucocorticoid-induced apoptosis. Together, these data define TDAG8 as a regulator of glucocorticoid-induced apoptosis and suggest that agonists of TDAG8 may be valuable therapeutics for leukemia and lymphoma.

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