Inhibition of Glucocorticoid-induced Apoptosis by Targeting the Major Splice Variants of BIM mRNA with Small Interfering RNA and Short Hairpin RNA*

Glucocorticoids (GCs) induce apoptosis in lymphocytes and are effective agents for the treatment of leukemia. The activated glucocorticoid receptor initiates a transcriptional program leading to caspase activation and cell death, but the critical signaling intermediates in GC-induced apoptosis remain largely undefined. We have observed that GC induction of the three major protein products of the Bcl-2 relative Bim (BimEL, BimS, and BimL) correlates with GC sensitivity in a panel of human precursor B-cell (pre-B) acute lymphoblastic leukemia (ALL) cell lines. To test the hypothesis that Bim facilitates GC-induced apoptosis, we reduced BIM mRNA levels and Bim protein levels by RNA interference in highly GC-sensitive pre-B ALL cells. Reducing Bim proteins by either electroporation of synthetic small interfering RNA (siRNA) duplexes or lentivirus-mediated stable expression of short hairpin RNA inhibited the activation of caspase-3 and increased cell viability following GC exposure. We also observed that the extent of GC resistance correlated with siRNA silencing potency. siRNA duplexes that reduced only BimEL or BimEL and BimL (but not BimS) exhibited less GC resistance than a potent siRNA that silenced all three major isoforms, implying that induction of all three Bim proteins contributes to cell death. Finally, the modulation of GC-induced apoptosis caused by Bim silencing was independent of Bcl-2 expression levels, negating the hypothesis that the ratio of Bim to Bcl-2 regulates apoptosis. These results offer evidence that the induction of Bim by GC is a required event for the complete apoptotic response in pre-B ALL cells.

Glucocorticoids (GCs)1 are steroid hormones that maintain physiological homeostasis. Synthetic GCs such as dexamethasone and triamcinolone acetonide (TA) are widely prescribed pharmaceuticals for indications ranging from inflammation to cancer. GCs induce apoptosis in numerous lymphoid and myeloid tissues and have been successful in the treatment of childhood leukemias (1). The mechanism of GC-induced apoptosis involves the hallmarks of the intrinsic pathway of apoptosis, i.e. the release of cytochrome c and Smac (second mitochondria-derived activator of caspase) from the mitochondria and the activation of caspase-9 (2). Determining the molecular trigger(s) that commit the cell to the activation of intrinsic apoptosis might enable strategies to combat GC-resistant leukemias. Precursor B-cell (pre-B) acute lymphoblastic leukemia (ALL) is the most common childhood cancer and a useful model to investigate the mechanism of GC-induced apoptosis (3).

GC signaling occurs through the glucocorticoid receptor (GR), a nuclear receptor superfamily member that dissociates from a large Hsp70-containing complex and translocates to the nucleus upon GC binding (reviewed in Ref. 4). The activated GR initiates a tissue-specific transcriptional program through both direct DNA binding and interaction with other transcription factors. Numerous studies with actinomycin D and cycloheximide have demonstrated that new transcription and protein synthesis, respectively, are required for GC-induced apoptosis (5, 6). Microarray profiling has shown that a short GC exposure induces or represses the transcription of >100 genes by a factor of 3-fold or greater in multiple models of GC-induced apoptosis (7–9). Among these genes are universal regulators of intrinsic apoptosis such as BCL2 and BIM, a gene encoding several splice variants of the related Bcl-2 homology (BH) region 3 domain-containing protein Bim (7, 9). GC represses BCL2 transcription but induces BIM transcription. Lymphocytes containing overexpressed (7) or high endogenous levels (10) of Bcl-2 protein are partially GC-resistant, underscoring the significance of intrinsic pathway regulation in GC signaling. Bim protein is in the “BH3-only” subset of Bcl-2 relatives, a group that also includes Bid, Bad, Puma, and Noxa. BH3-only proteins are transcriptionally activated, post-translationally modified, or released from sequestration in response to death stimuli and promote apoptosis by interacting with Bcl-2 family members that contain multiple BH domains (BH1–BH4) (reviewed in Ref. 11).

Bim is a critical regulator of immune cell homeostasis as well as apoptosis in several tissue types and has been recently investigated in preclinical models as a potential cancer therapeutic agent (12, 13). Mice lacking Bim contain strikingly high numbers of leukocytes and eventually succumb to autoimmune disease (14). Importantly, thymocytes isolated from these mice demonstrate a delayed apoptotic response to the GC dexamethasone (14). Loss of a single allele of BIM accelerated the rate of murine c-myc-induced lymphoma development, leading
to classification of Bim as a tumor suppressor protein (15). Bim expression is induced by growth factor withdrawal (16, 17), T-cell receptor ligation (18), paclitaxel treatment (19), and forced growth in suspension (20, 21) in various cell types, suggesting that the transcriptional regulation of Bim is an upstream event in apoptosis induced by diverse stimuli. Finally, lowering BIM mRNA by RNAi causes partial protection against apoptosis induced by the drugs paclitaxel (19) and imatinib (22), as well as apoptosis induced by forced suspension culture (anoikis) (20, 21).

The BIM gene is transcribed as three major splice variants, BIM EL, BIM L, and BIM S, encoding the functionally distinct proteins BimEL, BimL, and BimS, respectively (23). The BimS protein has been widely reported to be the most strongly pro-apoptotic of the three, although there may be exceptions (16). Only BimEL is likely to be regulated by phosphorylation and caspase-3 cleavage (24). Both BimEL and BimL (but not BimS) may be sequestered by LC3, a subunit of the dynein motor complex (25), although the significance of this interaction has recently been questioned (26).

Previous studies in our laboratory (7) and others (9, 27) have led to speculation that the transcriptional induction of Bim is required for GC-induced apoptosis. Only correlative evidence has thus far been generated in leukemic cells. To test this hypothesis directly, we used RNAi (28) to reduce BIM mRNA levels and found that both synthetic siRNAs and lentivirally-expressed shRNAs rendered human 697 pre-B ALL cells partially resistant to GC and inhibited GC-induced caspase-3 activity. Using splice variant-specific siRNAs, we found that all three major isoforms contribute to GC-induced apoptosis. Finally, we demonstrate that the Bim/Bcl-2 ratio is not a critical parameter in this pathway, suggesting that Bim functions primarily in a Bcl-2-independent manner in pre-B ALL cells.

**Materials and Methods**

**Cell Culture and Reagents—**The pre-B ALL cell lines were cultured in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen). Nalm-6, Kasumi-2, and Kop-8 cells were acquired from DSMZ (Braunschweig, Germany). TA, Polybrene, and all other reagents were purchased from Sigma unless otherwise indicated. Horseradish peroxidase-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology.

**Electroporation of siRNA—**siRNAs were purchased from Dharmacon, Inc. (Lafayette, CO). The sense strand sequences of the RNA duplexes used were as follows: control siRNA (firefly luciferase), 5'-CGUACCCAGAAUCCUUCG(AU3dtdT3); BIM #1, 5'-ACCACAGAAAGGAGCAGAU(AU3dtdT3); BIM #2, 5'-CCUGUCCUCCAGUGGAU(AU3dtdT3); BIM EL, 5'-CCUGUCCUCCUCCAGUGGAU(AU3dtdT3); BIM L, 5'-CAGUGUCCUCCAGUGGAU(AU3dtdT3); BIM S, 5'-CCUGUCCUCCUGAGGAU(AU3dtdT3).

**Cell Cycle Analysis and Viability Assays—**Electroporation and drug-treated 697 cells were harvested, washed with PBS, and fixed with 70% ethanol. After fixing for 8 h at 4 °C, cells were collected by centrifugation and resuspended in 1 ml of staining solution containing 50 μg/ml propidium iodide, 1 mg/ml RNase A, and 1 mg/ml glucose in PBS. Cells were analyzed for DNA content using a Beckman Coulter XL flow cytometer after 2 h of staining. 10,000 cells were counted per sample.

**Immunoblot Analysis—**Cells were washed once in PBS and lysed with radioimmunoprecipitation assay buffer containing 1× Complete protease inhibitors (Roche Applied Science). 30–50 μg of each whole cell extract was electrophoresed on Novex 12% Tris-Gly polyacrylamide gels (Invitrogen) and transferred to nitrocellulose membranes. Immunoblots were performed using antibodies against Bim (1:20,000 dilution; catalog number 202000, Calbiochem), Bcl-2 (1:10,000; clone 100, Upstate Biotechnology, Lake Placid, NY), and GAPDH (1:20,000; clone 6C5, Research Diagnostics, Flanders, NJ) using standard methods and detected using chemiluminescent horseradish peroxidase substrates (Pierce). Densitometry analyses were performed by generating net intensity values using Kodak 1D software (Rochester, NY).

**Lentivirus Generation and Infection—**The lentiviral transfer and packaging vectors were a generous gift from Dr. Xiao-Feng Qiu. Lentivirus production was conducted by co-transfection of HEK293T cells with four plasmids, i.e. a packaging defective helper construct (pMDLg/pRRE, 3 μg), a Rev-expressing construct (pRSV-Rev, 3 μg), a construct encoding a heterologous envelop protein envelope pseudotyped with 5 μg, and a transfer vector harboring a specific shRNA sequence under control of the H1 RNA polymerase III promoter (pHLug/Lac-shRNA or pHLug/Bim-shRNA; 10 μg). Briefly, 3 × 10⁶ HEK293T cells were seeded on 10-cm plates 24 h before transfection. For each shRNA, 100 μl of FuGENE-6 (Roche Applied Science) was combined with the four plasmids, 10⁴ cells were added dropwise onto the cells after a 30-min incubation, and the cells were analyzed for green fluorescent protein (GFP) expression by fluorescence microscopy after 24 h. At 48 h, virus-containing cell supernatants were collected and centrifuged twice to eliminate transfer of cells. shRNA-encoding pHUG vectors were created by cloning annealed complementary oligonucleotides into BamHI and XhoI sites at the 3′ of the H1 RNA polymerase III promoter. The coding strand sequences of the shRNA-encoding oligonucleotides are 5′-GATCCCC-CGTATGGCAACTTGTAGATTACAGATGTCGACG-3′ for control (luciferase) and 5′-GATCCCCCAGCTGAGATTACAGATGTCGACGACGACGACG-3′ for BIM #1 siRNA sequence. The 697 cells were infected by adding 1 ml of viral supernatant supplemented with 4 μg/ml Polybrene to 5 × 10⁶ cells in 24-well plates. The viral supernatant was replaced with standard growth medium after 24 h, and infection efficiency was monitored by GFP expression after 48 h. Viral titers were calculated by counting GFP-positive cells after infecting with serial dilutions of the viral supernatant.

**Total RNA Extraction—**Total RNA was prepared from shRNA-expressing cell lines using TRI reagent (Sigma) according to the vendor’s protocol. 30 μg of RNA were electrophoresed on a Tris borate-EDTA-urea 15% acrylamide gel (Invitrogen) and transferred to a Zeta Probe nitrocellulose membrane (Bio-Rad). The membranes were UV light-crosslinked and probed with an 32P-labeled Bim antisense probe (5′-AAATTCTGCTACCTTCGCGTGC-3′) at 42 °C for 18 h. For the two hybridization control oligonucleotides (perfect match and two-base mismatches).
match), the two complementary BIM shRNA-encoding oligonucleotides were used because wobble base-pairing in the shRNA design (29) created two mismatches in the sense sequence of the coding strand oligonucleotide.

**RESULTS**

The pro-apoptotic Bcl-2 relative Bim has been shown to be up-regulated by GC in GC-sensitive lymphocytes, suggesting that Bim may function as an early sensor for intrinsic apoptosis (7, 9, 27). To determine whether there is a correlation between Bim induction and GC sensitivity in human pre-B ALL cells, we monitored a panel of human pre-B ALL cell lines for viability in response to the potent GC triamcinolone acetonide after 48- and 72-h exposures (Fig. 1A and B). Significant cell death was observed in the cell line 697 at low doses (5 nM) after 48 h, but the other lines tested (Kasumi-2, Kopn-8, and Nalm-6) showed no effect after 48 h and only slight (Kasumi-2, Kopn-8) or moderate (Nalm-6) cell death at the 72-h time point. We then compared the effect of TA on Bim protein levels in the sensitive and resistant cells (Fig. 1C). We observed that the three major Bim proteins (BimEL, BimL, and BimS) (23) increased after 24 h in the 697 cells but not in the other pre-B ALL cell lines. In addition, basal levels of the Bim proteins were low or undetectable in the GC-resistant lines. To further explore the kinetics of Bim induction by GC, the 697 cells were also exposed to GC for shorter time points, showing rapid up-regulation of the three Bim proteins after a 4 or 8 h exposure to 100 nM TA (Fig. 1D). Taken together, these data suggest that Bim expression and induction are factors in determining the pro-apoptotic response to GC in pre-B ALL.

We then sought to reduce Bim expression in 697 cells by RNAi. The use of synthetic siRNAs has not been widely reported for lymphocytes, primarily because of the technical difficulty of nucleic acid transfection in non-adherent cultures. It has previously been shown that, for lymphocytes, electroporation is a more efficient delivery method for synthetic nucleic acids than liposome-mediated transfection (30). However, the cytotoxicity caused by the cellular stress of electroporation has often been too significant to perform downstream viability
measurements. It has been reported (30) that an siRNA against the KIF11 gene, which encodes the mitotic kinesin KSP (also known as Eg5), is an effective tool to optimize transfection conditions because of its ability to induce mitotic block. To determine conditions for electroporating siRNAs into human 697 pre-B lymphocytes, we tested several RNA concentrations and capacitance settings using a KIF11-targeting siRNA. After 48 h, cell cycle analysis was performed using propidium iodide staining and flow cytometry. Under optimized settings (220 V/500 microfarads), the percentage of cells blocked in the G2/M stage was 2-fold higher for the KSP siRNA as compared with that for control siRNA (Fig 2A), indicating that the level of transfection was sufficient to explore biological phenotypes. We also verified that RNA uptake occurred in ~90% of the cells under these transfection conditions using a nonspecific siRNA labeled with Cy3 (data not shown). Importantly, the relatively mild electroporation conditions resulted in a minimal accumulation of sub-G1/G0 cells, indicating that this RNA delivery method caused negligible cell death and is therefore suitable for performing downstream viability studies. These electroporation conditions were used for all subsequent siRNA experiments. Finally, increasing the capacitance setting or the siRNA concentration did not further increase the percentage of cells blocked in G2/M (Fig. 2B).

24 h after transfection with two BIM-targeting siRNAs, steady state levels of BimEL, BimL, and BimS were markedly reduced compared with those of a control siRNA sequence (Fig. 3A). Neither of the BIM-targeting siRNAs affected expression of the housekeeping protein GAPDH (Fig. 3A) or the other Bcl-2 family members Bax and Bad (data not shown). To determine whether the siRNA can reduce the level of Bim even after its expression is up-regulated by GC, we treated the cells with TA (100 nM) 24 h after siRNA electroporation. The 24-h recovery after electroporation allows for degradation of the existing Bim protein, the rapid turnover rate of which can be attributed in part to ubiquitin-proteasome processing (31). Both siRNAs caused a marked reduction in not only basal Bim expression but also in the TA-induced levels of the three major splice variants relative to a control sequence (Fig 3A, right). The more potent Bim siRNA (sequence 2) caused a 70% reduction in basal BimEL (n = 4) and a 30% reduction in TA-induced BimEL (n = 2) (Fig. 3A, right, compare lanes 1 and 2 to lanes 5 and 6, counting from the left). To explore the RNAi effect at the

![Figure 2. Optimization of siRNA electroporation in 697 cells. A, cells were electroporated with siRNAs targeting the control firefly luciferase (Ctrl siRNA) or KSP at 220 V/500 microfarads. The cells were fixed after 48 h and subjected to propidium iodide staining and flow cytometry. The G1 and G2/M peaks of the cell cycle are indicated. B, quantitation of cells in G2/M arrest after electroporating cells with control (white bars) or KSP (black bars) siRNAs under different conditions; results shown are representative of two trials.](image)

![Figure 3. BIM-targeting siRNAs reduce basal and GC-induced BimEL, BimL, and BimS proteins and the corresponding mRNAs in 697 pre-B ALL cells. A, cells were transfected with two unique BIM siRNA sequences (BIM#1 and BIM#2) and the control luciferase siRNA (Ctrl). For the experiment shown at the left, cells were harvested and lysed 24 h after transfection. On the right, cells were treated with vehicle or 100 nM TA as indicated 24 h after transfection and harvested after a 24-h treatment. The densitometry data for BimEL are above the image, showing that both siRNAs reduced the basal as well as the TA-induced protein levels and that BIM#2 is the more potent of the two RNA duplexes. B, cells were treated with vehicle or 100 nM TA for 2.5, 5, or 7.5 h immediately after transfection with BIM-targeting siRNA (BIM#1). Total RNA was isolated and subjected to RT-PCR using primers specific for BIM (left) or GAPDH (right). The positions of the three major splice variants are indicated.](image)
mRNA level, we performed RT-PCR on total RNA isolated from siRNA-transfected 697 cells. The use of end point RT-PCR allows for visualization of all three major splice variants. Immediately following a 20-min post-electroporation incubation (see “Materials and Methods”), the transfected cells were treated with vehicle or TA (100 nM) and then harvested 2.5, 5, or 7.5 h later. The mRNAs corresponding to the three major splice variants were effectively reduced by the BIM-targeting siRNA only 2.5 h after electroporation and reached near maximal silencing by that early time point, as further incubation to 7.5 h had little additional effect. Treatment of the cells with TA caused BIM mRNA induction relative to vehicle-treated controls, which was effectively abrogated by the siRNA (Fig 3B).

To determine whether targeting Bim affects the apoptotic machinery in the 697 cells, we analyzed cells for caspase-3 activation (cleavage) and caspase-3 enzyme activity. Cells were electroporated as above, allowed to recover for 24 h (to allow for the degradation of pre-existing Bim protein), and then treated with TA (5 nM) for 24 h. This low, physiologically-relevant GC concentration corresponds to the approximate $K_d$ of TA for the receptor (32). After the 24-h drug exposure, cells were immuno-stained with an antibody specific for the active (cleaved) form of caspase-3. Silencing of Bim reduced TA-induced caspase-3 activation, reflecting a decrease in activity of its upstream activator, caspase-9 (Fig 4A). To confirm these results, we also monitored the enzymatic activity of caspase-3 by assaying for cleavage of the fluorescent caspase-3 substrate DEVD-rhodamine-110. Error bars represent S.E. of three assays. Ctrl, control; Fold induction represents the ratio of caspase-3 activity in TA-treated cells over that of vehicle-treated cells. C, triplicate transfections were performed as described above with the control siRNA (white bars), BIM#1 siRNA (striped bars), or BIM#2 siRNA (black bars), followed 24 h later by the addition of vehicle or 5 nM TA. Cells were fixed after a 48-h treatment and subjected to propidium iodide staining and flow cytometry. Apoptotic (sub-G0) cells were scored using WinMDI software. Error bars represent the S.E. of three measurements, each originating from an independent siRNA transfection.

To determine the effect of BIM-targeting siRNAs on GC sensitivity, we transfected both siRNAs (si#1 and si#2) into the 697 cells and then treated siRNA-transfected 697 cells with TA (5 nM) 24 h after electroporation. The cells were fixed 48 h after the addition of the drug (a time point chosen to result in 60% cell death; see Fig. 1A), and apoptotic cells were detected by staining with propidium iodide followed by flow cytometry (Fig 4B). The more potent of the two sequences (si#2; see Fig. 3A) decreased the percentage of cells containing sub-G0/G1 by ~50%, whereas the less potent siRNA (si#1) showed less of an effect. These data were also verified by trypan blue exclusion and by staining live cells with another viability dye, 7-aminoactinomycin-D (data not shown). Taken together, these data demonstrate that GC-mediated induction of Bim is an essential signaling event in GC-induced apoptosis in a human pre-B ALL model.
Previous work has made functional distinctions among the major Bim isoforms (23–25). To determine the relative contributions of BimEL, BimL, and BimS in GC-induced apoptosis, we designed siRNAs that target only BIM EL or BIM EL and BIM L, but not BIM S. It is not possible to target BIM S independently because of the lack of a unique sequence. On the other hand, we designed siRNAs that target only BIM EL or BIM L, but not BIM S. These data suggest that all three major splice variants contribute to GC-induced apoptosis of human pre-B ALL cells.

To explore the possibility of creating a stable cell line in which partial GC resistance is acquired through a reduction in Bim levels, we designed a shRNA targeting BIM and a control sequence. These sequences were used to produce lentiviral vectors that express shRNA under the control of the H1 RNA polymerase III promoter (34). An infection rate of nearly 100% was achieved in the 697 cells, as indicated by the expression of a GFP marker that was encoded by the lentivirus under control of an independent cytomegalovirus promoter (Fig. 6A). GFP expression remained constant and complete in the population 8 weeks after infection, indicating that the provirus is stably integrated (data not shown). A Northern hybridization using a 32P-labeled probe specific for the BIM shRNA revealed the presence of the Dicer-processed 21-nucleotide RNA in cells infected with the BIM shRNA virus, but not in cells infected with a virus encoding an shRNA against firefly luciferase (Fig. 6B). In addition, the 62-nucleotide unprocessed shRNA was not detected, indicating that processing of the shRNA to the mature siRNA was rapid. Similar to the data generated with the BIM-targeting synthetic siRNA (Fig. 3A), immunoblot analysis revealed effective Bim silencing in both the basal state and after inducing Bim expression with TA (Fig. 6C). In addition, the BIM shRNA-infected cell line also reduced TA-induced caspase-3 activation (Fig. 6D).

The ratio of pro-apoptotic and anti-apoptotic Bcl-2 family members (the “Bcl-2 rheostat”) (35) often controls the decision of a tumor cell to commit to irreversible intrinsic apoptosis. Bim was first isolated as a Bcl-2-bound protein (23), and its deletion prevents defects caused by Bcl-2 deficiency in mice (36). Given the previous observation that Bcl-2 (but not Bcl-XL) was reduced in response to GC in 697 cells (2), we sought to
explore the role of the Bim/Bcl-2 ratio in RNAi-mediated GC resistance. We targeted BCL2 by electroporation of synthetic siRNA, achieving a marked reduction in Bcl-2 protein (Fig. 7A). BCL2-targeting siRNA was then electroporated into the 697 cells stably expressing control or BIM-targeting shRNAs. GC sensitivity was assayed by 48 h of TA exposure followed by cell cycle analysis as in previous figures (Fig. 7B). In the absence of TA, the BCL2 siRNA had a modestly toxic effect on both lentiviral-infected cell lines. In the drug-treated cells, silencing Bcl-2 did not significantly modulate the level of GC resistance caused by BIM shRNA. These data suggest that Bim may function independently from Bcl-2 in GC-induced apoptosis.

DISCUSSION

In this study, we have presented data demonstrating that GC-mediated induction of Bim is a critical signaling event in the GC-induced apoptosis of human pre-B ALL cells. Under conditions where the levels of Bim proteins were reduced by 70% in the basal state and 30% in the GC-induced state (Figs. 3A and 6C), TA treatment in 697 pre-B ALL cells resulted in ~50% fewer apoptotic cells (Figs. 4C and 7B) as well as the inhibition of caspase-3 (Figs. 4B and 6D). Using the powerful tool of splice variant-specific siRNAs, we have also demonstrated that all three major Bim proteins apparently contribute to apoptosis induction. Because GC-mediated Bim up-regulation occurs in other GC-sensitive leukemic cell lines as well (namely, S49, CEM-C7 and WEHI 7.2 cells) (9, 27), we propose that this transcriptional event is a primary mechanism of apoptotic induction in this clinically important signaling pathway. In this respect, this mechanism parallels many DNA damage pathways during which p53 induces apoptosis by up-regulating Noxa and Puma (37, 38). Our findings do not exclude roles for other BH3-proteins in this pathway; indeed, it must be noted that deletion of the PUMA gene causes a delayed response to dexamethasone in mouse thymocytes (39). However, PUMA does not appear to be a GR target gene in 697 pre-B ALL cells (7) or murine S49 T-cells (9).

Interestingly, this study suggests that Bim may be functioning independently of Bcl-2 in GC-induced apoptosis. Reducing the Bcl-2 protein ~50% did not suppress the GC resistance caused by BIM shRNA. This was somewhat unexpected, given the importance of the Bim/Bcl2 ratio in apoptotic pathways in a mouse knock-out model (36) and the fact that Bcl-2 overexpression prevents Bim-induced cell death in mouse fibroblasts (40). The BH3-only proteins promote apoptosis by directly in-

**Fig. 6. Lentiviral delivery of Bim shRNA.** A. 697 cells were infected with a lentivirus encoding Bim shRNA and analyzed by fluorescence microscopy after 48 h for the expression of GFP. B. total RNA (30 μg) from cells infected with lentivirus encoding Bim or the luciferase control (Ctrl) shRNAs was subjected to northern hybridization with a probe complementary to the antisense strand of Bim shRNA. The gel was stained with ethidium bromide (EtBr) as a loading control before transfer. PM (perfect match), control oligonucleotide with complete complementarity to the probe; MM (mismatch), control oligonucleotide that is complementary to the probe except for two mismatched bases. nt, nucleotides. C, cell lines stably expressing control (Ctrl) or Bim shRNAs were analyzed for effects on basal (left) and TA-induced (right) Bim protein levels by immunoblotting. Each cell line was treated with vehicle or 5 or 100 nM TA as indicated, and the densitometry data for BimEL (above image) show that the BIM shRNA-expressing cells induced Bim protein to a level that was ~30% lower than the control siRNA. D, control (white bars) and BIM shRNA (black bars) expressing cells were subject to caspase-3 activity assay after 24 h vehicle or TA treatment as described for Fig. 4B.
Bim and Glucocorticoid-induced Apoptosis

Although it has been established that GC regulates BIM mRNA transcription, there is also the possibility that GC might regulate the Bim protein through post-translational mechanisms. Indeed, BimEL phosphorylation is an early event in growth factor deprivation (21). However, it is unlikely that BimEL phosphorylation is a factor in the 697 cells, given that GC treatment does not affect its gel migration (Fig. 1).2 It has also yet to be determined whether GC treatment affects the subcellular location of BimEL and BimL, which have been shown to be released from sequestration to the dynein motor complex upon cytokine withdrawal or ultraviolet radiation exposure (25). Although Bim subcellular localization may be investigated in the future, it is worth noting that the concept of mitochondrial translocation of Bim is controversial, as it has recently been shown that most of the Bim protein in healthy resting T cells is already localized to the mitochondria (26).

Previous studies demonstrated that the up-regulation of BIM is indirect, i.e. not caused by direct binding of the GR to the BIM promoter. First, the promoter does not contain a glucocorticoid response element (43). More significantly, new protein synthesis is required for BIM mRNA induction by GC (9). Given that a functional DNA binding GR is required for GC-induced apoptosis (44), it is likely that induction or repression of (an)other GR target gene(s) must occur as a prerequisite to the effects on Bim. Indeed, the forkhead transcription factor FHKL1, which is known to target the BIM gene (45), is up-regulated by GC in 697 cells (7). However, it must be noted that targeting FHKL1 by siRNA did not affect GC-induced apoptosis in 697 cells.2

Consistent with the combinatorial nature of GR-mediated transcriptional effects (46), other GR target genes might also serve as apoptotic signaling intermediates in addition to BIM. Although RNAi inhibition of GR target genes has not previously been reported to our knowledge, it has been reported that forced overexpression of a number of known GR target genes, including catalase (47) and DIG2 (48), modulates the apoptotic response. Now that the GR transcriptome has been characterized by microarray experiments in numerous model systems (46), it will be possible to perform systematic RNAi screens toward uncovering the other critical signaling intermediates in this pathway. Because glucocorticoid resistance (49) and poor prognosis (50) are associated with low GR content in ALL, bypassing the receptor by targeting such downstream genes may become an effective therapeutic strategy.

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