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# Selective glucocorticoid receptor translational isoforms reveal glucocorticoid-induced apoptotic transcriptomes

I Wu[1,5], SC Shin[1], Y Cao[1], IK Bender[1], N Jafari [2], G Feng[3], S Lin[3,6], JA Cidlowski[4], RP Schleimer[1] and NZ Lu*,[1]

**Induction of T-cell apoptosis contributes to the anti-inflammatory and antineoplastic benefits of glucocorticoids. The glucocorticoid receptor (GR) translational isoforms have distinct proapoptotic activities in osteosarcoma cells. Here we determined whether GR isoforms selectively induce apoptosis in Jurkat T lymphoblastic leukemia cells. Jurkat cells stably expressing individual GR isoforms were generated and treated with vehicle or dexamethasone (DEX). DEX induced apoptosis in cells expressing the GR-A, -B, or -C, but not the GR-D, isoform. cDNA microarray analyses of cells sensitive (GR-C3) and insensitive (GR-D3) to DEX revealed glucocorticoid-induced proapoptotic transcriptomes. Genes that were regulated by the proapoptotic GR-C3, but not by the GR-D3, isoform likely contributed to glucocorticoid-induced apoptosis. The identified genes include those that are directly involved in apoptosis and those that facilitate cell killing. Chromatin immunoprecipitation assays demonstrated that distinct chromatin modification abilities may underlie the distinct functions of GR isoforms. Interestingly, all GR isoforms, including the GR-D3 isoform, suppressed mitogen-stimulated cytokines. Furthermore, the GR-C isoforms were selectively upregulated in mitogen-activated primary T cells and DEX treatment induced GR-C target genes in activated T cells. Cell-specific expressions and functions of GR isoforms may help to explain the tissue- and individual-selective actions of glucocorticoids and may provide a basis for developing improved glucocorticoids.**
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**Subject Category:** Cancer

Glucocorticoids are frequently used in the treatment of inflammatory conditions such as asthma, transplant rejection, and autoimmune disorders, as well as certain cancers such as Hodgkin's lymphoma, multiple myeloma, and acute lymphoblastic leukemia (ALL).[1] The anti-inflammatory and antineoplastic actions of glucocorticoids can be partially attributed to glucocorticoid-induced apoptosis.[1] Glucocorticoids activate their cognate receptor, the glucocorticoid receptor (GR), that can upregulate proapoptotic genes and downregulate antiapoptotic genes. Proapoptotic B-cell CLL/lymphoma 2 (BCL-2)-associated agonist of cell death (BAD), BCL-2-interacting mediator of cell death (BIM, BCL2L11), and BCL-2-associated X protein (BAX)[2–4] are induced and prosurvival BCL-2 and BCL-XL proteins are reduced[5,6] in T cells by glucocorticoids. It has been proposed that the balance between pro- and antiapoptotic factors regulated by glucocorticoids defines the apoptotic threshold of a cell.[7] Careful analyses have revealed that multiple candidate genes may underlie glucocorticoid-induced apoptosis in ALL cells.[8,9]

Despite the wide range of gene targets of the GR, only one single *GR* gene has been discovered to date. This single *GR* gene generates several GR isoforms, however, including GR$\alpha$ and $\beta$ via alternative splicing, with GR$\alpha$ being expressed at relatively higher levels in the majority of tissues examined.[10] We have reported that each GR transcript generates additional isoforms via alternative translation initiation

[1]Division of Allergy-Immunology, Department of Medicine, Feinberg School of Medicine, Northwestern University, Chicago, IL, USA; [2]Genomics Core Facility, Chicago, IL, USA; [3]Biomedical Informatics Center, Feinberg School of Medicine, Northwestern University, Chicago, IL, USA and [4]Laboratory of Signal Transduction, NIEHS, NIH, Research Triangle Park, NC, USA
*Corresponding author: N Lu, Division of Allergy-Immunology, Department of Medicine, Feinberg School of Medicine, Northwestern University, McGaw, Room M301, 240 East Huron, Chicago, IL 60611, USA. Tel: +312 503 1310; Fax: +312 503 0078; E-mail: nickzlu@northwestern.edu
[5]Current address: Department of Anesthesiology, College of Physicians and Surgeons, Columbia University, NY City, NY, USA.
[6]Current address: Biomedical Informatics Research Center, Marshfield Clinic Research Foundation, Marshfield, WI, USA.
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**Abbreviations:** ANOVA, analysis of variance; AKAP13, A kinase anchor protein 13; TUBA, $\alpha$-tubulin; ALL, acute lymphoblastic leukemia; ATG12, autophagy-related 12 homolog (*Saccharomyces cerevisiae*); BCL-2, B-cell CLL/lymphoma 2; BAD, BCL-2-associated agonist of cell death; BAX, BCL-2-associated X protein; BBC3, PUMA, BCL-2-binding component 3; BIM, BCL2L11, BCL-2-interacting mediator of cell death; ChIP, chromatin immunoprecipitation; GM-CSF, colony-stimulating factor 2 (granulocyte–macrophage); CDKN2D or p19, cyclin-dependent kinase inhibitor 2D; DAPL1, death-associated protein-like 1; DEX, dexamethasone; FDR, false discovery rate; FBS, fetal bovine serum; GR, glucocorticoid receptor; GZMA, granzyme A; ICOS, inducible T-cell costimulator; ING1, inhibitor of growth family, member 1; ITPR1, inositol 1,4,5-triphosphate receptor, type 1; IL-8, interleukin-8; IR-GRE, inverse repeat negative glucocorticoid response elements; LTB, lymphotoxin B; MAQC, microarray quality control; miRNAs, microRNAs; PMA, phorbol 12-myristate 13-acetate; PIM3, pim-3 oncogene; PARP, poly-ADP-ribose polymerase; SATB1, SATB homeobox 1; SOCS, suppressor of cytokine signaling; TSC22D, GILZ, transforming growth factor b1-stimulated clone 22 domain family member 3; TNFRSF21, TNF-related death receptor-6, tumor necrosis factor receptor superfamily, member 21; TNFSF, tumor necrosis factor (ligand) superfamily 2 (TNF$\alpha$); MYC, v-myc myelocytomatosis viral oncogene homolog (avian)
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mechanisms.[11] Ribosomal leaky scanning and ribosomal shunting are responsible for the GR$\alpha$-A, -B, -C1, -C2, -C3, -D1, -D2, and -D3 isoforms. These GR($\alpha$) isoforms have distinct tissue distribution patterns.[11] Pancreas and colon have the highest amount of the GR-C isoforms, whereas spleen and lungs have the highest amount of the GR-D isoforms. When individual GR isoforms were expressed at comparable levels in U-2 OS cells, an osteosarcoma cell line lacking endogenous GR, they regulated unique sets of genes and mediated glucocorticoid-induced apoptosis in distinct manners.[12] The GR-C3 (a representative of C1, C2, and C3) isoform has enhanced activity as demonstrated by the earlier onset and higher percentage of cell death in GR-C3 isoform-expressing cells compared with cells expressing other GR isoforms, whereas GR-D3 (a representative of the D1, D2, and D3) isoform-expressing cells are insensitive to glucocorticoid killing. The GR-C3 isoform induces the expression of proapoptotic molecules, such as granzyme A (GZMA), more efficiently than the GR-D3 isoform. It was also found that the GR-C3 isoform recruits coactivators, acetylates histone H4, and recruits activated RNA polymerase II more efficiently than the GR-D3 isoform on GZMA promoter.[12]

To further identify the physiological functions of GR translational isoforms, we produced stable clones of Jurkat cells expressing individual GR isoforms and examined the apoptosis-inducing activity of individual GR isoforms. As in U-2 OS cell lines, dexamethasone (DEX), a potent glucocorticoid, induced apoptosis most efficiently in Jurkat cells expressing the GR-C3 isoform, whereas the GR-D3 isoform-expressing cells were resistant to glucocorticoid killing. Thus, a new model system to identify genes mediating glucocorticoid-induced apoptosis was established. The GR-C-sensitive and GR-D-insensitive genes are likely mediators for glucocorticoid-induced apoptosis. In addition, we found that components of cytoskeleton may facilitate glucocorticoid-induced apoptosis. Furthermore, activated primary T cells selectively increased the expression of the GR-C isoforms and certain GR-C target genes. These studies improve our understanding of the role of GR isoforms in apoptosis and in regulation of Jurkat and primary T cells and may provide a basis for the development of new anti-inflammatory and antineoplastic glucocorticoids.

## Results

**Selective GR translational isoforms induce apoptosis in Jurkat cells.** Individual GR isoforms were stably transfected into Jurkat (clone E6.1) cells. This parental clone is resistant to glucocorticoid killing due to low expression of endogenous GR.[13] The expression level of individual GR isoforms among stable clones was comparable to that of the endogenous GR level in CEM-7 cells, another T ALL cell line (Figure 1). DEX treatment for 48 h killed approximately 30% of the cells expressing the GR$\alpha$, -A, or -B isoforms and up to 50% of the cells expressing the GR-C3 isoform, whereas the GR-D3 isoform-expressing cells were insensitive to DEX killing. Correspondingly, various apoptotic markers, including Annexing-V labeling, DNA degradation, and poly ADP-ribose polymerase (PARP) cleavage, were induced most efficiently by the GR-C isoform followed by the GR$\alpha$, -A, and -B isoforms. The induction of apoptosis markers by the GR-D3 isoform was minimal, although detectable, and significantly less than that by the GR$\alpha$. The selective apoptosis-inducing activity of GR isoforms in Jurkat cells confirmed the observations made previously in two sets of osteoblast U-2 OS clones.[12]

**Selective GR translational isoforms inhibit MYC.** We hypothesized that the GR isoform-selective apoptosis-inducing activity is due to selective regulation of apoptotic factors by the apoptosis-inducing GR isoforms, for example, the GR-C3 isoform, distinctly from the GR-D3 isoform. To test this, we determined the regulation of several previously reported mediators of glucocorticoid-induced apoptosis by these GR isoforms. The proto-oncogene v-myc myelocytomatosis viral oncogene homolog (avian, MYC) has been demonstrated to promote the survival and proliferation of both Jurkat and normal T cells.[14] Overexpression of MYC inhibits, whereas repression of MYC enhances, glucocorticoid-induced apoptosis of CEM cells.[15] The DEX-mediated repression of MYC was significantly more effective by GR$\alpha$, -A, -B, or -C3 isoform than by the GR-D3 isoform (Figure 2a). MYC suppression by DEX in GR-C3 or -A isoform-expressing cells occurred as early as 2 h after DEX exposure, whereas in GR-D3 isoform-expressing cells, MYC protein was not significantly reduced 24 h after DEX treatment (Figure 2b). Consistent with previous reports that MYC is antiapoptotic in ALL cells,[14] ectopic expression of MYC in GR-C3-expressing cells protected, whereas knockdown of MYC in GR-D3-expressing cells sensitized the cells to DEX killing (Figure 2c). MYC is suppressed by DEX at the transcriptional level.[16] Using chromatin immunoprecipitation (ChIP) analyses of the MYC gene regulatory region, we found that all GR isoforms bound to the inverse repeat negative glucocorticoid response elements (IR-GRE)[17] in the presence of DEX (Figures 2d and e). Consistent with the observation that DEX did not significantly inhibit the expression of MYC in GR-D3 isoform-expressing cells, histone H4 acetylation and recruitment of polymerase II on IR-GRE were not changed by DEX treatment in GR-D3 cells (Figure 2f), in contrast to the observations in GR-B and -C3 cells. Histone H4 acetylation and/or recruitment of polymerase II on IR-GRE were not changed by DEX treatment in GR$\alpha$ and GR-A cells, suggesting that DEX repression of MYC in these cells are mediated by additional GREs and/or cofactors.

**Selective GR translational isoforms regulate proapoptotic BIM and certain microRNAs.** BIM is a proapoptotic BH3-only member of the BCL-2 family that activates BAX and neutralizes BCL2-like antiapoptotic proteins.[18,19] It has been demonstrated that BIM induction is necessary for glucocorticoid-induced cell death in mouse thymocytes and CEM cells.[3,20–23] We found that the induction of BIM was significantly more effective by the GR-C3 isoform than by the GR-D3 isoform (Figure 3). BIM-S (the most potent proapoptotic BIM isoform) and BIM-L were induced by the GR-C3 isoform significantly more than by the GR-D3 isoform.

Certain microRNAs (miRNAs) have recently been implicated in glucocorticoid-induced apoptosis.[24–27] Consistent

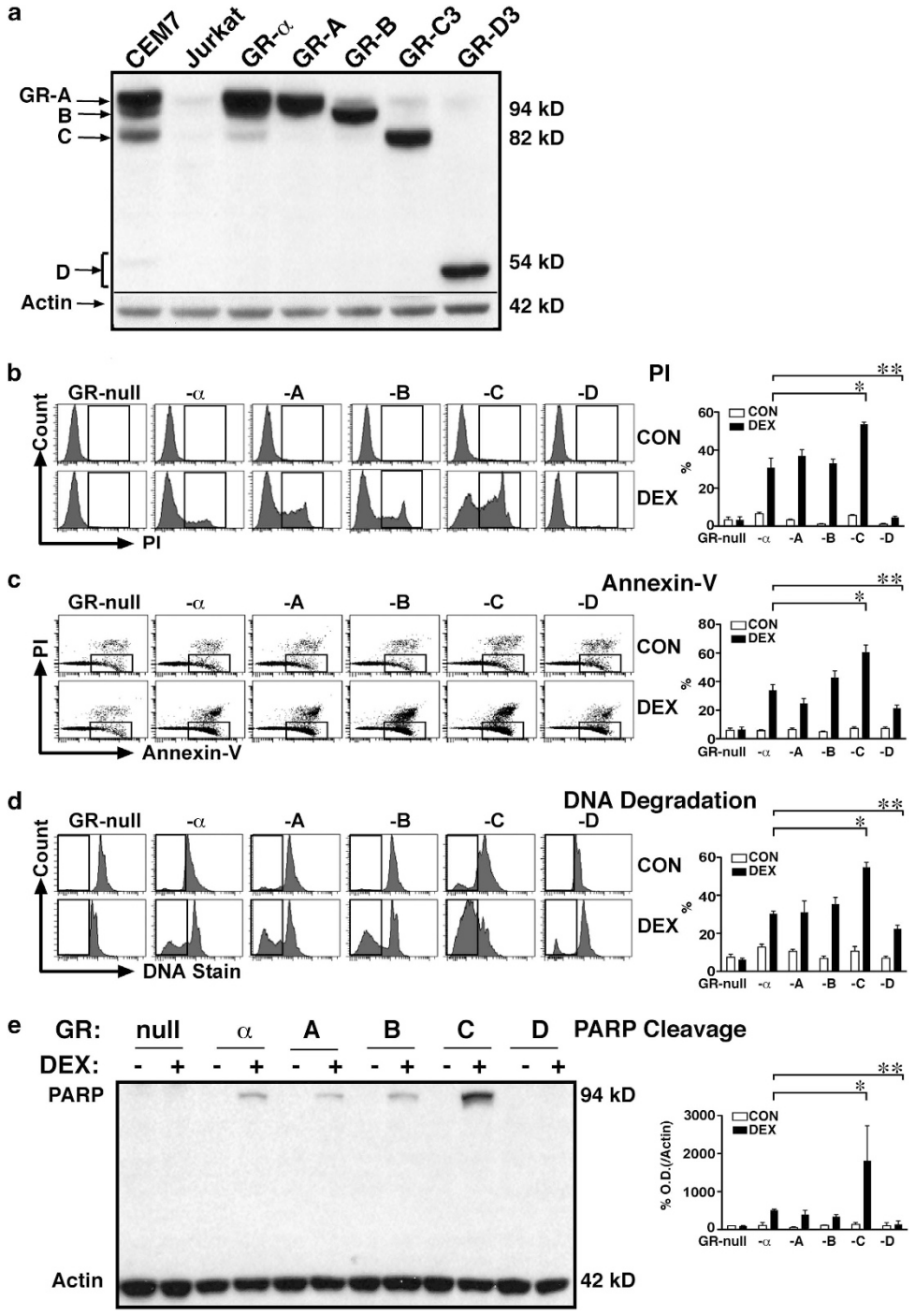


**Figure 1** GR isoforms mediate Jurkat cell apoptosis selectively. (**a**) Individual GR isoforms were expressed stably in Jurkat cells. Expression level of GR isoforms in each clone is similar as indicated by western blot analysis. Vehicle- or DEX- (100 nM, 48 h) treated cells were examined on parameters including PI staining (**b**), Annexin-V staining (**c**), DNA degradation (**d**), and the level of cleaved PARP (**e**). Averages ( ± S.E.M.) of at least three experiments are shown in the bar graphs. *Significantly greater than GRα; **significantly less than GRα, $P<0.05$, ANOVA followed by Tukey's *post hoc* test

with the literature, we found in our Jurkat model that the potentially proapoptotic miR-15b was selectively increased by DEX in the GR-C3 isoform-expressing cells, whereas in GR-D3 cells, miR15b was not induced (Supplementary Figure 1). In contrast, the putative antiapoptotic miR-17 was decreased in the GR-C3, but not in the GR-D3 isoform-expressing cells by DEX. GR isoform-selective regulation of miRNAs is target-specific as miR-195 was upregulated by both GR isoforms and let-7a was not regulated by either GR isoforms in this Jurkat model system.

**Using GR translational isoforms to identify novel apoptosis mediators.** Glucocorticoid-induced apoptosis in T cells is likely a multifactor-mediated event. Various

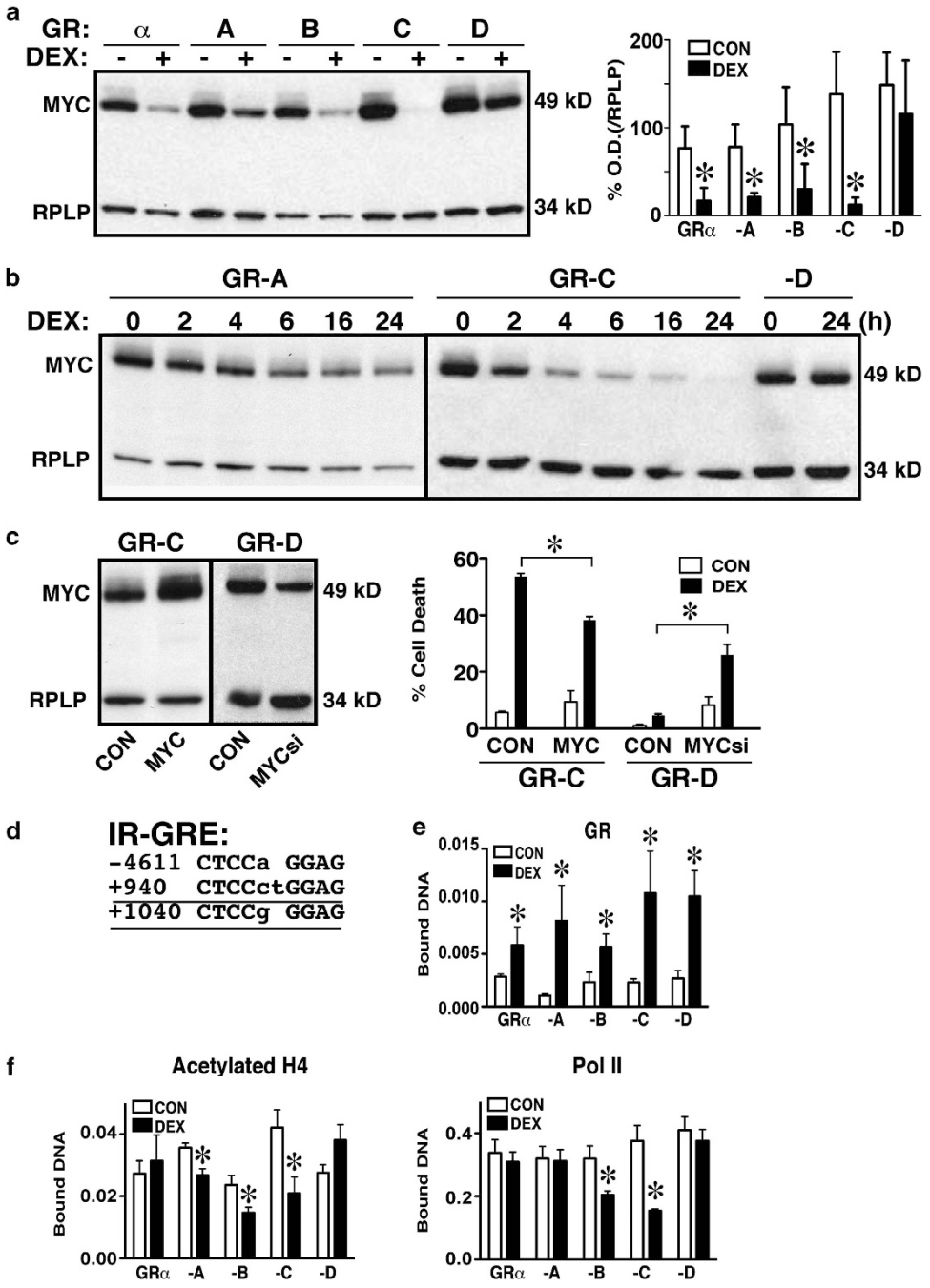


**Figure 2** MYC was suppressed by selective GR isoforms. (**a**) Vehicle- or DEX- (100 nM, 24 h) treated cells showed reduction of MYC protein levels. RPLP was used as a loading control. The average (±S.E.M.) of three experiments are shown in the bar graph. *Significantly less than CON, $P<0.05$, Student's *t*-test. (**b**) Time course of DEX regulation of MYC protein levels in GR-A- or -C3-expressing cells. MYC levels in GR-D3-expressing cells are shown as a control. (**c**) The role of MYC in DEX sensitivity. Ectopic expression of MYC increased the level of MYC in GR-C-expressing cells and MYCsi knocked down the expression level of MYC in GR-D-expressing cells. Ectopic expression of MYC partially protected, whereas knockdown of MYC sensitized the cells to DEX killing. *Significantly different, $P<0.05$, Student's *t*-test. (**d**) Three IR-GREs (positions are relative to transcription start site) in the MYC regulatory region. (**e**) ChIP assays demonstrated that DEX (100 nM, 6 h) increased binding of all GR isoforms to two of the three MYC IR-GREs (underlined). *Significantly greater than CON, $P<0.05$, Student's *t*-test, $n=4$ (±S.E.M.). (**f**) However, DEX reduced the amount of histone H4 acetylation and Pol II activation in cells expressing selective GR isoforms. *Significantly less than CON, $P<0.05$, Student's *t*-test, $n=4$ (±S.E.M.)

mediators likely trigger apoptosis in a coordinated manner as knockdown of key mediators such as BIM[3,20–23] or overexpression of MYC[15] only partially reduces the glucocorticoid sensitivity. To identify additional mediators of GR isoform-selective induction of apoptosis, cDNA microarray analyses of gene targets of the GR-C3 and GR-D3 isoforms were performed using the Jurkat model system. In all, 35 genes previously reported to be involved in mediating

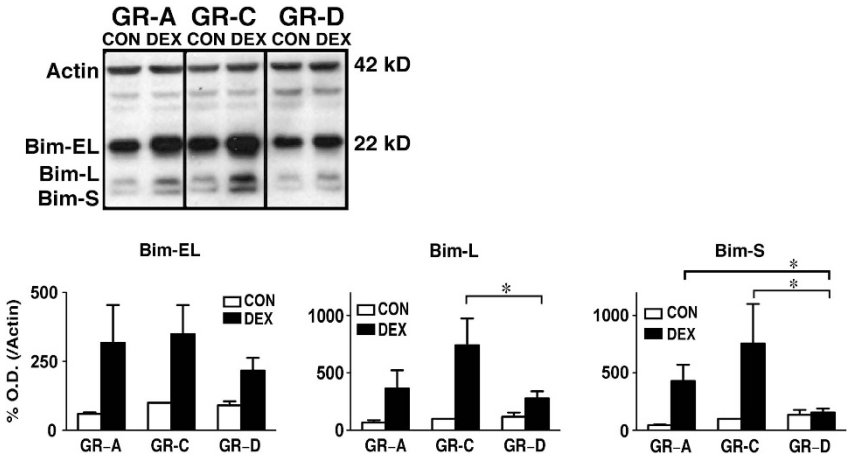


**Figure 3** BIM was induced by selective GR isoforms. Vehicle- or DEX- (100 nM, 24 h) treated cells showed induction of three forms of BIM proteins, EL, L, and S. The averages ( ± S.E.M.) of three experiments are shown in the bar graphs. *Significantly different, $P<0.05$, ANOVA followed by Tukey's *post hoc* test

**Table 1** Genes involved in apoptosis (GO: 0043067) and regulated by the GR-C3, but not the GR-D3, isoform

| Entrez i.d. | Symbol | Description | GR-C | GR-D |
|---|---|---|---|---|
| *Induced (DEX/CON)* | | | $P<0.01$ | NS |
| 330 | *BIRC3* | *Baculoviral IAP repeat-containing 3* | 3.74 | 1.09 |
| 11 040 | *PIM2* | *Pim-2 oncogene* | 3.11 | 1.35 |
| 92 196 | *DAPL1* | *Death-associated protein-like 1* | 2.74 | 1.15 |
| 3708 | *ITPR1* | *Inositol 1,4,5-triphosphate receptor, type 1* | 2.39 | 1.17 |
| 9140 | *ATG12* | *Autophagy-related 12 homolog (S. cerevisiae)* | 2.36 | 1.16 |
| 27 242 | *TNFRSF21* | *Tumor necrosis factor receptor superfamily, member 21* | 2.16 | 1.20 |
| 3621 | *ING1* | *Inhibitor of growth family, member 1* | 2.07 | 1.28 |
| 23 513 | *SCRIB* | *Scribbled homolog (Drosophila)* | 2.01 | 1.30 |
| 3001 | *GZMA* | *Granzyme A (granzyme 1, cytotoxic T-lymphocyte-associated serine esterase 3)* | 1.94 | 1.16 |
| 50 650 | *ARHGEF3* | *Rho guanine nucleotide exchange factor (GEF) 3* | 1.90 | 1.32 |
| 6446 | *SGK1* | *Serum/glucocorticoid-regulated kinase 1* | 1.89 | 1.34 |
| 11 214 | *AKAP13* | *A kinase (PRKA) anchor protein 13* | 1.84 | 1.28 |
| 8531 | *CSDA* | *Cold-shock domain protein A* | 1.83 | 1.17 |
| 5295 | *PIK3R1* | *Phosphoinositide-3-kinase, regulatory subunit 1 (alpha)* | 1.81 | 1.24 |
| 10 018 | *BCL2L11* | *BCL2-like 11 (apoptosis facilitator)* | 1.78 | 1.41 |
| 5590 | *PRKCZ* | *Protein kinase C, zeta* | 1.74 | 1.02 |
| 5921 | *RASA1* | *RAS p21 protein activator (GTPase-activating protein) 1* | 1.72 | 1.45 |
| 7852 | *CXCR4* | *Chemokine (C–X–C motif) receptor 4* | 1.69 | 1.33 |
| 5728 | *PTEN* | *Phosphatase and tensin homolog (mutated in multiple advanced cancers 1)* | 1.65 | −1.11 |
| 4908 | *NTF3* | *Neurotrophin 3* | 1.57 | 1.38 |
| 57 019 | *CIAPIN1* | *Cytokine-induced apoptosis inhibitor 1* | 1.56 | 1.07 |
| 10 276 | *NET1* | *Neuroepithelial cell transforming gene 1* | 1.56 | −1.08 |
| 329 | *BIRC2* | *Baculoviral IAP repeat-containing 2* | 1.55 | 1.02 |
| 55 179 | *FAIM* | *Fas apoptotic inhibitory molecule* | 1.55 | 1.07 |
| 10 370 | *CITED2* | *Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy terminal domain, 2* | 1.51 | 1.28 |
| *Repressed (DEX/CON)* | | | | |
| 41 5116 | *PIM3* | *Pim-3 oncogene* | −1.53 | 1.07 |
| 60 675 | *PROK2* | *Prokineticin 2* | −1.53 | −1.24 |
| 83 593 | *RASSF5* | *Ras association (RalGDS/AF-6) domain family member 5* | −1.55 | −1.26 |
| 79 156 | *PLEKHF1* | *Pleckstrin homology domain containing, family F (with FYVE domain) member 1* | −1.56 | −1.24 |
| 2769 | *GNA15* | *Guanine nucleotide-binding protein (G-protein), alpha 15 (Gq class)* | −1.66 | −1.13 |
| 6304 | *SATB1* | *SATB homeobox 1* | −1.83 | −1.20 |
| 6659 | *SOX4* | *SRY (sex determining region Y)-box 4* | −1.97 | −1.14 |
| 1032 | *CDKN2D* | *Cyclin-dependent kinase inhibitor 2D (p19, inhibits CDK4)* | −1.99 | −1.18 |
| 5896 | *RAG1* | *Recombination activating gene 1* | −2.03 | −1.12 |
| 4609 | *MYC* | *v-Myc myelocytomatosis viral oncogene homolog (avian)* | −3.39 | −1.32 |

Abbreviation: NS, not significant.

apoptosis, based on Gene Ontology (GO: 0043067, regulation of programmed cell death), were found to be regulated (25 induced and 10 repressed) by the GR-C3, but not by the GR-D3, isoform (Table 1). Among the induced genes are *death-associated protein-like 1* (*DAPL1*), *tumor necrosis factor receptor superfamily, member 21* (*TNFRSF21, TNF-related death receptor-6*), *inhibitor of growth family, member 1* (*ING1*), *GZMA*, and *BIM*. In contrast, antiapoptotic

molecules, such as MYC, pim-3 oncogene (PIM3), and cyclin-dependent kinase inhibitor 2D (CDKN2D or p19), were suppressed more by the GR-C3 isoform than by the GR-D3 isoform. A small number of genes (e.g., *BIRC3* and *FAIM* in Table 1) previously thought to be antiapoptotic were found to be induced by the GR-C3 isoform, suggesting that if these genes were antiapoptotic in Jurkat cells, their induction was overpowered by the proapoptotic programming. Real-time RT-PCR was performed to verify the observations from microarray studies (Figure 4). In addition, we determined the ability of GR-A to regulate genes involved in apoptosis along with the GR-C3 and GR-D3 isoforms. Reflecting the proapoptotic potency, the GR-C3 isoform appeared to be as effective as the GR-A isoform in upregulating proapoptotic ING1, whereas the GR-C3 isoform was most effective in inhibiting antiapoptotic PIM3. Seven of the 35 genes regulated uniquely by the GR-C3 isoform in Jurkat cells were also regulated in U-2 OS cells expressing the GR-C3 isoform.[12] They are *A kinase anchor protein 13* (*AKAP13*), *autophagy-related 12 homolog* (*Saccharomyces cerevisiae*) (*ATG12*), *CDKN2D*, *GZMA*, *ING1*, *inositol 1,4,5-triphosphate receptor, type 1* (*ITPR1*), and *SATB homeobox 1* (*SATB1*). Whereas proteins encoded by these genes may have a role in glucocorticoid-induced apoptosis in multiple cell types, the majority of the apoptosis-related genes were uniquely regulated in either Jurkat or U-2 OS cells.

Over 200 additional targets were regulated selectively by the GR-C3, but not by the GR-D3, isoform. These additional genes encode mediators of cell cycle, gene expression, cellular metabolism, signaling, cytoskeleton, and cell structure (Supplementary Table 1). These genes could have a role in glucocorticoid-induced apoptosis as they were selectively regulated by the proapoptotic GR-C3 isoform. These additional mediators could disturb cell signaling or gene expression or they could have a facilitatory role in apoptosis. For example, microtubule organization has been suggested to have a role in apoptosis. Disturbance of microtubules upregulates the proapoptotic proteins BAD, BCL-2-binding component 3 (BBC3, PUMA), BAX, and BCL-2 antagonist/killer,[28,29] and activates BAX through the induction of conformational change.[28,30]

We found that multiple components of the cytoskeleton system were selectively regulated by the GR-C3, but not by the GR-D3, isoform. Three members of the tubulin family, $\alpha$-tubulin (TUBA)3D, TUBA3E, and TUBA4A, belong to this GR-C3 isoform-selective category. We further investigated the mechanisms of the selective regulation and potential role in apoptosis of TUBA3E. Real-time RT-PCR experiments confirmed that TUBA3E mRNA was induced in GR-C3, but not in GR-D3 or GR-A, isoform-expressing cells (Figures 4 and 5). The induction of TUBA3E mRNA by the GR-C3 isoform does not require *de novo* protein synthesis as cycloheximide addition did not inhibit the induction (data not shown). To examine the role of TUBA3E in GR-C3 isoform-induced apoptosis, we used vector-based shRNAs to knockdown TUBA3E in the GR-C3 isoform-expressing cells (Figure 5a). Glucocorticoid sensitivity was compared among two separate stable clones expressing TUBA3E shRNA vectors and one clone expressing the empty vector. All three clones had equal amounts of cell death after DEX treatment despite approximately 60–90% reduction of TUBA3E mRNA levels by TUBA3E shRNA (Figure 5b). These results indicate that TUBA3E upregulation was not necessary for the GR-C3 isoform-induced apoptosis. To determine whether TUBA3E is facilitatory to apoptosis, we overexpressed TUBA3E in the GR-D3 isoform-expressing cells (Figure 5a). Overexpression of TUBA3E alone did not induce apoptosis in GR-D3 isoform-expressing cells compared with control vector-expressing cells (Figure 5b). However, in the presence of DEX, GR-D3 isoform-expressing cells became sensitive to glucocorticoid-killing. These results indicate that TUBA3E, even though not an initiator of apoptosis, facilitates the completion of apoptosis. Furthermore, the hypothesis that TUBA3E has unique functions was supported by its diffuse cellular distribution pattern that is distinct from the spindle-like distribution of the majority of tubulins (Figure 5c).

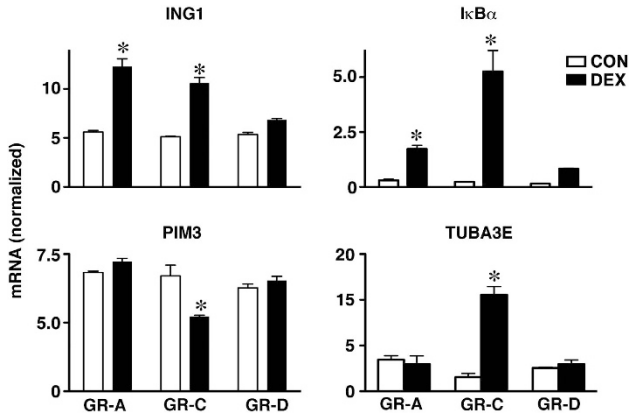


**Figure 4** Real-time RT-PCR confirmation of cDNA microarray results. Averages ($\pm$S.E.M.) of at least three real-time RT-PCR experiments comparing vehicle (CON) or DEX (100 nM, 6 h) treatments. *Significantly different from CON, $P<0.05$, Student's *t*-test

**Cytokine suppression by GR isoforms.** Additional microarray analyses using cells treated with phorbol 12-myristate 13-acetate (PMA) and ionomycin in the presence or absence of DEX revealed that 38 genes were commonly repressed by the GR-C3 and GR-D3 isoforms (Table 2). *Interleukin-8* (*IL-8*)*, inducible T-cell costimulator* (*ICOS*)*, colony-stimulating factor 2 (granulocyte–macrophage)* (*GM-CSF*), and *tumor necrosis factor (ligand) superfamily* (*TNFSF*) *2* (*TNF*$\alpha$)*, 3, 10, 14*, and *lymphotoxin B* (*LTB*; Supplementary Figure 2) belong to this category. In addition, both the GR-C3 and the GR-D3 isoforms induced the anti-inflammatory molecules suppressor of cytokine signaling (SOCS) 1 and 2 (Supplementary Table 2), both of which attenuate cytokine signaling in adaptive and innate immunity.[31,32] The transforming growth factor b1-stimulated clone 22 domain family member 3 (TSC22D, GILZ), which has been implicated in the immunosuppressive function of glucocorticoids by interfering with NF-$\kappa$B-mediated gene transcription in T cells,[33] was also induced by both GR isoforms (Supplementary Table 2 and Supplementary Figure 2).

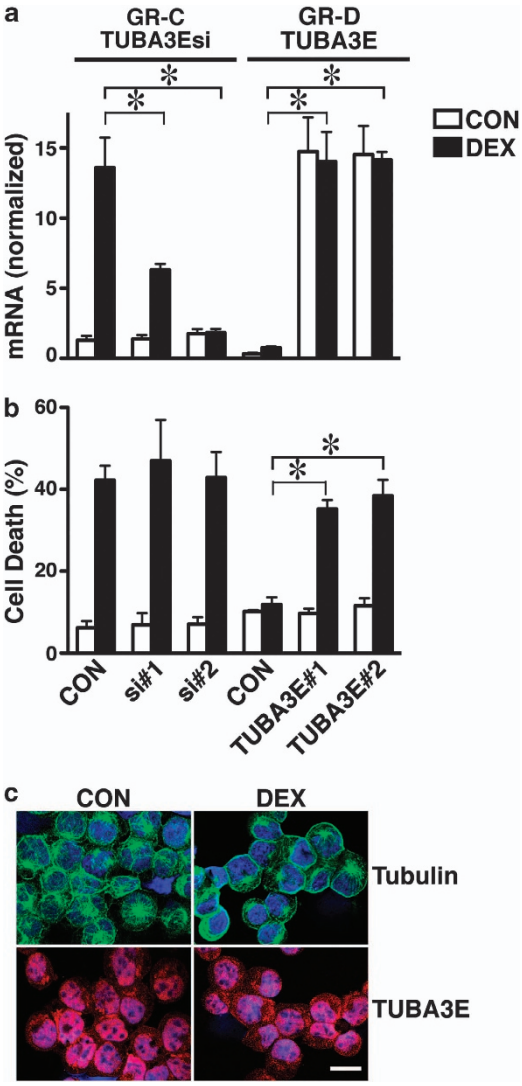


**Figure 5** Facilitatory role of TUBA3E in apoptosis. (**a**) Verification of knockdown of TUBA3E ($n=2$, average ± S.E.M.) in GR-C3-expressing cells and overexpression of TUBA3E in GR-D3-expressing cells. DEX, 100 nM, 6 h. *Significantly different, $P<0.05$, ANOVA followed by Tukey's *post hoc* test. (**b**) DEX (100 nM, 48 h) sensitivity of cells with altered TUBA3E levels. Cell death was examined using PI staining ($n=4$, average ± S.E.M.). *Significantly different, $P<0.05$, ANOVA followed by Tukey's *post hoc* test. (**c**) Localization of tubulin (green) and TUBA3E (red) in GR-C-expressing cells treated with vehicle (CON) or DEX (100 nM, 6 h). Nuclei are indicated in blue (DAPI). Scale bar, 20 $\mu$m

**GR translational isoforms are selectively expressed in T cells.** To determine whether GR translational isoforms are expressed in primary CD3+ T cells, we purified the CD3+ T cells (>97%) from peripheral blood samples using magnetic beads. The GR isoforms were examined on freshly isolated cells and cells cultured with the mitogen concanavalin A (ConA). The GR-A and -B isoforms were the predominant isoforms in primary CD3+ T cells, whereas the GR-C and -D isoforms contributed approximately 10–20 and 5% of the total GR protein, respectively (Figure 6a). Activation by ConA maintained the level of the GR-A/B and -D isoforms and selectively increased the GR-C isoforms. The elevated level of the GR-C isoforms did not enhance the glucocorticoid sensitivity of the ConA-activated cells (Figure 6b), an observation in line with previous reports indicating that T-cell activation cross-talks with GR signaling.[34] Thus, the glucocorticoid sensitivity of activated T cells is likely a product of T-cell activation and elevated expression of GR-C isoforms. The selective elevation of the GR-C isoforms in activated T cells may have additional impact on T-cell functions. We found that GR-C target genes (*TUBA3E* and *AIM1*) were induced by DEX in ConA-treated T cells, while DEX alone had no effects on the expression of these genes in resting cells (Figure 6c).

## Discussion

**Selective regulation of genes responsible for cell death by GR translational isoforms.** Using Jurkat cells expressing individual GR translational isoforms, we discovered a novel approach to identify glucocorticoid target genes responsible for cell death. These genes are regulated by the GR-C3, but not by the GR-D3, isoform. GR target genes involved in apoptosis fall into three categories: (1) genes already known to be directly involved in programmed cell death; (2) genes that indirectly contribute to cell death, that is, those involved in cell structural integrity, growth, and metabolism; and (3) genes that have unknown functions in cell death. BIM and MYC belong to the first category, whereas TUBA3E falls into the second, and those in Supplementary Table 1 belong to the third category. These findings are consistent with the observations made in glucocorticoid-sensitive and -resistant CEM model systems.[9,35–37] GR-D3 isoform-expressing cells, even though not sensitive to glucocorticoid killing, are capable of undergoing apoptosis as evidenced after ectopic expression of TUBA3E. Despite the differences in experimental protocols or bioinformatics analysis, current and previous studies using different T-cell apoptosis model systems revealed common GR targets potentially important in glucocorticoid-induced apoptosis. Overall, GR isoforms directly target multiple initiation and execution enzymes for apoptosis as well as apoptosis facilitators such as TUBA3E. It has been demonstrated that microtubules have a key role in BIM translocation to mitochondria and BIM activation.[38,39] Our data support that selective isoforms of tubulin, for example, TUBA3E, are involved in this pathway and selective antibodies are needed to further determine the role of TUBA3E. Blockade of some of the GR targets individually only partially blocks glucocorticoid-induced apoptosis. Thus, GR isoform-selective induction of apoptosis in Jurkat cells is likely mediated by multiple factors in concert rather than by the action of a single master gene.

**Cell-specific gene regulation by GR translational isoforms.** It has been reported that the glucocorticoid-induced apoptotic programs have common as well as cell type-specific features. For example, caspase-3, -8, and -9 activities are essential for glucocorticoid-induced apoptosis in murine thymocytes but not in splenic T cells.[40] The GR-C3 isoform is more effective than the other GR isoforms in killing

**Table 2** Genes commonly repressed by the GR-C3 and -D3 isoforms in the presence of PMA and ionomycin

| Entrez i.d. | Symbol | Description | GR-C | GR-D |
|---|---|---|---|---|
| *(PMA + ionomycin + DEX)/(PMA + ionomycin)* | | | $P<0.01$ | $P<0.01$ |
| 4050 | *LTB* | *Lymphotoxin beta (TNF superfamily, member 3)* | −4.81 | −3.07 |
| 3576 | *IL-8* | *Interleukin-8* | −8.25 | −2.65 |
| 677 | *ZFP36L1* | *Zinc-finger protein 36, C3H-type-like 1* | −5.11 | −2.57 |
| 4319 | *MMP10* | *Matrix metallopeptidase 10 (stromelysin 2)* | −2.58 | −2.41 |
| 1026 | *CDKN1A* | *Cyclin-dependent kinase inhibitor 1A (p21, Cip1)* | −2.79 | −2.36 |
| 8740 | *TNFSF14* | *Tumor necrosis factor (ligand) superfamily, member 14* | −2.21 | −2.18 |
| 8743 | *TNFSF10* | *Tumor necrosis factor (ligand) superfamily, member 10* | −1.63 | −2.18 |
| 6004 | *RGS16* | *Regulator of G-protein signaling 16* | −2.03 | −2.15 |
| 79 156 | *PLEKHF1* | *Pleckstrin homology domain containing, family F (with FYVE domain) member 1* | −1.89 | −2.02 |
| 1959 | *EGR2* | *Early growth response 2 (Krox-20homolog, Drosophila)* | −2.56 | −2.02 |
| 50 617 | *ATP6V0A4* | *ATPase, H+ transporting, lysosomal V0 subunit a4* | −1.54 | −1.94 |
| 7124 | *TNF* | *Tumor necrosis factor (TNF superfamily, member 2)* | −1.72 | −1.91 |
| 3002 | *GZMB* | *Granzyme B (granzyme 2, cytotoxic T-lymphocyte-associated serine esterase 1)* | −5.23 | −1.80 |
| 1647 | *GADD45A* | *Growth arrest and DNA-damage-inducible, alpha* | −1.71 | −1.75 |
| 10 135 | *NAMPT* | *Nicotinamide phosphoribosyltransferase* | −1.90 | −1.74 |
| 1437 | *CSF2* | *Colony-stimulating factor 2 (granulocyte-macrophage)* | −1.61 | −1.73 |
| 2669 | *GEM* | *GTP-binding protein overexpressed in skeletal muscle* | −1.90 | −1.71 |
| 29 851 | *ICOS* | *Inducible T-cell costimulator* | −1.58 | −1.71 |
| 4851 | *NOTCH1* | *Notch homolog 1, translocation-associated (Drosophila)* | −1.87 | −1.70 |
| 3659 | *IRF1* | *Interferon regulatory factor 1* | −3.06 | −1.69 |
| 22 822 | *PHLDA1* | *Pleckstrin homology-like domain, family A, member 1* | −4.65 | −1.67 |
| 8459 | *TPST2* | *Tyrosylprotein sulfotransferase 2* | −2.68 | −1.61 |
| 1649 | *DDIT3* | *DNA-damage-inducible transcript 3* | −2.74 | −1.61 |
| 2841 | *GPR18* | *G-protein-coupled receptor 18* | −1.94 | −1.57 |
| 3775 | *KCNK1* | *Potassium channel, subfamily K, member 1* | −1.80 | −1.57 |
| 3399 | *ID3* | *Inhibitor of DNA-binding 3, dominant-negative helix–loop–helix protein* | −2.56 | −1.55 |
| 54 739 | *XAF1* | *XIAP-associated factor 1* | −1.53 | −1.55 |
| 444 | *ASPH* | *Aspartate beta-hydroxylase* | −1.52 | −1.54 |
| 6304 | *SATB1* | *SATB homeobox 1* | −1.84 | −1.54 |
| 5366 | *PMAIP1* | *Phorbol-12-myristate-13-acetate-induced protein 1* | −2.35 | −1.54 |
| 15 3769 | *SH3RF2* | *SH3 domain-containing ring-finger 2* | −1.76 | −1.53 |
| 2731 | *GLDC* | *Glycine dehydrogenase (decarboxylating)* | −1.83 | −1.52 |
| 490 | *ATP2B1* | *ATPase, $Ca^{2+}$ transporting, plasma membrane 1* | −1.64 | −1.51 |
| 51 561 | *IL-23A* | *Interleukin-23, alpha subunit p19* | −2.15 | −1.51 |
| 3156 | *HMGCR* | *3-Hydroxy-3-methylglutaryl-coenzyme A reductase* | −1.77 | −1.51 |
| 8795 | *TNFRSF10B* | *Tumor necrosis factor receptor superfamily, member 10b* | −1.70 | −1.50 |
| 326 | *AIRE* | *Autoimmune regulator* | −1.56 | −1.50 |
| 26 191 | *PTPN22* | *Protein tyrosine phosphatase, non-receptor type 22 (lymphoid)* | −1.50 | −1.50 |

both Jurkat cells and U-2 OS cells. However, few genes selectively regulated by the GR-C3 isoform are common between U-2 OS cells[12] and Jurkat cells. *GZMA* and *MYC* are examples in the small category of common genes. In contrast, the majority of the genes selectively regulated by the GR-C3 isoform are different between the two cell types. Cell-specific gene profiles could be partially due to the difference in the expression level of GR in these two cell types. Expression levels of the GR isoforms in Jurkat cells are at the physiological level, comparable to that of the endogenous GR level in CEM cells, whereas expression levels of the GR isoforms in U-2 OS cells are two times as high as those in Jurkat cells. Cell type-specific actions of glucocorticoids contribute to their anti-inflammatory effects. In addition to killing proinflammatory cells, glucocorticoids protect several other cell types, such as certain epithelia, hepatocytes, ovarian follicular cells, and fibroblasts.[41,42] Delineating the role of GR isoforms in these cells will advance our understanding of various actions of glucocorticoids including their benefits as well as side effects, such as osteoporosis, hypothalamic-pituitary-adrenal axis suppression, and metabolic syndrome.

**Gene repression by GR translational isoforms.** We found that the GR-C3 isoform is more efficient in suppressing MYC in Jurkat cells than the GR-D3 isoform. This is consistent with the previous report that the GR-C3 isoform is more efficient than the GR-D3 isoform in repressing antiapoptotic genes, such as *BCL-XL*, *cellular inhibitor of apoptosis protein 1*, and *survivin* in U-2 OS cells.[43] The mechanisms for the GR isoform-selective repression of antiapoptotic genes, however, appeared different in these two model systems. In Jurkat cells, the GR-C3 and -D3 isoforms both bind to the IR-GRE in *MYC* gene, whereas in U-2 OS cells,[43] the GR-C3, but not the GR-D3, isoform was recruited to the promoter regions of the antiapoptotic genes. In Jurkat cells, the acetylation of histone H4 and recruitment of activated RNA polymerase II was repressed by the GR-C3 more than by the GR-D3 isoform. In U-2 OS cells, the DNA-associated GR-C3, but not the GR-D3, isoform inhibits NF-$\kappa$B-mediated gene expression.[43] Thus, it seems that cell-specific mechanisms mediate the repression of antiapoptotic genes by the GR translational isoforms. This is consistent with previous reports indicating that all GR isoforms inhibit NF-$\kappa$B reporter activity in COS-1 cells[12]

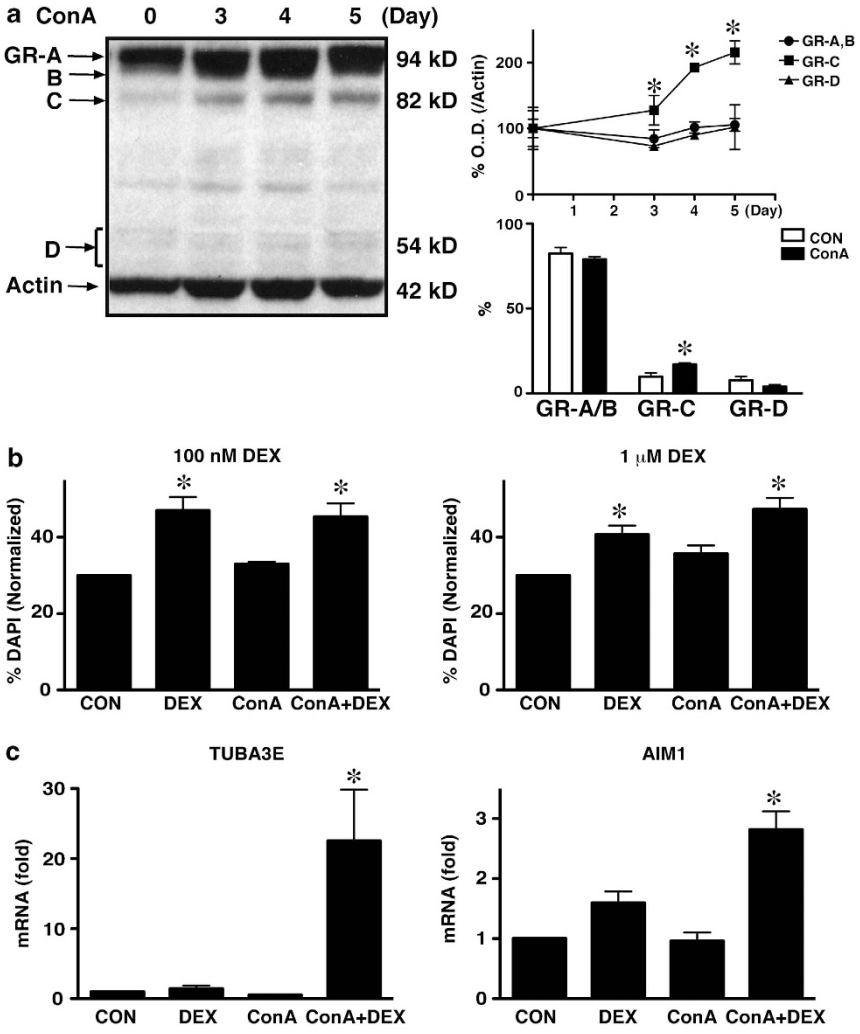


**Figure 6** Selective expression of GR isoforms in primary T cells. (**a**) A representative western blot analysis of the GR isoforms in CD3 + T cells treated with ConA (7.5 $\mu$g/ml, 0–5 days). Averages ($n=3$, average ± S.E.M.) of the level of GR isoforms are shown in the line graph (*significantly greater than day 0, $P<0.05$, one-way ANOVA followed by Tukey's *post hoc* tests, $n=3$) and the relative proportions of GR isoforms on day 3 are shown in the bar graph (*significantly greater than day 0, $P<0.05$, Student's *t*-test, $n=3$, ± S.E.M.). (**b**) DEX (100 nM or 1 $\mu$M, 48 h) sensitivity of vehicle (CON) or ConA-treated cells as indicated by DAPI staining. *Significantly greater than CON, $P<0.05$, one-way ANOVA followed by Tukey's *post hoc* tests, $n=3$, ± S.E.M. (**c**) Real-time RT-PCR analysis of TUBA3E and AIM1 mRNA levels in vehicle (CON)- and DEX- (100 nM 6 h) treated control or ConA- (2.5 $\mu$g/ml, 3 days) treated cells. *Significantly greater than CON, $P<0.05$, one-way ANOVA followed by Tukey's *post hoc* tests, $n=3$, ± S.E.M.

and selective GR isoforms inhibit NF-$\kappa$B reporter activity in U-2 OS cells.[43]

In addition to the cell specificity, transrepression by GR translational isoforms seems to be gene-specific. In both U-2 OS cells and Jurkat cells, antiapoptotic genes are spared by the GR-D3 isoform. In contrast, proinflammatory cytokines, such as IL-8, GM-CSF, and TNF in both Jurkat cells and U-2 OS cells,[12] are suppressed by all GR isoforms including the GR-D3 isoform. DNA- and ligand-binding domains, critical to transrepression activity, are present in all GR translational isoforms. In addition to transrepression of cytokines, another anti-inflammatory mechanism for both the GR-C and -D isoforms is induction of anti-inflammatory molecules such as SOCS and GILZ. Although apoptosis of proinflammatory T cells contributes to the anti-inflammatory actions of glucocorticoids, killing of immune cells may increase the susceptibility to infection. The ability of the GR-D3 isoform to dissociate cytokine-suppressing and proapoptotic functions of DEX, thus, may be beneficial in inhibiting inflammation and preventing immunosuppression. It will also be interesting to examine the role of GR translational isoforms in other cytokine suppression mechanisms such as RNA turnover.[44]

**Cell-specific expression of GR translational isoforms.** It is unclear why glucocorticoid responses among tissues and individuals can be drastically different. Cell-specific expression of glucocorticoid-metabolizing enzymes[45,46] and GR coregulators[47,48] has been proposed to contribute to the tissue-selective actions of glucocorticoids, although neither mechanism fully explains the tissue specificity of glucocorticoid responses. We propose that GR isoforms have a key role in tissue-specific glucocorticoid responses. Several lines of evidence support this notion. First, GR isoforms have tissue-specific distribution patterns.[11] Second, GR isoforms

have unique gene targets.[11,12] We found that the GR-C isoforms are selectively upregulated in activated T cells, possibly to counteract the antiapoptotic effects of T-cell activation and maintain glucocorticoid sensitivity.

Glucocorticoids are essential in treating inflammation and certain cancers, although severe side effects limit their use. Understanding the mechanisms mediating the cell-selective actions of GR isoforms will help us in designing new glucocorticoid drugs with increased efficacy and reduced side effects. GR isoform-selective compounds and drugs that selectively regulate the expression of GR isoforms may provide a new direction for designing glucocorticoids with tissue-selective actions. Our findings support the conclusion that GR isoforms have distinct functions in Jurkat and probably primary T cells.

## Materials and Methods

**Reagents and antibodies.** DEX was obtained from Steraloids (Newport, RI, USA). Rabbit anti-GR antibodies were from ThermoFisher (Waltham, MA, USA). Rabbit anti-CBP, anti-acetyl histone H4, and mouse antiphosphorylated RNA polymerase II (CTD4H8) antibodies were from Millipore (Billerica, MA, USA). Rabbit anti-MYC antibodies were from Cell Signaling (Danvers, MA, USA). Mouse anti-PARP was from BD Pharmingen (San Diego, CA, USA). Goat anti-rabbit and anti-mouse antibodies conjugated with horseradish peroxidase were from Jackson Immunoresearch (West Grove, PA, USA). Mouse monoclonal anti-FLAG (clone M2, unconjugated and Cy3-conjugated), mouse anti-TUBA (clone B-5-1-2), rabbit anti-RPLP0, rabbit anti-actin, and all other reagents were obtained from Sigma (St. Louis, MO, USA).

**Cell culture.** Jurkat cells (clone E6.1; ATCC, Manassas, VA, USA) and primary T cells were maintained in RPMI medium (Invitrogen, Carlsbad, CA, USA) containing 10% US-defined fetal bovine serum (FBS) (HyClone Laboratories, Logan, UT, USA), 4 mM glutamine, 50 U/ml penicillin, and 50 $\mu$g/ml streptomycin in a 7% $CO_2$ atmosphere at 37 °C. Jurkat cell lines stably expressing individual GR isoforms were generated by transfection using Amaxa (Lonza, Walkersville, MD, USA) kits and pTRE-GR plasmids.[11] In all, 500 and 200 $\mu$g/ml of hygromycin were used for selection and maintenance, respectively, of positive clones. Receptor levels in the stable clones were compared using western blot analysis. FLAG-TUBA3E, pSingle-TUBA3Esi, pcDNA3-cmyc, and pRetrosuper-Myc shRNA were transfected into Jurkat cells using Amaxa and selected using geneticin or puromycin. Human CD3+ cells were purified from peripheral blood mononuclear cells (American Red Cross, St. Paul, MN, USA) using Miltenyi Biotec (Auburn, CA, USA) kits. Cells were cultured in growth media supplemented with 10% charcoal-dextran-stripped FBS to remove endogenous glucocorticoids for 2 days before experiments.

**DNA plasmids.** GR isoform-expressing pTRE plasmids were described previously.[11,12] pcDNA3-cmyc (Addgene plasmid 16011)[49] and pRetrosuper-Myc shRNA (Addgene plasmid 15662)[50] were described previously. pCDNA-TUBA3E was made by subcloning TUBA3E into pCDNA3.0 (Invitrogen, Carlsbad, CA, USA). pSingle (Clontech, Mountain View, CA, USA)-TUBA3Esi contains TUBA3E shRNA targeting the sequence 5′-CGGTTGAGGTCTGGAAGTA-3′. Cloned sequences were verified at the Genomics core facility at Northwestern University.

**Western blot analysis.** Procedures for preparing cell lysates for western blot analysis were described previously.[11] Lysates containing 10–50 $\mu$g of proteins were resolved on 4–12% NuPage bis–tris gels (Invitrogen) and titers for antibodies were: anti-GR antibodies (1 : 500), anti-BIM (1 : 1000), anti-MYC (1 : 5000), anti-FLAG (1 : 5000), anti-RPLP0 (1 : 2000), anti-PARP (1 : 1000), anti-TUBA (1 : 300 000), and anti-actin (1 : 100 000). Secondary antibodies were used at 1 : 10 000 dilution for 30 min. The membranes were probed with ECL detection reagent (GE Amersham, Pittsburgh, PA, USA) and exposed to ECL Hyperfilm (GE Amersham).

**Immunofluorescent staining.** Jurkat cells expressing the GR-C3 isoform and FLAG-TUBA3E were treated with vehicle or DEX (10 nM, 20 h), transferred onto slides via cytospin, and fixed with 4% paraformaldehyde. To stain FLAG-TUBA3E, slides were blocked with 0.5% blocking reagent (Perkin-Elmer, Waltham, MA, USA) in PBS containing 0.1% Triton X-100 for 30 min and incubated with 1 : 100 Cy3-conjugated mouse anti-FLAG for 2 h. To stain tubulin, slides were blocked with 10% normal goat serum before incubating with 1 : 100 mouse anti-TUBA overnight. After blocking, slides were incubated with 1 : 200 dilution of Dylight488 anti-mouse antibodies (Vector Laboratories, Burlingame, CA, USA) in 5% normal human serum (Jackson ImmunoReseach). Slides were then incubated with 1 $\mu$g/ml of DAPI and mounted with Floromount. Images were obtained on a Nikon C1Si confocal inverted microscope. Dylight488 was imaged using the 488 nm excitation line of an argon laser and an emission bandpass filter of 518 nm. DAPI was excited with the 402 nm argon UV laser and imaged through a 461 nm bandpass filter. Cy3 was imaged using the 561 nm excitation line of an argon laser and an emission bandpass filter of 570 nm. A $\times$60 NA 1.25 PlanApochromat objective was used for all images.

**Microarray.** Approximately 10 million cells expressing the GR-C3 or GR-D3 isoforms were treated with vehicle, DEX (100 nM, 6 h), PMA (10 ng/ml) + ionomycin (50 $\mu$M) for 5 h, or DEX for 1 h followed by PMA + ionomycin + DEX for an additional 5 h. Total RNA was harvested and treated with DNaseI using Stratagene Absolute RNA kits (Stratagene, San Diego, CA, USA) according to the manufacturer's protocol. Biotin-labeled cRNA was generated using the Illumina TotalPrep RNA Amplification kit (Ambion, Foster City, CA, USA). Briefly, 250 ng of total RNA with 260/280 absorbance ratio of 1.8–2.0 and RNA integrity number of more than 9 were reverse transcribed with oligo (dT) primer bearing T7 promoter. The first-strand cDNAs were used to make the second strand. Purified second-strand cDNA along with biotin UTPs were used to generate biotinylated antisense RNA of each mRNA in an *in vitro* transcription reaction. Size distribution profiles for the labeled cRNA samples were evaluated on a bioanalyzer. After purification, labeled cRNA (1.5 $\mu$g) from each sample was hybridized with HumanRef-8 expression Beadchips (Illumina, San Diego, CA, USA) at 55 °C overnight. After washing, signals were developed with streptavidin-Cy3 and the chip was scanned with an Illumina BeadArray Reader. EachRef-8 Beadchip can profile eight samples on a single slide in parallel, and therefore samples from three biological replicates were randomly assigned onto different slides. Two MicroArray quality control (MAQC) RNA samples (Stratagene Universal RNA and Ambion Brain RNA) were added on each slide for normalization. Thus, a total of 32 arrays (2 cell lines $\times$ 4 treatments $\times$ 3 biological replicates + 8 MAQC samples) were performed. Raw signal intensities of each probe were obtained using data analysis software (Beadstudio; Illumina) and imported to the Lumi package of Bioconductor for data transformation and normalization.[51,52] A/P call detection was performed based on detection *P*-value. A total of 16 961 out of 45 281 probes with $<0.01$ in at least one sample were considered as valid signals. Differentially expressed genes were identified using an analysis of variance (ANOVA) model with empirical Bayesian variance estimation. The problem of multiple comparisons was corrected using the false discovery rate (FDR). Genes were identified as being differentially expressed when having a statistically significant change (up or down) in expression levels in different treatment groups (raw *P*-value $<0.01$, FDR $<0.05$, and change $>1.5$-fold).

**Real-time RT-PCR.** RNA samples were extracted as above. The level of individual mRNA in each sample was measured using the predesigned expression assays and one-step RT-PCR procedure on a Prism 7500HT thermocycler (Applied Biosystems, Foster City, CA, USA) as described previously.[11] Each experiment was performed in duplicate at least two times. Quantification was achieved using the Sequence Detection Software 2.0 Absolute Level subroutine (Applied Biosystems). Human RPLP0 mRNA levels were measured using primers 5′-TGCAGCTGATCAAGACTGGAGACA-3′ and 5′-TCCAGGAAGCGAGAATGCA GAGTT-3′ and the probe 5′-AAGCCACGCTGCTGAACATGCTCAACAT-3′ labeled with FAM reporter at the 5′ end and Iowa Black quencher at the 3′ end. RPLP0 mRNA levels were used to normalize the levels of genes of interests. For miRNA and small nucleolar RNA measurement, total RNAs were isolated using MirVana kits (Ambion). Taqman kits specific to let-7a, miR-15b, miR-17, miR-195, and RNU48 (Applied Biosystems) were used for RT reaction and amplification. The level of miRNAs was normalized to that of RNU48.

**ChIP assays.** ChIP assays were performed using ChIP assay kits from Millipore according to the manufacturer's protocol and have been described previously.[12] Briefly, $5 \times 10^6$ cells were cultured in steroid-free medium for 48 h

and then were treated with vehicle or DEX (100 nM) for 3 h. The amount of total input DNA for each immunoprecipitation reaction was 140 $\mu$g. The amount of antibodies (in $\mu$l) per immunoprecipitation reaction was 5 for anti-GR, 4 for anti-Pol II, and 0.5 for anti-acetylated H4. The level of precipitated DNA containing a 109 bp fragment in the first intron of *MYC* gene was quantified using real-time PCR analysis as described previously.[12] Each experiment was performed in duplicate at least two times. Quantification was achieved using the Sequence Detection Software 2.0 Absolute Level subroutine (Applied Biosystems). The primer sequences were 5′-TTGCATCCTGAGCTCCTTGGAGTA-3′ and 5′-AGCCGAAATTTAAATGCCCTCCCG-3′. The probe (FAM labeled) sequence was 5′-ATATCGCCTGTGTGAGCCAGATCGCT-3′. All measurements were normalized to the level of input DNA.

**Flow cytometry analysis.** Annexin-V and propidium iodide (PI) labeling was processed using the Annexin-V-FITC apoptosis detection kit (Biovision, Mountain View, CA, USA) according to the manufacturer's instructions. A total of $1.5 \times 10^5$ control or treated cells were processed and $1 \times 10^4$ cells were analyzed using a BD LSRII flow cytometer (Franklin Lakes, NJ, USA) with an excitation of 488 nm and emission at 530 nm (for Annexin-V-FITC) or 585 nm (for PI).

**Statistical analysis.** For comparison of two groups, Student's *t*-test was performed. For comparison of three or more treatment groups, one-way ANOVA was performed followed by Tukey's *post hoc* comparison using Prism software (GraphPad, San Diego, CA, USA). A $P$-value $< 0.05$ was considered significant.

## Conflict of Interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Cell Death and Disease website (http://www.nature.com/cddis)