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Use of Recombinant Cell-Permeable Small Peptides To Modulate Glucocorticoid Sensitivity of Acute Lymphoblastic Leukemia Cells[†]

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ABSTRACT: Glucocorticoid (GC) hormones induce apoptosis in T-cell and pre-B-cell acute lymphoblastic leukemia (ALL) cells. Steroid-mediated apoptosis requires a threshold level of the glucocorticoid receptor (GR) protein, and increasing the intracellular GR levels in ALL cells would augment their hormone sensitivity. A protein transduction domain (PTD) approach was used to accomplish this. We produced an HIV Tat PTD domain fusion protein (Tat-$GR_{554-777}$) that potentially competes for the degradation of GR protein by the ubiquitin−proteasome system and should thus increase its intracellular levels by "stabilizing" the GR. We also designed a fusion peptide for the c-Myb DNA binding domain, Tat-c-Myb DBD, since the biological function of this peptide as a dominant negative inhibitor of the c-Myb protein was already known. Purified, bacterially expresssed Tat-c-Myb DBD and Tat-$GR_{554-777}$ exhibited highly efficient transduction into cultured ALL cell lines including 697 (pre-B-ALL) and CEM-C7 (T-ALL) cells. As expected, the transduced Tat-c-Myb DBD peptide inhibited steroid-mediated stimulation of a GR promoter-luciferase reporter gene. Significantly, transduced Tat-$GR_{554-777}$ effectively increased intracellular GR levels in the GC-resistant T-ALL cell line, CEM-C1, and in the pre-B-ALL 697 cell line. Furthermore, transduction of Tat-$GR_{554-777}$ rendered GC-resistant CEM-C1 cells sensitive to steroid killing and further sensitized 697 cells to steroid. The use of Tat-fusion peptide transduction may eventually lead to innovative therapeutic modalities to improve the clinical response of patients suffering from T-cell and pre-B-cell acute lymphoblastic leukemia by increasing steroid responsiveness and perhaps converting steroid-resistant leukemia to a hormone-responsive phenotype.

The use of highly potent synthetic analogues of corticosteroids, together with other cytotoxic agents, has greatly improved the remission rate of acute lymphoblastic leukemia (ALL)[1] patients to up to 60−80% (*1*, *2*). However, about 20% of ALL patients fail to respond to currently available treatment protocols, for reasons that are still largely undefined. Furthermore, frequent relapse and the emergence of clones that are highly resistant to the original treatment protocol are observed (*3*−*6*). How to improve the efficacy and outcome of treatment in these resistant ALL patients is of significant interest in this field (*7*, *8*). Comprehensive clinical studies reveal that ALL patients who are sensitive to corticosteroid therapy (responders) have a much better outcome and event-free survival compared to glucocorticoid-resistant ALLs (nonresponders) (*9*). Thus, glucocorticoids play a central role in all protocols for the treatment of ALL.

The function of glucocorticoid (GC) steroid hormones depends upon the presence and activity of their intracellular binding protein, the glucocorticoid receptor (GR), a member of the nuclear receptor family of ligand-activated transcription factors. The hormone-activated GR triggers GC-induced ALL cell apoptosis in conjunction with the specific activation and suppression of gene expression. However, to date, the detailed pathways and molecular basis involved in this process are incompletely understood. Interestingly, during the apoptotic response in these ALL leukemic cells, among those genes that are regulated by corticosteroids, the GR gene itself is substantially autoupregulated immediately upon GC treatment (*10*−*14*). This finding is significant, because GR protein is usually downregulated to a low level in a glucocorticoid- and GR-dependent manner in most cell types and tissues, and this is believed to be a protective (homeostatic) response of these cells to possible adverse effects of chronic high levels of GCs (*13*, *15*−*19*). Thus, it is possible that GC-induced GR expression in sensitive ALL cells is essential, or at least beneficial, for triggering the apoptotic pathway, whereas a lowered GR expression level may result in apoptosis resistance. The critical association between GR expression levels and GC-mediated apoptosis in ALL cells is directly supported by results obtained using various cell line models as well as in patient primary leukemia cell samples (*12*, *13*, *20*−*23*), although there is some controversy regarding how important upregulation of GR is to the clinical response (*24*). Our studies have shown that the autoupregulation of the GR depends on the direct transcriptional activation of hGR gene promoters, and this involves the GR itself as well as certain transcription factors (c-Myb, selective Ets family members) whose expression is limited to immature hematopoietic lineage precursors (*25*−*32*). Therefore, because

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[1]Abbreviations: ALL, acute lymphoblastic leukemia; Dex, dexamethasone; GC, glucocorticoid; GR, glucocorticoid receptor; GRU, glucocorticoid response unit; pre-B-ALL and T-ALL, B- and T-cell acute lymphoblastic leukemia.

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GR expression levels can directly control the sensitivity of ALL blasts to the killing effect of GCs, directly targeting intracellular GR expression may provide therapeutic benefit to ALL patients.

A large number of diseases, including cancer, result from defects in endogenous proteins (e.g., kinases, transcription factors, key enzymes) or to alterations in their intracellular concentrations (for comprehensive reviews see refs (*33*−*36*)). Among the various strategies to modulate the intracellular levels of these proteins, an attractive idea is protein therapy, that is, the exogenous supplementation of proteins to the target cells. Due to the hydrophobic barrier of the plasma membrane that is largely impermeable to most proteins and peptides, a direct introduction of such molecules into mammalian cells is problematic. However, since the first report of the ability of the human immunodeficiency virus-1 transactivator protein (HIV 1-Tat) to translocate across the mammalian cell membrane in 1987, large advances in protein transduction technology have been made, mostly in the past several years (reviewed in ref *37*). The ability of the Tat protein to cross the cell membrane was mapped to the 11 amino acid long Tat *p*rotein *t*ransduction *d*omain (Tat PTD) (aa47−aa57). Thereafter, other PTD peptides, which are similarly capable of penetrating the cell membrane, were identified and characterized. All of these PTDs are able to carry along attached molecules, including proteins, nucleotides, and even large iron beads, into cells, and the transfer occurs in nearly any tissue and cell type. These observations have led to the proposed use of PTDs to deliver biologically active cargo, especially functional proteins and peptides, directly across the cell membrane. Furthermore, a recent approach that holds promise for the utilization of this technique involves the expression of proteins or peptides of interest fused to the Tat transduction domain using bacterial recombinant expression vectors. After purification and solubilization of the fusion proteins, the purified fusion protein can be directly used to treat cells in culture or *in vivo*. These Tat-fusion proteins can transduce the target cell population, and importantly, the biological functions of these peptides and proteins are manifested in the transduced cells (for comprehensive reviews see refs (*37*−*39*)). These experiments, especially those demonstrating the capability of certain proteins to efficiently correct pathological conditions in the transduced cells, raise hopes for the effective translation of this technology to clinical situations.

We evaluated the possibility of using PTD fusion peptides as potential agents of "protein therapy" to modulate the GC sensitivity of ALL cells. First, we used a dominant negative inhibitor Tat-c-Myb DNA binding domain (Tat-c-Myb DBD) in proof of principle studies, as its action using a cDNA transfection approach had been well characterized by us previously (*25*, *26*, *40*). Next, we designed a GR Tat fusion peptide (Tat-$GR_{554-777}$). Transduction of cell-permeable Tat-$GR_{554-777}$ into ALL cells led to the elevation of intracellular GR levels in CEM-C1 T-ALL and 697 pre-B-ALL cells. Transduction of Tat-$GR_{554-777}$ converts CEM-C1 cells from corticosteroid resistance to GC sensitivity. Similarly, the increase in GR concentration by Tat-$GR_{554-777}$ in GC-sensitive 697 pre-B-ALL cells effectively further sensitized these cells to GC-induced apoptosis. Thus, corticosteroid treatment along with Tat-fusion peptide transduction may result in a hypersensitive response of ALLs to the killing effect of these hormones. Potentially, this might lead to "protein therapy" to elicit glucocorticoid responsiveness in ALL that is hormone resistant due to low (insufficient) GR expression.

MATERIALS AND METHODS

Purification of Recombinant Tat-Fusion Proteins from Bacterial Cultures. Competent cells from the recombinant protein *Escherichia coli* expression strain BL21 (DE3) Gold were obtained from Agilent Biotechnologies/Stratagene (La Jolla, CA). Cells were transformed with expression vectors following manufacturer's instructions and transformants selected via antibiotic resistance. After inoculation with a single transformed clone and culturing overnight in a shaking incubator, 0.5 mM IPTG was added to induce recombinant protein expression. After 4 h, the cells were collected by centrifugation and washed with PBS. The cell paste was lysed with a denaturing lysis buffer (8 M urea, 50 mM phosphate buffer, pH 7.8, 10 mM imidazole, 2 mM PMSF), sonicated, and centrifuged to remove debris. The supernatant was loaded onto a preequilibrated (denaturing lysis buffer) nickel nitrilotriacetic acid (Ni-NTA) agarose column, and the recombinant proteins were eluted with lysis buffer containing imidazole. Eluted proteins were loaded onto an SP-Sepharose ion-exchange column (Amersham Biosciences, Pittsburgh, PA) equilibrated with wash buffer (50 mM phosphate buffer, 20 mM NaCl, pH 7.8). After extensive washing to remove the urea, the recombinant protein was eluted by increasing concentrations of NaCl in the wash buffer. The purified proteins were desalted on a G-25 column (Pierce Protein Research Products, Rockford, IL), which involved a buffer change to storage buffer (50 mM phosphate buffer, pH 7.8, 100 mM NaCl, 5% glycerol). Protein aliquots were flash frozen in liquid nitrogen and stored at −80 °C until use. The purification scheme for the recombinant proteins is shown in Figure 1A. SDS−polyacrylamide gel electrophoresis (SDS−PAGE) and silver staining were used to monitor and analyze the recovery of the proteins. The concentration of proteins was determined using a D_C protein assay kit II (catalog no. 500-0112; Bio-Rad, Hercules, CA). To label the peptides with fluorescein isothiocyanate (FITC), the EZ-Label fluorescein isothiocyanate (FITC) protein labeling kit (catalog no. 53004; Pierce, Rockford, IL) was used. FITC-labeled peptides were used to determine the transduction efficacy of purified recombinant Tat-fusion peptides into the ALL cells.

Acute Lympholastic Leukemia Cell Culture and Treatment. The human pre-B acute lymphoblastic leukemia cell line 697 (a generous gift from Dr. Noreen M. Robertson, Drexel University School of Medicine, Philadelphia, PA) and the T-cell acute lymphoblastic leukemia cell lines CEM-C7 and CEM-C1 (kind gifts from Dr. E. Brad Thompson, University of Texas Medical Branch at Galveston, Galveston, TX) were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA). Cells were grown at 5% CO_2 and 37 °C. To transduce cells, purified recombinant peptides were added directly to the culture medium and incubated for various times. Dexamethasone (Dex; Sigma, St. Louis, MO) at a final concentration of 1 μM or an equivalent volume of the ethanol vehicle (control) was added for an additional 18−24 h.

Plasmid DNA Constructs. High-fidelity polymerase chain reaction (PCR) synthesis used Pfu Ultra high-fidelity DNA polymerase (Stratagene) to generate the cDNA coding sequences for recombinant expression constructs. The primers used to amplify the c-Myb DNA binding domain (amino acids 1−201) coding sequences (from pcDNA3-c-Myb DBD) were 5′-CCGCGTCGACATGGCCCGAAGACCCCGGCACAGCA-3′ (forward, *Sal*I) and 5′-GCCTCGAGGTCGACGGTATCGATAAG-3′ (reverse, *Xho*I). The PCR-amplified DNA fragment

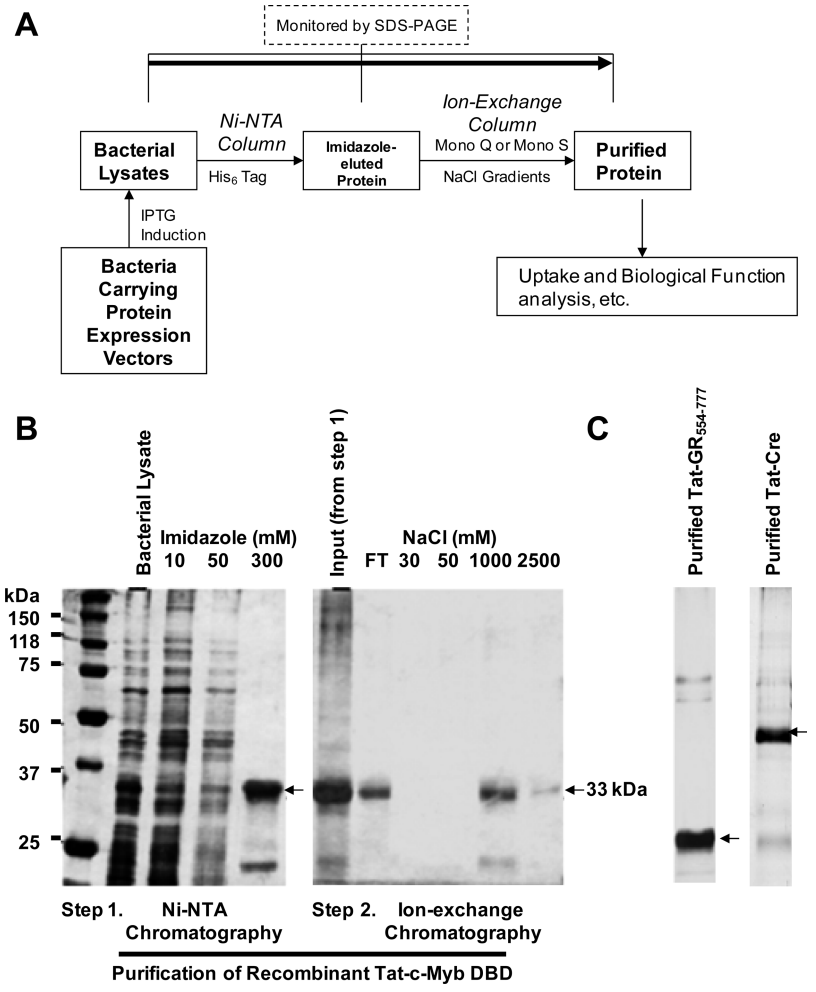

FIGURE 1: Recombinant expression and purification of Tat-fusion proteins and peptides. The *E. coli* strain BL21 (DE3) Gold carrying pTAT vectors for fusion proteins or peptides was induced by IPTG to express the Tat-fusion protein as described in Materials and Methods. The bacterial lysates were loaded onto a Ni-NTA column to purify 6×His-tagged fusion proteins. The Ni column purified proteins were loaded onto an ion-exchange column and eluted with increasing NaCl concentrations. (A) Diagram of the process to obtain purified recombinant Tat-fusion proteins or peptides. (B) SDS−PAGE of protein aliquots from each step of purification of recombinant Tat-c-Myb DBD. The SDS gels were visualized by silver staining. (C) Purified Tat-$GR_{554-777}$ and Tat-Cre protein.

was digested with *Sal*I/*Xho*I, gel purified, and ligated into the pET28.2 TAT vector (a generous gift from Dr. S. F. Dowdy, UCSD, La Jolla, CA) that had been linearized with the same restriction enzymes, to give the pTat-cMyb DBD expression vector. For Tat-$GR_{554-777}$, the cDNA coding sequence for the human GR peptide (amino acids 554−777) was PCR amplified from the human GRα protein expression vector pCYGR (a kind gift from Dr. J. A. Cidlowski, NIEHS, Research Triangle Park, NC) using the forward primer 5′-AGACTCAACTTGGAG-GATCATG-3′ and the reverse primer 5′-TGCTCGAGCTTTT-GATGAAACAGAAGTTTTTTGAT-3′ (*Xho*I). After digesting with *Xho*I, the PCR-amplified $GR_{554-777}$ cDNA coding fragment was inserted into the pET28.2 TAT vector. The resulting construct is pTat-$GR_{554-777}$. The GR protein sequence coded for by this construct includes the entire ligand binding domain but lacks the amino-terminal domain and the DNA binding domain. A forward primer, 5′-TCTGGTTTTGTCAAGCCCCAGTAATG-3′, and a reverse primer, 5′-CA*CTCGAG*AGCTTCATCAGAGCACAC-CAG-3′ (*Xho*I), were used to amplify the cDNA coding sequence for amino acids 263−428 of the human GRα protein by PCR (from the pCYGR vector). The resultant PCR fragment was digested with *Xho*I and inserted in the pET28.2 TAT vector to create pTat-$GR_{263-428}$. The GR protein sequence coded for by this construct comprises the region in the amino-terminal domain that follows the tau 1 region plus the first seven amino acids of the DNA binding domain, but it lacks the rest of the DNA binding domain and the entire ligand binding domain. All constructs were sequenced to confirm the in-frame insertions of the designed target sequences. The human c-Myb DNA binding domain (DBD) expression construct, pcDNA3-c-Myb DBD, was kindly provided by Dr. Giuseppe Raschellà [Ente Nuove Tecnologie Energia Ambiente (ENEA), Rome, Italy]. The human GR promoter luciferase reporter constructs, pXP-1D (−4525/−4898) and pXP-1C (−2523/−2986), were described previously (*25*, *26*, *40*).

RNA Purification and Real Time Quantitative Reverse Transcription Polymerase Chain Reaction (*QRT-PCR*). To prepare total RNA, cells were extracted with Tri Reagent (Molecular Research Center, Cincinnati, OH). RNA was precipitated with 2-propanol, washed with 70% EtOH, and air-dried. After being dissolved in nuclease-free water, the RNA samples were used for RT-PCR or stored at −80 °C. The primers and Taqman probes used for each hGR transcript (1A3, 1C, 1D, or exon 8−9 hGR α) were designed and synthesized. The sequences are listed in Table 1. The Taqman one-step RT-PCR reagents (Applied Biosystems, Foster City, CA) were used for RT-PCR detection of transcripts as described previously (*13*).

Table 1: Probes and Primers Used in QRT-PCR Analysis

targeted sequences	forward primer	Taqman probe	reverse primer
hGR exon 1A3	5′GCCTGGCTCCTTTCCTCAA3′	5′CTCCAAAGAATCATTAACTCCTG-GTAGAGAAGAAAACC3′	5′CCTGAGCAAGCAA-GCACACTGCTG3′
hGR exon 1C	5′TGTCTGTGACGGGAGCCC3′	5′CTCCAAAGAATCATTAACTCCTG-GTAGAGAAGAAAACC3′	5′CCTGAGCAAGCAA-GCACACTGCTG3′
hGR exon 1D	5′CCGCACAAGTTGATATTCACTGAT3′	5′CTCCAAAGAATCATTAACTCCTG-GTAGAGAAGAAAACC3′	5′CCTGAGCAAGCAA-GCACACTGCTG3′
hGR exon 8−9α	5′GGCAGCGGTTTTATCAACTGA3′	5′TTTCAACCACTTCATGCATAGAA-TCCAAGAGTTT3′	5′AATGTTTGGAAGCAA-TAGTTAAGGAGA3′

Each transcript measurement was normalized to the 18S rRNA of its total RNA sample, respectively.

Transient Transfection and Luciferase Reporter Gene Assay. Electroporation was used for transient transfection of 697 pre-B-ALL cells with the luciferase reporters, based upon the modifications of an unpublished protocol (kindly provided by Dr. E. Brad Thompson, UTMB, Galveston, TX). Briefly, cells in log phase growth were collected by centrifugation and washed. The cells were resuspended at 1×10^7 cells/mL in serum-free RPMI 1640 medium containing 1.25% DMSO. Aliquots of 400 μL (4×10^6 cells) were mixed with 3 μg of the reporter construct vectors plus 3 μg of a β-galactosidase expression vector (to normalize for transfection efficiency), and the whole was transferred into 0.4 cm gap electroporation cuvettes (Bio-Rad). The cuvettes were electroporated using 975 μF and 250 V with a Gene Pulser II (Bio-Rad) electroporator and then cooled on ice for 5 min. Electroporated cells from each cuvette were diluted into 3 mL of RPMI 1640 supplemented with 10% FBS and 1.25% DMSO and cultured overnight. Cells were washed two times with RPMI 1640 containing 10% FBS and were resuspended in 3 mL of the fresh medium to remove the DMSO. The cells were cultured for 4 h before recombinant peptides were added for transduction. After 24 h, ethanol vehicle (control) or Dex (1 μM final concentration) was added and incubation continued for another 24 h. The cells were collected, washed, and analyzed for luciferase reporter activity and β-galactosidase activity as described previously (*40*). For each sample, the luciferase signal was normalized to its own β-galactosidase value to normalize for sample variations in transfection efficiency. Experiments were replicated three to five times to allow for statistical analysis.

Western Blotting. Cells were collected and lysed with 1× Laemmli sample buffer containing a protease inhibitor cocktail (Sigma, St. Louis, MO). Proteins were resolved on 10% SDS−PAGE and transferred to immobilon nitrocellulose (NC) membranes (Millipore, Bedford, MA). ECL (Amersham Biosciences, Pittsburgh, PA) detection was then performed. Rabbit polyclonal antibodies for the hGR (H-300), c-Myb (H141), and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) were obtained from Santa Cruz Biotechnologies (Santa Cruz Biotechnology, Santa Cruz, CA).

Viability Analysis. The Vybrant apoptosis assay kit no. 4 (Invitrogen) was used to determine the viability of cells. The cells were collected by centrifugation and washed two times with phosphate-buffered saline (PBS, pH 7.4, without Ca^{2+} and Mg^{2+}). They were resuspended in 1 mL of PBS, the reagents were added, and the whole was incubated according to the manufacturer's instruction. Two-channel flow cytometry (BD Biosciences Instrument, Franklin Lakes, NJ) was used to monitor the intracellular accumulation of the YO-PRO dye and propidium iodide. The numbers of live cells (those that did not take up either dye) were determined. The results were plotted as the percentage of viable cells in Dex-treated samples versus the EtOH controls.

RESULTS

Recombinant Expression and Purification of Tat-Peptide-Fused c-Myb DBD and $GR_{554-777}$ Peptides. The recombinant Tat fusion peptides were purified from bacteria as described in Materials and Methods (Figure 1A). Fractions from each step of purification were analyzed by SDS−PAGE and silver staining of proteins on the gel. Figure 1B shows the samples from each step during the purification process for the recombinant Tat-c-Myb DNA binding domain (Tat-c-Myb DBD). The built-in 6×histidine tag in the pET28.1 TAT vectors is fused at the C-terminus of the peptides, which provides for a convenient one-step purification of these recombinant proteins with a Ni-NTA resin. The silver-stained gel shows that Ni-NTA agarose affinity chromatography effectively removes almost all contaminant bacterial proteins from the recombinant peptides. Cation ion-exchange chromatography and NaCl elution further eliminated trace contaminant proteins as well as the denaturant (urea) from the Tat-c-Myb DBD peptide. As shown by the stained gel, the purified Tat-c-Myb DBD peptide was eluted from the ion-exchange column with 1 M NaCl (Figure 1B). Using a similar purification process, highly purified Tat-$GR_{554-777}$ (Figure 1C), Tat-Cre (Figure 1C), and pTat-$GR_{263-428}$ (data not shown) were obtained in the 1 M NaCl eluted fractions. The authenticity of the purified fusion proteins was further confirmed by Western blotting with protein specific antibodies (data not shown). Tat-Cre, a bacterial protein, should not have any functions in the ALL cell lines, and it was used as a nonfunctional, cell-permeable, Tat fusion protein control in our experiments.

Transduction of Recombinant Tat-Fusion Peptides into Lymphoblastic Leukemia Cells. Using FITC-labeled peptides, we tested the capability and the efficacy of the purified recombinant Tat fusion peptides to transduce across the membranes of CEM-C7 (T-cell acute lymphoblastic leukemia) and 697 (pre-B-cell acute lymphoblastic leukemia) cells. The cells were inspected via fluorescence microscopy for the specific intracellular accumulation of a fluorescent signal after 15 min, 4 h, 24 h, 2 days, 3 days, and 4 days of incubation. The Tat-c-Myb DBD (dominant negative DNA binding domain mutant), Tat-$GR_{554-777}$, Tat-Cre, and Tat-$GR_{263-428}$ recombinant peptides can all efficiently transduce these lymphoid cell lines to a level of almost 100% after 36 h (697 cells) or 48 h (CEM-C7 cells) of incubation, while FITC-labeled BSA (which lacks the Tat, protein transduction domain) cannot traverse the cell membrane (Figure 2A). After the first 4 h of transduction about 30% of the CEM-C7 and 697 cells were transduced (data not shown). While

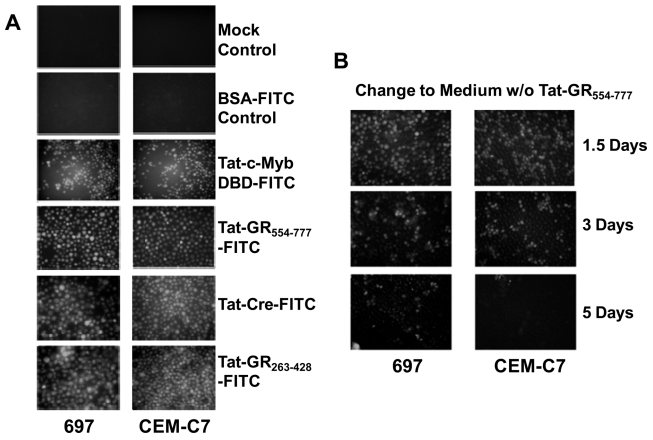

FIGURE 2: Transduction of Tat-fusion peptides into lymphoid cells. (A) The purified recombinant Tat-fusion peptides, Tat-c-Myb DBD, Tat-$GR_{554-777}$, Tat-Cre, and Tat-$GR_{263-428}$, were labeled with FITC and incubated with cultured cells for 36 h (697 pre-B-ALL cells) or 48 h (CEM-C7 T-ALL cells). The samples were then observed via fluorescence microscopy. (B) Reversibility of transduction of recombinant Tat-fusion peptides in lymphoid cells. The cultured cells were transduced with the Tat-$GR_{554-777}$ fusion peptide for 48 h, such that virtually 100% of the cells in the two cell lines contained the fluorescent peptide. The cells were washed and incubated in peptide-free medium for the indicated times. The FITC signal for the labeled peptide was gradually lost from the cells, indicating the reversibility of the process.

a large accumulation of the fluorescent signal was observed in the 697 cell line as soon as 24 h after transduction, CEM-C7 cells tended to need a longer time to be equivalently transduced (2 days; data not shown). Similar results were obtained for both the FITC-labeled Tat-c-Myb DBD and Tat-$GR_{554-777}$ peptides, suggesting that this is a function of the cell type rather than of the particular Tat-fusion peptide. These data demonstrate the efficient transduction of Tat-fusion proteins into lymphoid cells and perhaps indicate subtle permeability differences for different recipient cell types. Results similar to those seen for the 697 cell line were obtained for the IM-9 B-lymphoblastoid cell line, and results similar to the CEM-C7 cell line were obtained for its hormone-resistant derivative line, CEM-C1 (data not shown).

Replacement of the culture medium with fresh medium without Tat-fusion peptides resulted in a gradual elimination of the fluorescent label over time, with the eventual complete loss of all label from inside of the cells. A gradual loss of Tat-$GR_{554-777}$ from equivalently transduced lymphoid cells is observed (Figure 2B). Interestingly, the loss of the fluorescent signal was also not at the same rate for the different cell lines, as CEM-C7 cells tended to lose the label somewhat faster than 697 cells (Figure 2B). These data indicate that the transduction of these recombinant peptides in these lymphoid cells is a transient process and is reversible when Tat-fusion peptide was removed from the medium. Furthermore, cell type differences in both uptake and loss were noted.

Biological Function Analysis of Transduced Recombinant Tat-Fusion Peptides. Our previous studies showed that the autoupregulation of hGR gene expression depends on a class of composite GRUs (containing binding sites for the GR, c-Myb, and Ets protein family members) in GR promoters (1A, 1B, 1C, 1D, and 1F), and the molecular mechanism controlling the autoregulation of hGR promoters involves (besides the GR) the transcription factor c-Myb and/or Ets protein family members. Binding of the GR protein together with c-Myb at the composite GRU robustly stimulates the upregulation of hGR promoter activity and gene expression, while binding of the GR and an Ets protein (e.g., PU.1) at the same GRU suppresses promoter transcription in GC-resistant cells (*25*). (PU.1 is a transcription factor involved in granulocyte and lymphoid cell development and that binds to a *PU*rine-rich DNA sequence.) Transfection of a plasmid cDNA construct coding for a c-Myb DNA binding domain (DBD) dominant negative inhibitor blunts the transcriptional response of GR promoters to Dex upregulation (*25*, *26*, *40*). In 697 pre-B-ALL cells, the 1C and 1D promoters appear to be the major GR promoters that control GR expression, and they are significantly upregulated by hormone treatment (C. Geng and W. V. Vedeckis, in preparation). Therefore, we examined the biological function of the transduced Tat-c-Myb DBD peptide by assessing its ability to modify Dex-mediated autoupregulation of GR transcript expression in 697 cells. Also, using a luciferase reporter gene assay, we directly assessed the hormone responsiveness of GR promoters 1C and 1D in 697 cells transduced with Tat-c-Myb DBD.

Tat-Cre was used as a control for these and subsequent experiments. To determine if any transduction with any Tat-fusion protein might affect GR gene expression, we compared GR 1C- and 1D-luciferase activity in mock-transduced cells (no Tat-fusion protein) to that in Tat-Cre transduced cells (Figure 3A). There was no significant effect of Tat-Cre on GR promoter 1C or 1D activity, as the stimulation of the luciferase reporter gene was similar to that in the mock-transduced sample. However, incubation with the purified Tat-c-Myb DBD fusion peptide (400 ng/mL final concentration) caused significant suppression of hGR promoter 1C- and 1D-driven expression of the luciferase reporter gene in 697 cells (Figure 3A). Also, using QRT-PCR, we monitored the response of endogenous GR transcript expression to hormone treatment in 697 cells transduced with the Tat-c-Myb DBD peptide (400 ng/mL). As seen in the promoter−luciferase reporter assay, transduction of the Tat-c-Myb DBD suppresses steroid stimulation of GR gene expression of total GRα transcripts (hGR 8/9α), as well as the transcripts originating from GR promoters 1C and 1D (Figure 3B). Tat-Cre, the nonspecific protein transduction control, has no effects on the hormone responsiveness of promoters in all these cases, nor did it have any effect of its own, as the steroid-mediated upregulation observed was very similar to that seen in the mock-transduced sample. Using Western blot analysis, we also examined the intracellular GR protein expression in 697 cells transduced with the Tat-c-Myb DBD peptide. As seen for the suppressed upregulation of GR transcripts by Tat-c-Myb DBD transduction, GR protein expression is also decreased in 697 cells incubated with Tat-c-Myb DBD and autoupregulation upon Dex treatment is severely blunted (Figure 4). These results are expected for the function of c-Myb DBD as a dominant negative inhibitor of the endogenous c-Myb protein. Because results using the Tat-c-Myb DBD recombinant peptide are the same as our previous transfection overexpression analysis using a c-Myb DBD plasmid cDNA construct (*25*, *26*, *40*), the biological function of recombinant Tat-c-Myb DBD in 697 pre-B-ALL cells is confirmed.

In published studies on the protein PGC-1, an increased concentration of endogenous protein was observed after overexpressing specific partial peptide domains that appear to interfere with the ubiquitin−proteasome-mediated degradation of the full-length endogenous protein (*41*). That is, these partial peptide domains may act as competitive inhibitors of the ubiquitin ligase required for the degradation of the native, full-length protein.

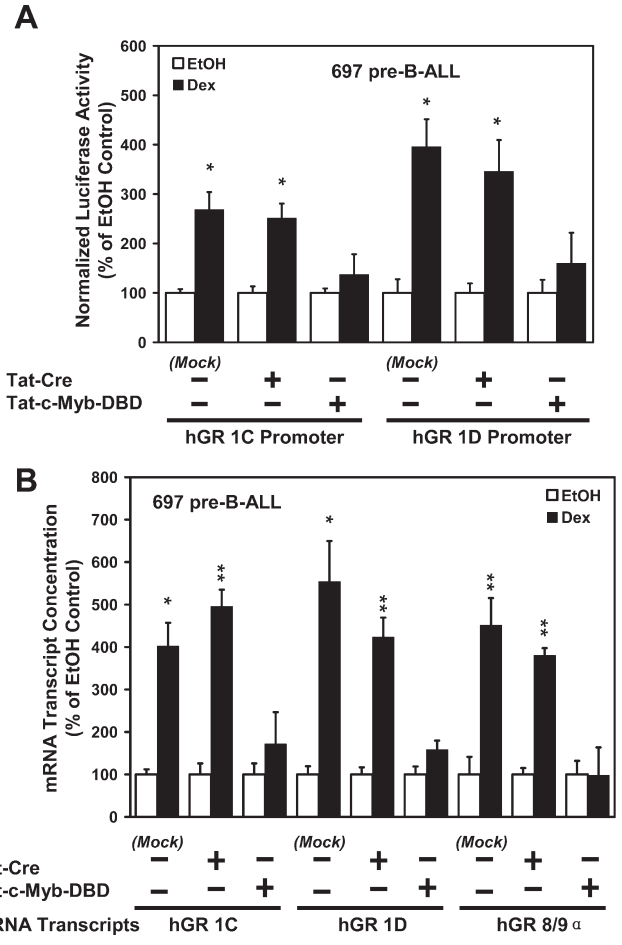

FIGURE 3: Transduction of the Tat-c-Myb DBD peptide effectively suppresses autoupregulation of GR gene expression in 697 pre-B-ALL cells. (A) Transient reporter gene analysis of hGR gene promoters in 697 cells transduced with the purified recombinant Tat-c-Myb DBD peptide and the GR promoter luciferase reporter genes. For each sample, the GR-luciferase reporter gene plasmid was cotransfected with a CMV promoter-β-galactosidase reporter gene. The luminescent signal of the sample was normalized to the value obtained for the β-galactosidase activity in the same sample, to adjust for variations in transfection efficiencies between samples. The normalized value for the Dex-treated sample was divided by the normalized value from its respective ethanol control sample, and this value was multiplied by 100 to give the percent of ethanol control value. (B) QRT-PCR analysis of the expression of endogenous hGR trancripts in Tat-c-Myb DBD transduced 697 pre-B-ALL cells. Purified recombinant Tat-Cre was used as the control Tat-fusion peptide to treat 697 cells. Three separate experiments were performed. The data represent the mean $\pm$ the SEM for Dex-treated samples versus the EtOH-treated control (value set at 100%). *, $P < 0.05$, and **, $P < 0.01$.

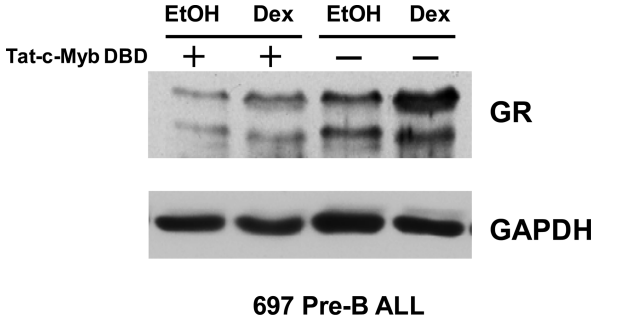

FIGURE 4: Transduction of the Tat-c-Myb DBD peptide leads to the suppressed expression of intracellular GR protein levels in 697 pre-B-ALL cells. 697 cells were incubated with the Tat-c-Myb DBD for 48 h, after which they were treated for an additional 18 h with 1 μM Dex or an equivalent volume of the ethanol vehicle. Western blotting was performed as described in Materials and Methods. GR levels were decreased in 697 cells as a result of Tat-c-Myb DBD transduction. GAPDH was used to monitor loading of total protein on the gel.

However, to date, only limited data on the use of such "stabilizers" have been reported. We identified the known subdomains of the GR that interact with ubiquitin ligases (see Discussion), designed and purified a corresponding recombinant Tat-fusion GR peptide (Tat-$GR_{554-777}$), and tested its capability to transduce ALL cells (Figure 2). We then tested if Tat-$GR_{554-777}$ causes an increase in the endogenous full-length GR protein levels, as expected from its predicted function. Tat-$GR_{554-777}$ was incubated with 697 and CEM-C1 cells (a steroid-resistant derivative of CEM-C7 cells containing reduced levels of functional GR (*42*)) for 48 h at different final concentrations (40, 80, and 400 ng/mL), and the endogenous GR protein concentration was evaluated using Western blotting with GR specific antibodies. The endogenous GR protein levels were elevated in Tat-$GR_{554-777}$-transduced CEM-C1 and 697 ALL cells, compared to controls that were transduced with the nonfunctional cell permeable protein control, Tat-Cre (Figure 5). Quantitation of the GR bands by densitometry revealed a dose-dependent correlation of endogenous, full-length GR protein levels with increasing concentration of added Tat-$GR_{554-777}$ (Figure 5). There was a greater than 4-fold increase of GR in CEM-C1 cells that were incubated with 400 ng/mL Tat-$GR_{554-777}$, while in 697 cells a 3-fold increase in GR was observed. These results indicate that (1) the purified recombinant Tat-$GR_{554-777}$ peptide can effectively transduce the lymphoblastic leukemia cells and (2) the designed Tat-$GR_{554-777}$ protein is functional in increasing endogenous GR protein concentration.

Together these data demonstrate that, upon transduction into lymphoblast cells, both of these two recombinant Tat-fusion peptides are functional and mediate the expected biological functions. The most important result, however, would be if Tat-$GR_{554-777}$ treatment could increase the sensitivity of ALL blasts to steroid-mediated killing.

Modifying GC Sensitivity of ALL Cells by Tat-$GR_{554-777}$ Transduction. The expression level of GR protein in ALL cells largely determines the sensitivity of these leukemia cells to GC-induced apoptosis. Although there is some diversity in opinion (*24*), numerous studies on cell lines and primary clinical leukemic cell analysis have shown that insufficient GR expression appears to be one of the important causes for GC resistance in ALL (*12*, *20*, *23*, *43*). CEM-C1 cells (a GC-resistant CEM-C7 derivative cell line) express a low level of GR protein and lack substantial autoupregulation of GR expression during hormone treatment ((*42*); C Geng and W. V. Vedeckis, unpublished). Increasing the GR level in these cells using stable transfection of a rat GR expression vector restored GC-sensitive apoptosis (*42*). Thus, we reasoned that increasing the GR protein in these cells by treatment with Tat-$GR_{554-777}$ (Figure 5A) might also render them sensitive to GC-mediated apoptosis. Compared to cells treated with the Tat-Cre control, transduction of Tat-$GR_{554-777}$ effectively converts the resistant CEM-C1 cells to Dex sensitivity in a dose-dependent manner (Figure 6A). There was a good correlation between elevated GR protein levels (Figure 5A) and increased GC sensitivity (Figure 6A). Moreover, in hormone-sensitive pre-B-ALL 697 cells, transduction of Tat-$GR_{554-777}$ further sensitized them to Dex treatment at the 40 and 400 ng/mL

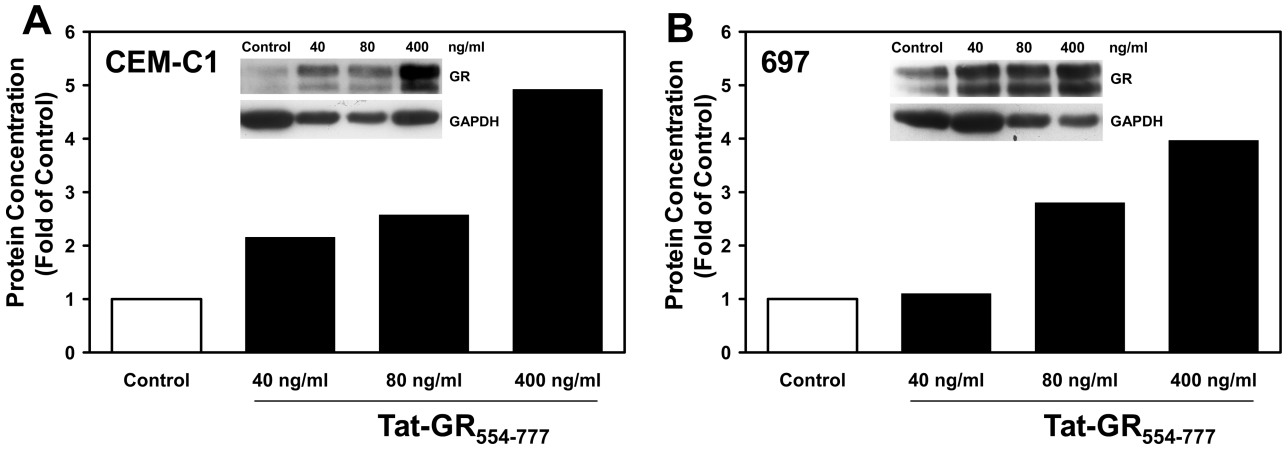

FIGURE 5: Transduction of Tat-$GR_{554-777}$ effectively increases the intracellular GR protein levels in CEM-C1 T-ALL cells and 697 pre-B-ALL cells in a dose-dependent manner. After 48 h of transduction, the expression of endogenous GR protein at each dose of transduced Tat-$GR_{554-777}$ was detected by Western blotting with a GR specific antibody that recognizes a sequence in the N-terminus (which is absent in Tat-$GR_{554-777}$). The amount of GR protein was determined by densitometry and normalized to GAPDH protein levels in the same sample. The plot displays the fold differences comparing to the Tat-Cre-treated control cell samples (which are set at 1).

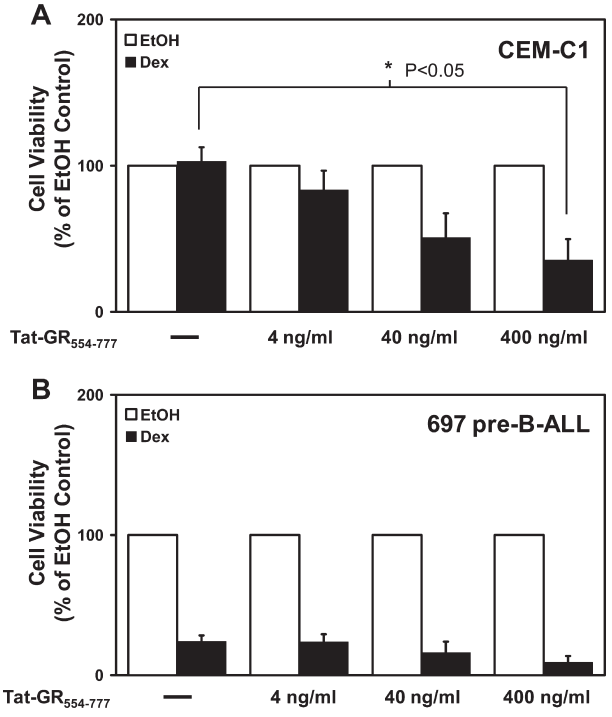

FIGURE 6: Sensitization of cells to glucocorticoid-induced cell death by transduction of purified recombinant Tat-$GR_{554-777}$ into ALL cells. Cells were transduced with the indicated concentrations of Tat-$GR_{554-777}$ for 48 h. They were then treated for an additional 36 h with 1 μM Dex or an equivalent volume of ethanol vehicle. Cell viability, determined using the Vybrant apoptosis assay kit no. 4 (Invitrogen), in the Dex-treated sample was compared to the ethanol vehicle-treated respective control sample. (A) Transduction of Tat-$GR_{554-777}$ results in conversion of the steroid-resistant CEM-C1 T-ALL cell to hormone sensitivity in a dose-dependent manner. (B) Tat-$GR_{554-777}$ further sensitizes the 697 pre-B-ALL to GC killing.

doses (Figure 6B), in line with the increased intracellular GR protein levels (Figure 5B). These data suggest that increased doses of the Tat-$GR_{554-777}$ caused increases in the endogenous GR levels, which then resulted in an increase in hormone-mediated apoptosis. Finally, we determined the specificity of these responses (Figure 7). Mock-treated cells, Tat-Cre-treated cells, and cells incubated with a region that incorporates part of the amino-terminal domain of the GR did not cause an increase in endogenous GR protein in either CEM-C1 cells (Figure 7A,B) or 697 cells (Figure 7D,E), while incubation with Tat-$GR_{554-777}$ did increase GR protein levels. The concept that the increased intracellular GR is responsible for increased steroid-sensitivity is supported by data for CEM-C1 cells (Figure 7C) and 697 cells (Figure 7F), where an enhanced corticosteroid-mediated cell killing was only seen for the fusion peptide that caused increased GR levels, namely, Tat-$GR_{554-777}$.

DISCUSSION

Glucocorticoids are routinely used to treat ALL because they are an effective component in combination chemotherapy, as they trigger apoptosis in sensitive blasts. Importantly, the initial response of the ALL patient to steroid treatment is the most accurate predictor for long-term prognosis (*9*, *44*, *45*). However, initial as well as acquired GC resistance is a troublesome clinical problem. While mutation in the coding sequence of the GR in patients is a rare event that does not contribute significantly to the development of steroid resistance (*46*−*48*), lowered levels of wild-type, intracellular GR appears to be a more common cause of acquired steroid resistance. Thus, directly targeting the GR concentration in these cells could cause an increase in the steroid responsiveness and lead to a better clinical response.

The successful design, production, and validation of the biological effects of the Tat-$GR_{554-777}$, which is permeable in ALL cells, appear to be promising developments to reach this goal. Even though we have successfully demonstrated the ability of Tat-$GR_{554-777}$ to increase the endogenous GR protein levels in ALL cells, the mechanism involved in the augmentation of the cellular levels of full-length protein is currently unclear. The concentration of cellular proteins is finely adjusted in the cell by their rates of synthesis and degradation. While gene transcription plays a critical role in regulating intracellular protein levels, the turnover rate of proteins is often equally as important, and this occurs mainly via the ubiquitin−proteasome system (*49*, *50*). For example, proapoptotic Bcl members like Bim tend to have very short half-life (*51*), and general inhibitors that block protein degradation efficiently cause apoptosis in treated cells, presumably by stabilizing these proapoptotic proteins (*52*, *53*). During the turnover process, the protein is recognized and physically interacts with a certain class of ubiquitin ligase (E3) through

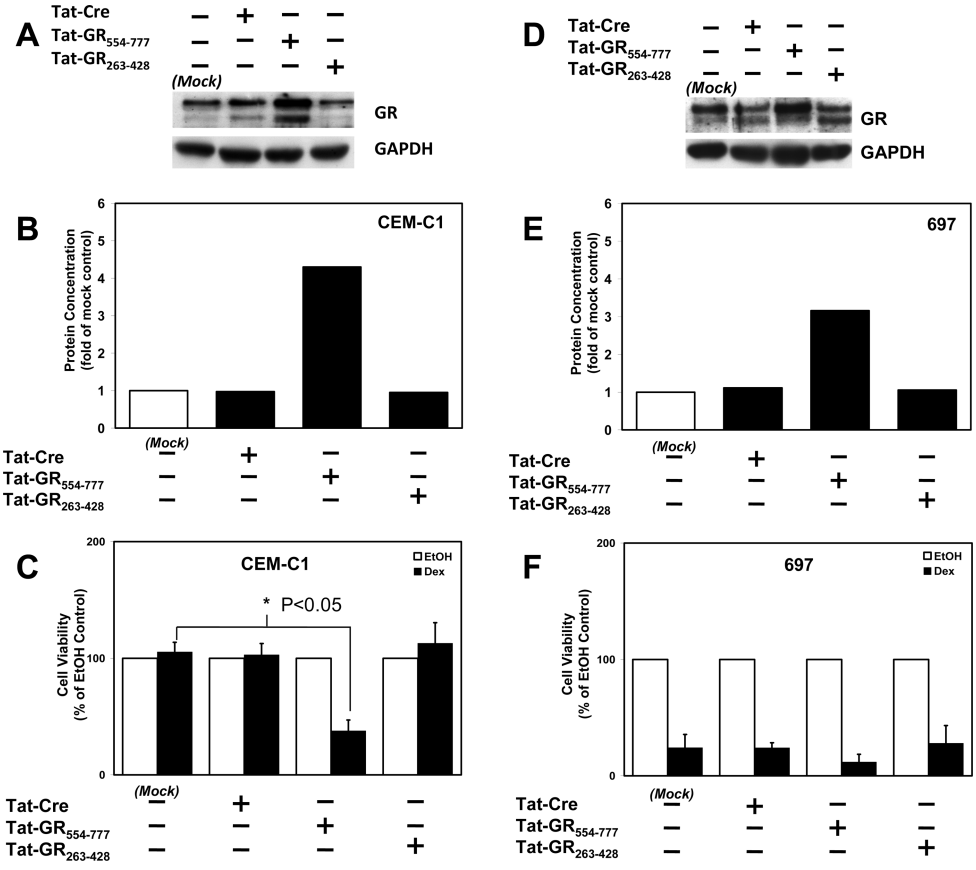

FIGURE 7: Transduction of Tat-GR554−777 specifically increases the intracellular GR protein levels in CEM-C1 T-ALL cells and 697 pre-B-ALL cells and sensitizes cells to glucocorticoid-induced cell death. (A, D) The expression of the endogenous GR protein was detected by Western blotting in ALL cells transduced by Tat-Cre, Tat-$GR_{554-777}$, and Tat-$GR_{263-428}$. (A) CEM-C1 T-ALL cells; (D) 697 pre-B-ALL cells. (B, E) The amount of GR protein in ALL cells transduced by recombinant Tat-fusion peptides was determined by densitometry and normalized to GAPDH protein levels. Figures show the fold differences of transduced samples versus the mock controls. (B) CEM-C1 cells; (E) 697 cells. (C, F) Cell cultures were transduced for 48 h with the respective Tat-fusion peptide and then were treated for an additional 36 h with 1 μM Dex or an equivalent volume of the ethanol vehicle. Cell viability, determined using the Vybrant apoptosis assay kit no. 4 (Invitrogen), in the Dex-treated sample was expressed as a percent of the respective ethanol vehicle-treated control sample. (C) Tat-$GR_{554-777}$ specifically results in conversion of the steroid-resistant CEM-C1 T-ALL cell to hormone-sensitivity; (F) Tat-$GR_{554-777}$, but not Tat-Cre or Tat-$GR_{263-428}$, further sensitizes the 697 pre-B-ALL cells to GC killing.

specific peptide domains located in the target molecule. The ubiquitin ligase adds ubiquitin groups at specific sites on the protein, and the modified protein is then sorted and routed to the proteasomic degradation machinery. Using transfected constitutive expression vectors, overexpression of the C-terminal peptide portion of c-Myb in cells results in significant accumulation of endogenous full-length c-Myb protein (*54*−*56*). Similar observations were made for PGC-1, where the intracellular overexpression of its C-terminal domain significantly increased the cellular concentration of full-length PGC-1 (*41*). These studies suggest a specifically suppressed turnover rate of the targeted proteins, presumably via the overexpressed peptide domains being recognized by and directly interacting with the ubiquitin ligases specific for the full-length proteins. Indeed, studies have shown that overexpressing these exogenous peptides strongly decreases ubiquitination of the endogenous protein, presumably via a competitive inhibition of the relevant ubiquitin ligases. Thus, these peptides function as "stabilizers" of their respective full-length proteins. Specific E3 ligases, such as CHIP (*57*) and Hdm2 (*58*), regulate ubiquitination of the GR. Furthermore, the carboxyl-terminal ligand binding domain of the GR physically interacts with the E2 ubiquitin ligases, Ubc9 (*59*) and UbcH7 (*60*). Thus, we focused our attention on the carboxyl terminus of the GR in the present studies when we generated Tat-$GR_{554-777}$, because it was a likely candidate to be targeted by the ubiquitin−proteasome system. Although it remains to be definitively demonstrated, it thus seems likely that transduced Tat-$GR_{554-777}$ increases endogenous GR protein levels in ALL cells by blocking endogenous GR protein ubiquitination via the competitive inhibition of the cognate ubiquitin ligase. Further studies and direct evidence are needed to determine if this putative "stabilizer peptide" mechanism of action is operative for Tat-$GR_{554-777}$ in ALL cells.

From a technical viewpoint, there are two potential advantages to using a stabilizer peptide approach. First, it is likely that this will have specificity in targeting the protein of interest by presumably inhibiting primarily the cognate E3 ubiquitin ligase. This might prevent problems with "off-target" effects seen with less specific drugs and inhibitors. The fact that a different GR fusion protein, Tat-$GR_{263-428}$, did not have any effects on protein level and cell viability supports a specific mechanism of action for Tat-$GR_{554-777}$. Second, these stabilizers are relatively small in size. In theory, these smaller fragments should be easier to produce and purify than the full-length proteins. In addition, it should be more likely that the smaller peptide would fold into a native state after extraction from bacteria with a denaturant. This could be important when it comes to determining the usefulness of these molecules as potential reagents in translational studies.

In summary, we have successfully obtained peptides fused to the Tat PTD. Using this technology, we have generated a small, cell-permeable recombinant peptide, Tat-$GR_{554-777}$, that efficiently elevates the level of endogenous GR proteins and sensitizes ALL cells to GC-induced apoptosis. These studies suggest a potential novel avenue of treatment for acute lymphoblastic leukemia: "stabilizer protein therapy". Because the "stabilizer" is designed to specifically target the endogenous expression of its respective full-length, functional protein, its use may limit nonspecific, "off target" effects on other cellular proteins, thus minimizing deleterious side effects. As opposed to gene therapy, where the biological treatment is irreversible, stabilizer protein therapy is not genetically stable. Rather, it is transient, and the transduced peptide diffuses out of the cell and/or is eventually degraded. Thus, any unwarranted side effects would only occur during therapy and depend upon the half-life and clearance rates of the stabilizer peptide. This approach may have eventual clinical applications to improve the treatment of ALL patients by providing novel, specific, low-toxicity treatment agents to supplement current treatment protocols.

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