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Inhibition of AP-1 by the Glucocorticoid-inducible Protein GILZ*

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The immunosuppressive effects of glucocorticoids arise largely by inhibition of cytokine gene expression, which has been ascribed to interference between the glucocorticoid receptor and transcription factors such as AP-1 and NF-κB as well as by competition for common coactivators. Here we show that glucocorticoid-induced inhibition of interleukin-2 mRNA expression in activated normal T cells required new protein synthesis, suggesting that this phenomenon is secondary to expression of glucocorticoid-regulated genes. One of the most prominent glucocorticoid-induced genes is glucocorticoid-induced leucine zipper (*GILZ*), which has been reported to inhibit activation-induced up-regulation of Fas ligand (FasL) mRNA. Indeed, transient expression of GILZ in Jurkat T cells blocked induction of a reporter construct driven by the FasL promoter. This could be accounted for by GILZ-mediated inhibition of Egr-2 and Egr-3, NFAT/AP-1-inducible transcription factors that bind a regulatory element in the FasL promoter and up-regulate FasL expression. GILZ also potently inhibited AP-1-driven and IL-2 promoter-driven reporter constructs, and recombinant GILZ specifically interacted with c-Fos and c-Jun *in vitro* and inhibited the binding of active AP-1 to its target DNA. Whereas homodimerization of GILZ required the presence of its leucine zipper, the interaction with c-Fos and c-Jun occurred through the N-terminal 60-amino acid region of GILZ. Thus, GILZ represents a glucocorticoid-induced gene product that can inhibit a variety of activation-induced events, at least in part by direct interference with AP-1, and is therefore a candidate for a mediator of glucocorticoid-induced immunosuppression.

Glucocorticoids are potent immunosuppressive agents that inhibit T cell activation and cytokine production. They are used clinically for treatment of autoimmune and inflammatory diseases, such as rheumatoid arthritis and asthma, and for preventing transplant rejection. Glucocorticoids inhibit transcriptional up-regulation of T cell-derived cytokines, such as IL[1]-2, IL-4, IL-10, and γ-interferon (1–3), and proinflammatory cytokines, such as IL-1, granulocyte-macrophage colony-stimulating factor, and tumor necrosis factor-α. Another target of glucocorticoids in T cells is Fas ligand (FasL), a membrane protein that triggers apoptosis of mature T cells by engaging Fas (4, 5). Inhibition of FasL-initiated apoptosis by glucocorticoids may help to stem the loss of immune cells in HIV-infected individuals (6). The apparent immune-enhancing effect of FasL suppression underscores the complexity of glucocorticoid biology and implies that there are numerous levels where glucocorticoid actions are controlled.

Glucocorticoids are small hydrophobic molecules that diffuse through the plasma membrane to reach the cytosolic glucocorticoid receptor (GR), which then translocates to the nucleus to regulate transcription. The GR contains an N-terminal hormone-independent activation domain, a DNA-binding domain containing a pair of zinc fingers, and a C-terminal ligand-dependent activation domain integrated with the ligand-binding domain (2, 7). The C terminus is also the site of interaction with coactivator proteins, including SRC-1, CBP/p300, and PCAF (8–10). Ligand-bound GR is mostly monomeric in solution but homodimerizes upon cooperative binding to a GRE, a region of DNA containing two inversely symmetrical GR-binding elements (11). Classic GREs regulate glucocorticoid-inducible genes that play roles in metabolism and cell growth, such as those encoding metallothionein, tyrosine aminotransferase, alanine aminotransferase, and phosphoenolpyruvate carboxykinase (7, 12). The activated GR can also suppress transcription upon binding negative GREs, whose sequences differ from the classic GRE, and are found in the genes encoding IL-1β (13), pro-opiomelanocortin (14), prolactin (15), α-subunit of glycoprotein hormone (16), and proliferin (17). GREs adjacent to other transcription factor-binding sites, such as for AP-1, can contribute to the function of composite elements where the GR can have either positive or negative effects, depending on the cellular context and the composition of AP-1 (18).

Increasing evidence indicates that the GR also represses gene activation without binding DNA. This mode of repression, termed "direct interference" (19), has been associated with glucocorticoid inhibition of AP-1 and NF-κB, transcription factors that contribute to the transcriptional induction of most cytokine genes. The first reported example of direct interference was glucocorticoid repression of the AP-1 response element (TRE) of the collagenase gene (20, 21). Both components of AP-1, c-Fos and c-Jun, were shown to interact with the GR in the absence of any GR to DNA interaction. Levels of AP-1 in the nucleus were not altered by glucocorticoids, and the ability of AP-1 to bind to the TRE was unaffected (20, 22). When overexpressed, however, the GR could displace c-Jun from the TRE (21), and c-Jun could displace the GR from the GRE (23). Consistent with the notion that the liganded GR was directly interfering with AP-1 function were the observations that repression could take place in the absence of new protein synthesis, occurred at a concentration of the synthetic glucocorticoid dexamethasone (Dex) lower than that required for transcriptional induction by the GR (20), and could be carried out by mutant GRs whose transactivation ability was disrupted (20,

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[1] The abbreviations used are: IL, interleukin; GR, glucocorticoid receptor; GRE, glucocorticoid-responsive element; TRE, TPA-responsive element; GST, glutathione *S*-transferase; PMA, phorbol myristate acetate; GAPDH, glyceraldyhyde-3-phosphate dehydrogenase; MOPS, 3-morpholinopropanesulfonic acid; CHX, cycloheximide; Dex, dexamethasone; PAGE, polyacrylamide gel electrophoresis; TCR, T cell receptor; Tet, tetracycline; Dox, doxycycline; GILZ, glucocorticoid-induced leucine zipper.

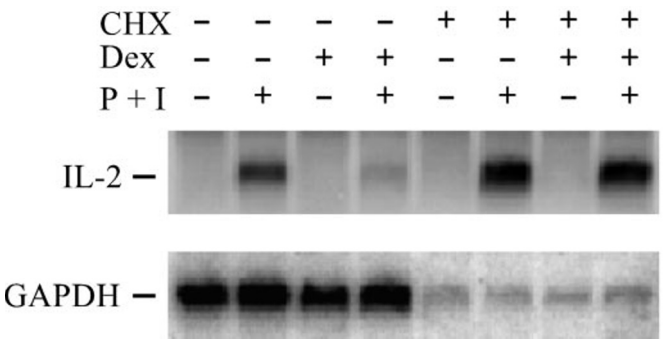

FIG. 1. **Repression of IL-2 mRNA induction by Dex requires new protein synthesis.** Human peripheral blood mononuclear cells were stimulated with PMA (20 ng/ml) and ionomycin (1 μg/ml) in the presence or absence of 1 μM Dex and 20 nM CHX. After 3 h, total RNA was extracted, and IL-2 mRNA was assessed by Northern blotting (*A*). Equivalence of loading among all samples was verified by ethidium bromide staining of the gel (data not shown). Equivalence of loading among CHX-treated and -untreated groups of samples was determined by re-probing the membrane with GAPDH cDNA (*B*). The experiment shown is representative of three independent experiments. *P + I*, PMA (20 ng/ml) + ionomycin (1 μg/ml).

21, 23). Direct interference by the GR with the p65 subunit of NF-κB has also been reported (24). The direct interference model for repression by nuclear receptors has been extended to the octamer-binding factors (25) and C/EBP (26) (repressed by the estrogen receptor). The direct interference model was further supported through the use of a C-terminal zinc finger point mutant GR whose homo-dimerization surface was disrupted, preventing cooperative binding to and transactivation through the classic GRE, which was able to repress AP-1 activity as a monomer (27). Mice whose normal GR was replaced by this mutant appeared grossly normal and had no obvious peripheral lymphoid defects, although their thymocytes did not undergo apoptosis upon treatment with Dex *in vitro* (28). In embryonic fibroblasts from these mice, Dex could block the TRE and the inflammatory mediators collagenase and gelatinase B, suggesting that direct interference is a major, if not the predominant, physiological mode of action of the GR.

Another transcription-independent model for repression by the GR invokes competition, or "squelching," between the GR and AP-1 or NF-κB for mutual coactivators such as p300/CBP. These coactivators possess histone acetyltransferase activity, which acts on chromatin to permit access to site-specific transcription factors. Limiting coactivators would thus serve to direct the cellular response toward the strongest signal (29). Evidence supporting this model is based on studies in which overexpression of coactivators (30) or treatment with histone de-acetylase inhibitors relieves glucocorticoid repression (31). However, this model does not satisfactorily account, for example, for the ability of the activated retinoic acid receptor to repress AP-1 but not NF-κB (32). Thus, additional mechanisms would be needed to confer specificity.

To determine if the immunosuppressive effects of glucocorticoids might be due at least in part to mechanisms other than direct interference or squelching, we asked whether the glucocorticoid-mediated inhibition of activation-induced upregulation of IL-2 mRNA requires new protein synthesis. Cycloheximide prevented the suppression, suggesting that a glucocorticoid-induced gene product might be responsible. A recent analysis of glucocorticoid-inducible genes in human peripheral blood mononuclear cells by cDNA gene chip array found that among the over 9,000 cDNA tested, the most highly induced gene was the DSIPI ("delta sleep-inducing peptide immunoreactor," named for its shared immunoreactivity with the sequence-unrelated nonamer neuropeptide DSIP (33)).[2] DSIPI shares nearly 100% nucleotide sequence homology with the C-terminal 75% of human glucocorticoid-induced leucine zipper (GILZ) (34). Because murine GILZ has been reported to inhibit the expression of FasL in activated T hybridoma cells, we investigated the possibility that this glucocorticoid-responsive gene might mediate the inhibitory effects of glucocorticoids on the expression of activation-induced genes.

EXPERIMENTAL PROCEDURES

Cells, Reagents, and Antibodies—The Jurkat human T cell line (ATCC) and the murine T cell hybridoma MA 5.8 (35) were maintained in RPMI 1640 (Biofluids Inc., Rockville, MD) supplemented with 4 mM glutamine, 50 μM β-mercaptoethanol, 100 units/ml penicillin, 150 μg/ml gentamicin, and 10% heat-inactivated fetal calf serum. Human peripheral blood mononuclear cells were obtained from the NIH Blood Bank and were isolated by density gradient centrifugation with the use of lymphocyte separation medium (Biofluids). Antisera against GILZ were prepared by immunizing with the GST-GILZ fusion protein. A rabbit was immunized with 500 μg of the GST-GILZ fusion protein mixed with complete Freund's adjuvant and was boosted with 100 μg of GST-GILZ in incomplete Freund's adjuvant at 2–4-week intervals. The monoclonal antibody against TCR-ζ was obtained from Ralph Kubo (La Jolla Institute of Allergy and Immunology, La Jolla, CA) (36). PMA, ionomycin, dexamethasone, and doxycycline were obtained from Sigma.

Plasmids—Luciferase reporters containing 511 nucleotides upstream of the FasL translation initiation site (−511) or containing the Egr-binding site (FLRE) (37) and Egr-2- (38) and Egr-3-driven (39) luciferase reporters have been described. The eukaryotic expression plasmid TAM-67, encoding a transactivation-defective c-Jun mutant, and the luciferase reporter constructs TRE-luc, driven by six copies of the proximal (−153) TRE element from the murine IL-2 promoter, NFAT-luc, driven by three copies of the distal (−287) NFAT/AP-1 element of the murine IL-2 promoter, and IL-2-luc, driven by 0.3 kilobase pairs of the human IL-2 promoter, have been described (40). c-*fos* and c-*jun* in pcDNA3 expression vectors were obtained from Nancy Colburn (NCI, National Institutes of Health). The GILZ cDNA was amplified from total RNA obtained from Dex-treated murine 2B4.11 hybridoma cells (41). Nucleotide 65 of GILZ derived from 2B4.11 cells (T) differed from the corresponding nucleotide in the original characterization of this gene (C) (GenBank NM 010286), resulting in a change in the predicted amino acid sequence from a threonine to an isoleucine. This difference was also found in the T cell hybridoma 3DO, as well as spleen cells from C57/BL6 mice, and is consistent with the published nucleotide sequences of rat (AB025431) and human (AB025432) GILZ. After adding a C-terminal Myc tag epitope, the cDNA was cloned into the *Eco*RI and *Xba*I sites of the expression vector pCI-neo (Promega, Madison, WI). The GILZ mutant ΔLZ, from which leucine zipper-spanning amino acids 76–96 were removed, was generated by polymerase chain reaction and subcloned into pCI-neo. GILZ-(C-terminal)Myc was transferred into the tetracycline-regulated expression vector pcDNA4/TO (Invitrogen). The plasmid pcDNA6, encoding the tetracycline repressor, was obtained from Invitrogen. Constructs encoding GST-GILZ fusion proteins were created by amplifying the GILZ cDNA and fragments 1–60, 61–104, 61–137, 105–137, and ΔLZ to incorporate *Bam*HI and *Eco*RI sites and transferring them into pGEX-4T2 (Amersham Pharmacia Biotech).

Transient Transfections—In transient reporter assays, 2×10^6 Jurkat cells in 0.2 ml of complete medium supplemented with 20 mM HEPES, pH 7.4, were electroporated with the indicated plasmids with a GenePulser (Bio-Rad) using 960 microfarads and 250 V. Empty parental expression plasmids were included to equalize the amount of DNA used in each cuvette. Where indicated, cultures were stimulated with 20 ng/ml phorbol myristate acetate (PMA) and 1 μg/ml ionomycin. Error bars represent the geometric error of duplicate transfections. Experiments using the tetracycline-regulated expression system were performed using tetracycline-free fetal calf serum (CLONTECH, Palo Alto, CA). 1 μg of the pcDNA4-GILZ constructs, 0.5 μg of the repressor-encoding plasmid pcDNA6, and 2.5 μg of the luciferase reporter plasmids were used. Doxycycline was added at a final concentration of 100 nM. The base-line activities of the reporter plasmids were generally in the range 300 to 6000 relative light units and did not change appreciably when GILZ was coexpressed.

[2] D. Franchimont, J. Galon, M. S. Vacchio, S. Fan, R. Visconti, D. M. Frucht, V. Geenen, G. P. Chrousos, J. D. Ashwell, and J. J. O'Shea, submitted for publication.

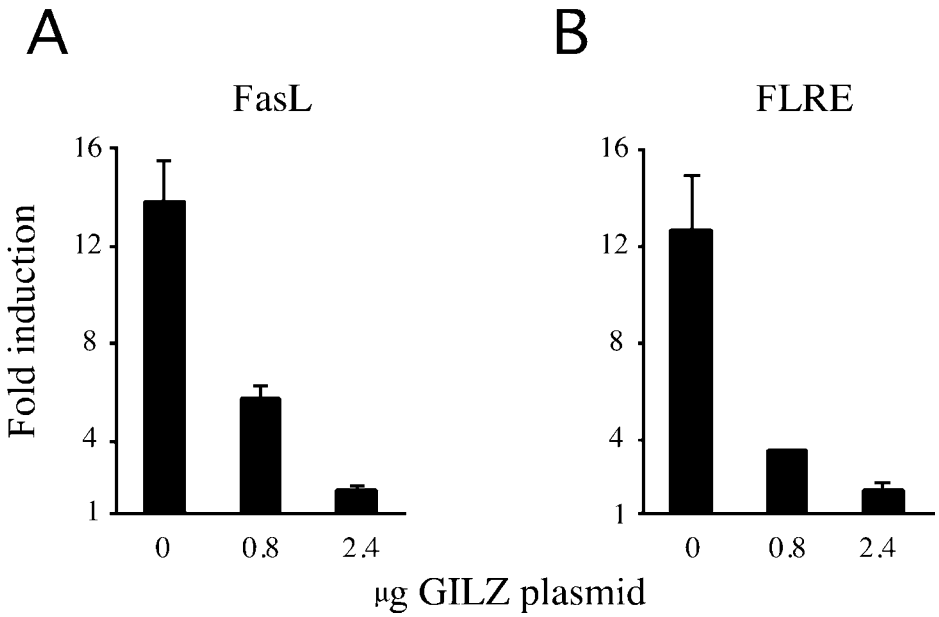

FIG. 2. **Expression of GILZ blocks induction of the FasL promoter and its Egr-regulated enhancer element.** Jurkat T cells were transiently transfected with luciferase-expressing reporter constructs driven by the 511 base pairs of human FasL promoter (*A*), the Egr-responsive element of the FasL promoter (FLRE) (*B*), and the indicated amounts of a GILZ-encoding expression plasmid. Empty expression vector was added to equalize the total amount of DNA in each transfection. Individual transfections were divided into two parts, one of which was left untreated, and the other was stimulated with PMA and ionomycin. After 15 h, cells were harvested, and luciferase activity was determined. Results shown are the mean and geometric error of duplicate transfections. The results are representative of three independent experiments.

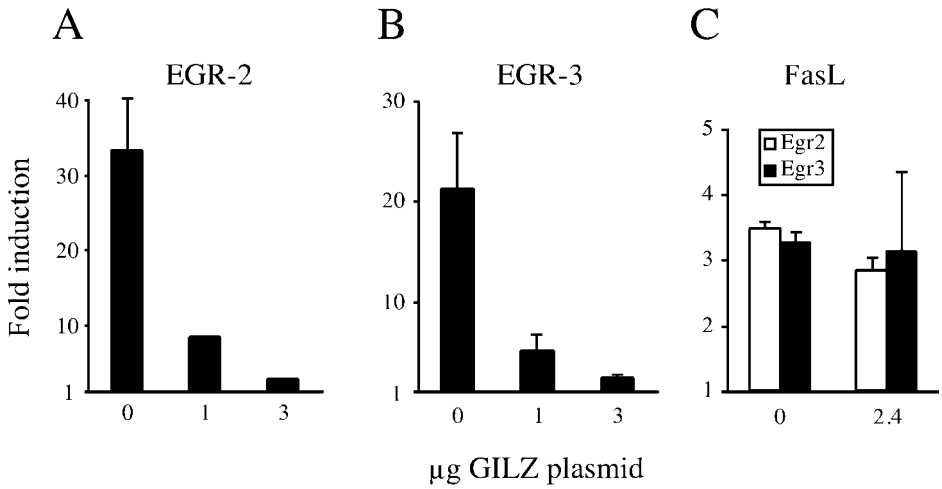

FIG. 3. **Expression of GILZ blocks induction of the Egr-2 and Egr-3 promoters.** Jurkat T cells were transiently transfected with luciferase-expressing reporter constructs driven by the 950 base pairs of Egr-2 promoter (*A*) and the 2.4 kilobase pairs of Egr-3 promoter (*B*) along with the GILZ-encoding plasmid and activated with PMA and ionomycin. *C*, the 511 base pairs of FasL-dependent reporter was activated with cotransfected Egr-2- (*open bars*) and Egr-3 (*filled bars*)-expressing plasmids. Results are expressed as fold activity determined by dividing the activity obtained with the Egr-expressing plasmid by that obtained with the parental plasmid pCB6. Results shown are representative of three independent experiments.

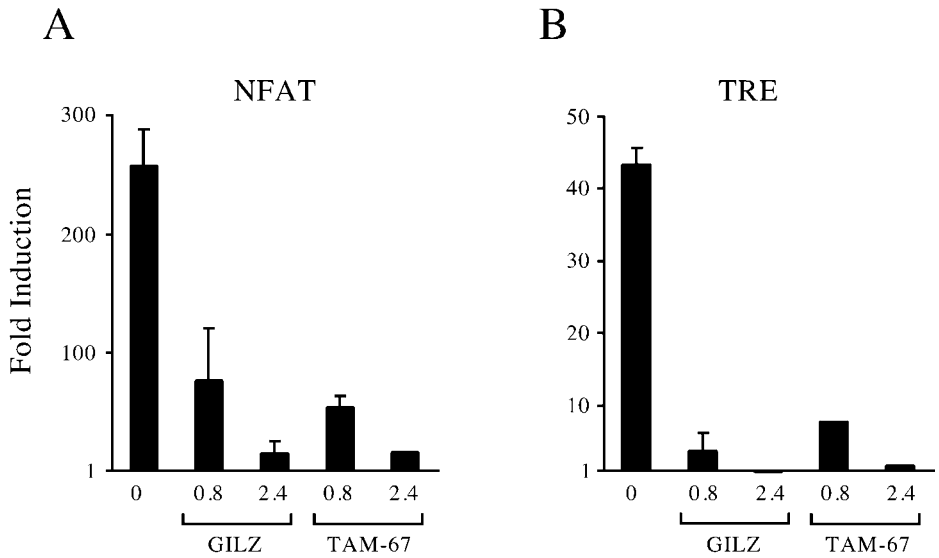

FIG. 4. **The NFAT element is sensitive to GILZ by virtue of the strong sensitivity of the associated AP-1 element to GILZ.** Jurkat cells were transiently transfected with luciferase reporter constructs for NFAT (*A*) or AP-1 (*B*). The cells were cotransfected with the indicated amounts (µg) of expression plasmids encoding GILZ or TAM-67. Results shown are representative of three experiments.

Northern Blot Analysis—Total RNA (3 µg) was separated by electrophoresis through a 1.5% agarose gel containing 6% formaldehyde and MOPS buffer (Quality Biological, Inc., Gaithersburg, MD). After transfer to a Genescreen membrane (PerkinElmer Life Sciences), RNA was covalently bound by UV cross-linking, and hybridization with ^{32}P-labeled cDNA probes was carried out at 42 °C in 5× SSPE, 1% SDS, 10% dextran sulfate, and 50% formamide (Analytical Biosciences). cDNAs encoding human IL-2 and glyceraldyhyde-3-phosphate dehydrogenase (GAPDH) were used as probes. Final washes were performed at 65 °C in 2× SSPE and 2% SDS. After exposing the blot to detect human IL-2

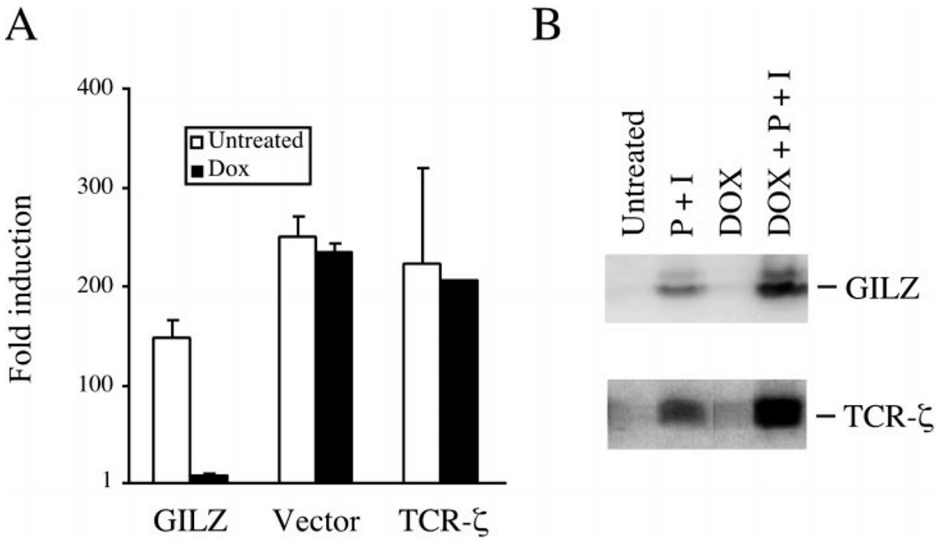

FIG. 5. **GILZ expressed from a doxycycline-regulated plasmid inhibited the TRE.** *A,* Jurkat cells were transfected with the TRE-dependent reporter, a plasmid encoding the Tet repressor protein, and the Dox-regulated expression plasmid either empty or encoding the 14-kDa protein TCR-ζ or the 17-kDa protein GILZ. Fold induction of luciferase activity after stimulation with PMA and ionomycin in the absence or presence of doxycycline (100 nM) was determined. *B,* the effect of Dox on GILZ and TCR-ζ expression in the presence of PMA and ionomycin was evaluated by Western blot. Results are representative of three independent experiments.

mRNA, the membrane was stripped by boiling in 0.1× SSC and 1% SDS and probed for GAPDH. Blots were visualized using a Storm PhosphorImager and ImageQuant software (Molecular Dynamics, Amersham Pharmacia Biotech).

In Vitro Binding Assays—Bacterially expressed glutathione *S*-transferase (GST) fusion proteins were immobilized on glutathione-Sepharose beads (Amersham Pharmacia Biotech). Binding reactions between the GST fusion protein-coated beads and ^{35}S-labeled proteins were performed in 20 mM HEPES, pH 7.9, 250 mM KCl, 0.5% Nonidet P-40, and 20% glycerol while rotating at 4 °C for 2 h. Proteins remaining after washing the beads with binding buffer were eluted by boiling in sample buffer, separated by electrophoresis through 10% or 13.5% SDS-PAGE gels, and visualized with a PhosphorImager.

Electromobility Shift Assays—To obtain AP-1, the murine T cell hybridoma MA 5.8 was stimulated for 30 min with 50 nM PMA. Extracts were prepared as described, as was the binding reaction buffer, except that 50 mM NaCl was included (37). The following reagents were added as indicated: 1 pmol (a 50-fold excess) of unlabeled AP-1 or NF-κB oligonucleotides, 2 μg of Fos-specific (K-25) or control Egr-2-specific antibodies (Santa Cruz Biotechnology), or 8 μM GST or GST-GILZ. After incubation for 30 min at 4 °C, 20 fmol of [^{32}P]dATP end-labeled AP-1 oligonucleotide was added to the reactions, which were allowed to incubate for an additional 30 min at room temperature. The samples were resolved by electrophoresis through a 5% native acrylamide gel run at 100 V in 0.5× TBE for 1 h and visualized with a PhosphorImager. The sequences of the oligonucleotides used were AP-1, 5′-CTAGTGATGAGTCAGCCGGATC-3′, and NF-κB, 5′-TAGTTGAGGGGACTTTCCCAG-GCA-3′.

Western Blotting—Samples from equivalent numbers of cells were lysed in sample buffer (50 mM Tris, pH 6.8, 2% SDS, and 10% glycerol) and denatured by boiling. After separation by SDS-PAGE, the proteins were transferred to nitrocellulose, probed with antibodies, and developed with ^{125}I-labeled protein A (Amersham Pharmacia Biotech). Anti-GILZ antibodies were used at a dilution of 1:200. The anti-TCR-ζ-probed blot was probed with rabbit anti-mouse Ig (H + L) antibodies (Jackson Laboratories, Bar Harbor, ME).

RESULTS

Suppression of IL-2 Induction by Glucocorticoids Requires New Protein Synthesis—Transcription-independent direct interference is accepted as a major, if not the major, mechanism by which glucocorticoids suppress transcription of responsive genes. A key feature of this phenomenon is that suppression should occur in the absence of new protein synthesis (provided that induction of the gene in question itself does not rely on factors that require *de novo* synthesis). IL-2 plays a pivotal role in the immune response, and its transcriptional induction in T cells activated *in vitro* can be inhibited by Dex (42, 43). Induction of IL-2 transcription largely depends on constitutively expressed transcription factors and thus does not require new protein synthesis (44). To determine whether glucocorticoid-mediated repression of IL-2 production requires new protein synthesis, human peripheral blood mononuclear cells were stimulated for 3 h with PMA and ionomycin in the presence or absence of Dex and/or the protein synthesis inhibitor cycloheximide (CHX), at which time IL-2 mRNA levels were determined by Northern blotting (Fig. 1*A*). PMA/ionomycin-induced IL-2 mRNA up-regulation was inhibited by cotreatment with Dex (compare *lanes 2* and *4*). Treatment with CHX did not induce expression of IL-2 message but did cause an increase in the activation-induced level (compare *lanes 2* and *6*). This increase, characteristic of early-response genes whose transiently elevated mRNA levels are prolonged by blocking synthesis of presumed destabilizing proteins, has been described as "superinduction" (45). Adding CHX to Dex-stimulated cells restored the level of IL-2 mRNA to that of the CHX-superinduced cells. Note that although CHX treatment itself caused a reduction in GAPDH, as reported previously (46), the GAPDH levels were the same within the group cultured without CHX and within the group cultured with CHX and therefore serves as a loading control. These data argue against a strict direct interference model, suggesting instead that in normal T cells the liganded GR induces a gene whose product inhibits transcription of IL-2.

GILZ Prevents Activation-induced Up-regulation of FasL and Inhibits Induction of Egr-2 and Egr-3 Transcription—Because *GILZ* is among the most responsive of the genes up-regulated by glucocorticoids,[2] we considered the possibility that it might be a general mediator of glucocorticoid-induced transcriptional repression. Stable expression of GILZ in T cell hybridoma cells has been shown to block activation-induced up-regulation of FasL (34). To begin to address the mechanism by which GILZ acts, we first determined whether its inhibitory effect on FasL induction is at the transcriptional level. GILZ cDNA was transiently expressed in Jurkat T cells together with FasL promoter-driven reporter plasmids. When cotransfected with an empty expression plasmid, the intact FasL reporter plasmid (bases −511 to −1 relative to the translation initiation site) was strongly induced by treatment with PMA and ionomycin (Fig. 2*A*). Cotransfection of a GILZ-expressing plasmid decreased the induction in a dose-responsive manner, indicating that GILZ acts on transcription. This region of the *fasL* gene contains a number of transcription factor binding sites that have been implicated in FasL regulation, including sites for Egr family members (−214 to −207), NFAT (−276 to −272), and NF-κB (−138 to −128) (37, 47–50). We have found that much of the activity of the FasL promoter depends upon the Egr-binding site (termed the FLRE), since mutation of this element abrogated activation-induced reporter up-regulation. To determine if the effect of GILZ on FasL promoter activity

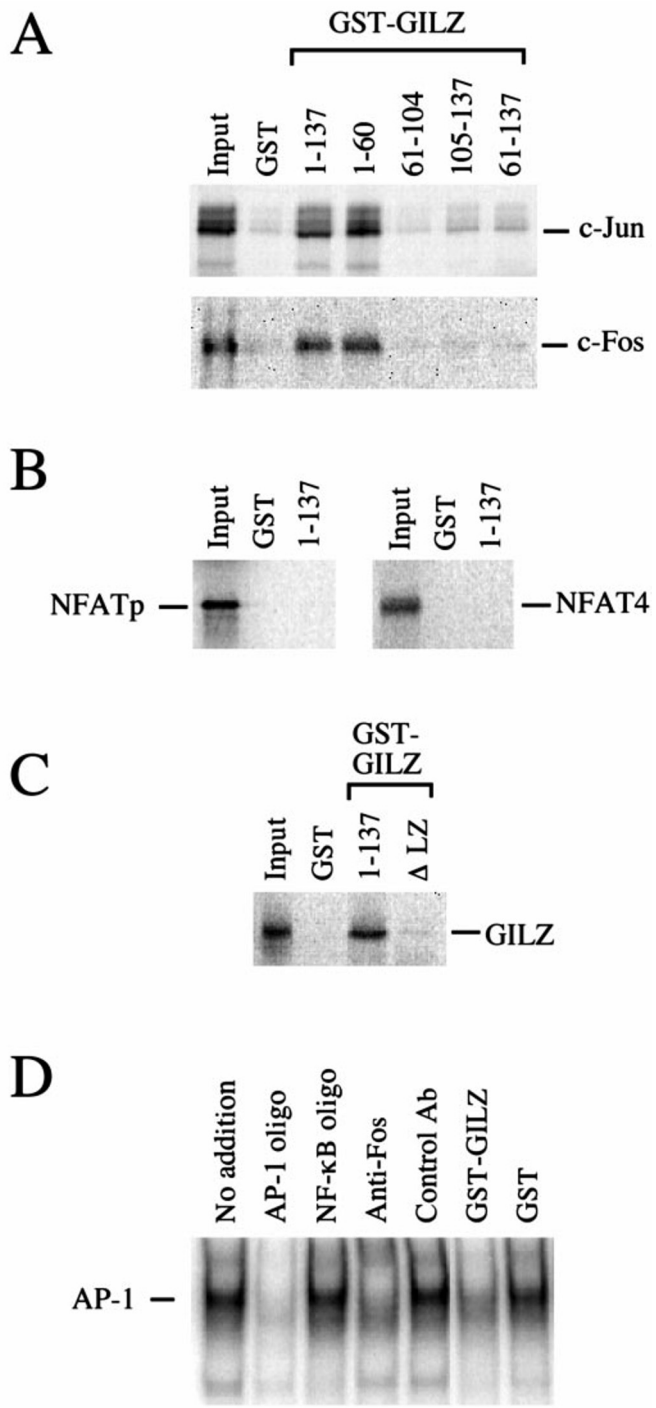

FIG. 6. **GILZ interacts with c-Fos and c-Jun through a site outside of its leucine zipper, which itself can mediate homodimerization.** *A–C,* the indicated ^{35}S-labeled *in vitro* translated proteins were incubated with the indicated GST-GILZ fusion proteins. Interacting proteins remaining after washing were resolved by SDS-PAGE and visualized by autoradiography. The lanes labeled *Input* represent 10% of the *in vitro* translated material offered to the GST-coated beads. Results are representative of six (c-Fos), four (c-Jun), three (NFATs), and 5 (GILZ) experiments. *D,* GILZ inhibits binding of AP-1 to its cognate DNA. The effect of the indicated reagents on binding of AP-1 to a consensus AP-1 element was determined by electromobility shift assay. Results are representative of three experiments. *Ab*, antibody.

was due to interference with the Egr-mediated pathway, a reporter driven by a promoter containing just the FasL FLRE (−220 to −205) driving the proximal 3′ region of the FasL promoter (37) was tested for sensitivity to the GILZ-expressing plasmid. GILZ inhibited the Egr-2/3-dependent FLRE-driven reporter as well as it did the entire FasL promoter region (Fig. 2*B*), indicating that GILZ exerts its effects, at least in part, by preventing the Egr-2/3-mediated pathway of FasL transcriptional induction.

Egr-2 and Egr-3 are synthesized *de novo* in activated T cells, and their enforced expression by transient transfection is sufficient to induce FasL reporter activity and induction of endogenous FasL mRNA (37). GILZ could exert its effect on the FasL transcription by preventing their *de novo* synthesis of Egr proteins, or it could block their activity at a posttranslational step, for example at the site of binding to the FasL promoter. To distinguish between these possibilities, the GILZ expression vector was cotransfected with luciferase reporter constructs driven by the Egr-2 or the Egr-3 promoter (Fig. 3, *A* and *B*). PMA and ionomycin induction of both Egr promoter-driven reporters was blocked by coexpressed GILZ. In contrast, when transcriptional up-regulation of the FasL promoter was induced by enforced expression Egr-2 or Egr-3 alone, coexpression of GILZ had no effect on reporter activity (Fig. 3*C*). These results indicate that GILZ does not interfere with Egr-mediated up-regulation of FasL directly but rather indirectly by preventing the transcriptional induction of Egr-2 and Egr-3.

GILZ Blocks NFAT/AP-1 Activity—The induction of Egr-2 and Egr-3 by T cell activation is mediated by NFAT-binding elements in their promoters (38, 39). To determine if the effect of GILZ on Egr-2/3 expression could be due to interference with NFAT signaling, Jurkat cells were cotransfected with an NFAT reporter with or without GILZ cDNA and activated with PMA and ionomycin. GILZ inhibited the induction of the NFAT reporter (Fig. 4*A*). NFAT-binding elements are typically found adjacent to binding sites for AP-1 (also referred to as the TRE or TPA-responsive element), and the affinity of each factor for its binding site is enhanced by binding of the other. All four NFAT family members (NFATp, -c, -2, and -4) can participate in cooperative binding to the TRE-adjacent NFAT sites. c-Jun most commonly occupies the side of the TRE nearest to NFAT (51), although NFAT makes contacts with both c-Jun and c-Fos (52). In fact, as reported previously (40), the NFAT reporter construct was also inhibited by a dominant-negative mutant of c-Jun (TAM-67) to a similar extent as GILZ (Fig. 4*B*), raising the possibility that GILZ in fact interferes with the AP-1 component of the NFAT·AP-1 complex. To explore this possibility, a reporter driven by the proximal TRE of the IL-2 promoter (40, 53) was introduced into Jurkat T cells; its induction by PMA and ionomycin was abolished by GILZ (Fig. 4*B*).

To characterize further the effect of GILZ on AP-1 activity, GILZ cDNA was cloned into a tetracycline (Tet)-regulated expression vector. Activation of the coexpressed Tet repressor protein with the Tet analog doxycycline (Dox) causes its dissociation from the operator of the expression vector and allows transcription by the full-length cytomegalovirus promoter. Jurkat cells were cotransfected with the Tet-regulated GILZ expression plasmid, the repressor-encoding plasmid, and the TRE reporter plasmid. In the presence of the empty Tet-responsive expression plasmid, PMA and ionomycin caused a 100-fold increase in luciferase activity, which was not affected by the addition of Dox (Fig. 5). When the GILZ-encoding Tet-responsive plasmid was cotransfected, there was a small reduction in activation-induced AP-1 activity in the absence of Dox. Adding Dox almost completely inhibited induction of AP-1 activity. The inhibitory effect of Dox was accompanied by increased expression of GILZ protein (Fig. 5*B*). An irrelevant protein (the ζ chain of the T cell antigen receptor) encoded by the Tet-regulated plasmid was similarly induced by Dox in the presence of PMA and ionomycin but had no effect on the stimulation of the

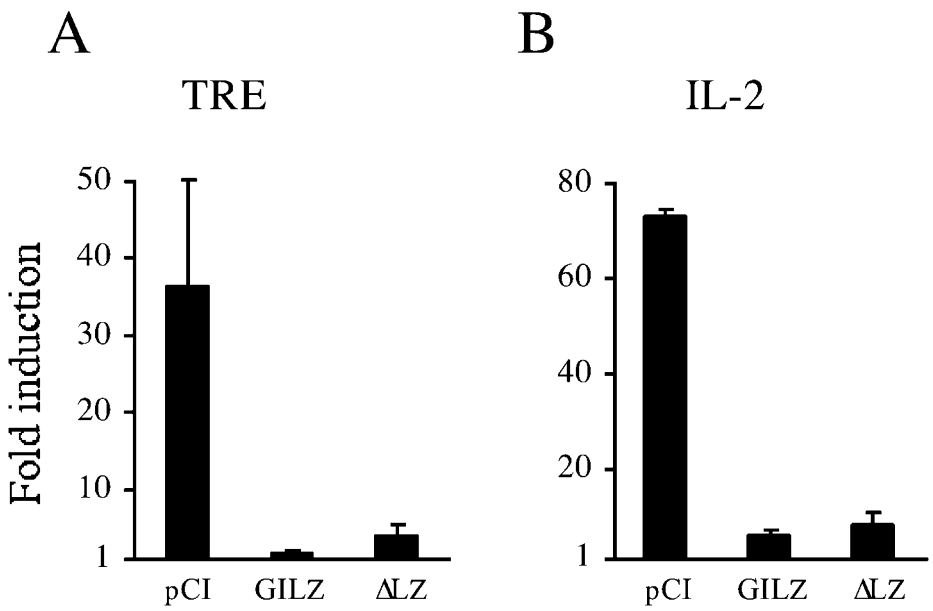

FIG. 7. **GILZ inhibits the IL-2 promoter and the TRE reporter in the absence of dimerization.** Jurkat cells were transiently transfected with luciferase reporter constructs driven by the TRE and the 0.4 kilobase pairs of human IL-2 promoter. Plasmids encoding full-length GILZ, a GILZ mutant in which the leucine zipper domain was deleted (amino acids 76–96), or the empty parental plasmid were cotransfected, and activity was analyzed as above. Results are representative of four independent experiments.

TRE. Taken together, these results are consistent with a model wherein GILZ inhibits the activity of AP-1, which impairs NFAT/AP-1 signaling and leads to reduced expression of Egr-2, Egr-3, and FasL.

GILZ Interacts with c-Jun and c-Fos through Its N-terminal Portion—Because GILZ inhibited activation-induced AP-1 activity and contains a leucine zipper domain, the possibility was considered that GILZ might dimerize with the leucine zipper-containing AP-1 constituents c-Fos and c-Jun. *In vitro* translated ^{35}S-labeled c-Fos or c-Jun were offered to immobilized GST fusion proteins. *In vitro* translated c-Jun is represented by the major lower band, with two minor upper bands that may be the products of in-frame upstream translation initiation from the polylinker region of the plasmid presumably containing c-Jun, and thus also bind GST-GILZ. There was only a small amount of nonspecific binding of c-Jun and c-Fos to GST protein alone (Fig. 6*A*). In contrast, both proteins were retained by immobilized GST-GILZ. GST-GILZ did not interact with ^{35}S-labeled NFATp or NFAT4, two isoforms of NFAT that have been implicated in Fas ligand regulation (38), supporting the notion that the target of GILZ is in fact AP-1 (Fig. 6*B*). To map the site of interaction in GILZ, fusion proteins were constructed using three non-overlapping segments of GILZ, each consisting of roughly a third of the full-length protein. The boundaries of these segments were determined by estimating each end of the α-helical region containing the leucine zipper domain with an α-helical prediction program ("The PSA Protein Structure Prediction Server," bmerc-www.bu.edu/psa/). Surprisingly, both c-Fos and c-Jun were efficiently retained by the N-terminal portion of GILZ-(1–60), which lacks the leucine zipper (Fig. 6*A*). Neither c-Fos nor c-Jun bound the C-terminal third (residues 105–137) or two-thirds (residues 61–137) of the molecule. GST-(61–137) retained the ability to dimerize with translated GILZ via the leucine zipper, arguing that the distal two-thirds of the molecule was properly folded (data not shown). As expected, given the lack of a requirement for the GILZ leucine zipper to bind c-Fos and c-Jun, the AP-1 components did not bind the isolated central domain of GILZ that contains the leucine zipper (residues 61–104).

It is possible that the GILZ leucine zipper is in fact nonfunctional. One way to test this is to determine if GILZ can form leucine zipper-dependent homodimers. ^{35}S-Labeled GILZ was incubated with GILZ fusion proteins. As shown in Fig. 6*C*, GILZ bound to GST-GILZ. Deleting GILZ residues 76–96, which constitute three turns of the leucine zipper, abrogated the interaction. GILZ also bound to the related product of the transforming growth factor-β-induced gene *TSC-22* immobilized by fusion to GST in a leucine zipper-dependent fashion (data not shown), suggesting that the leucine zipper of GILZ may be selective for a particular subclass of leucine zippers. The effect of GILZ on the interaction of AP-1 with its target DNA was tested in an electromobility supershift assay. As shown in Fig. 6*D*, extracts of a PMA-stimulated T cell hybridoma bound DNA containing a consensus AP-1-binding site. The shifted band was effectively competed for by unlabeled cognate AP-1-binding sequence (AP-1 oligonucleotides) but not an NF-κB-binding sequence (NF-κB oligonucleotides). Furthermore, anti-Fos antibody but not a control antibody (anti-Egr-2) reduced the amount of bound AP-1. Incubation of the extracts with GST-GILZ but not an equimolar amount of GST greatly reduced the intensity of the shifted band, indicating that, at least under these conditions, GILZ interferes with the binding of active AP-1 to its cognate DNA. Taken together, these data support a model in which a GILZ homodimer interacts with AP-1 components and prevents binding to DNA.

The Leucine Zipper Motif of GILZ Is Not Required for Transcriptional Inhibition—The initial observation in this study was that repression of IL-2 by Dex depended on new protein synthesis (Fig. 1). To determine whether GILZ could serve as such an induced protein, the ability of GILZ to block activity of the IL-2 promoter was tested. GILZ blocked the PMA- and ionomycin-induced up-regulation of the IL-2 promoter (Fig. 7*A*). Because the leucine zipper of GILZ did not directly participate in direct binding to c-Fos or c-Jun, its role in the repressive function of GILZ was examined. The GILZ mutant lacking the leucine zipper domain was nearly as effective as wild type GILZ at inhibiting both the TRE (Fig. 7*A*) and the IL-2 promoter (Fig. 7*B*). This indicates that the repressive activity of GILZ resides outside of the leucine zipper. Furthermore, because a leucine zipper-bound partner protein is not required for GILZ to exhibit inhibitory activity, it is likely that GILZ can function as a monomer.

DISCUSSION

Glucocorticoids influence metabolic and functional activities in almost all cell types. As such a versatile molecule it is likely to have diverse modes of action. Although the GR possesses strong transcriptional activity, its suppressive effects, in par-

ticular those mediated by passively obstructing other transcription factors, have recently received a great deal of attention. In this study we report that in addition to their ability to promote direct interactions between the GR and target transcription factors, glucocorticoid suppression of normal T cell cytokine production may also depend on a contribution from GR-mediated transactivation, leading to transcription of a gene whose product can itself carry out direct interference.

Direct interference between the GR and transcriptional activators has been implicated in glucocorticoid repression of inflammatory mediators such as collagenase (for AP-1) and IL-6 (for NF-κB) (24, 32), which can be produced by non-lymphoid cells. A similar mechanism was invoked to account for glucocorticoid repression of IL-2 in T cells, based on the observation that occupancy of a GR lacking the N-terminal transactivation domain was able to inhibit the IL-2 promoter (54). However, the study of the IL-2 promoter was performed with Jurkat T cells, in which some signaling pathways are aberrant, for example the lack of the phosphatase PTEN and the resulting constitutive activity of phosphatidylinositol 3-kinase (55). In fact, we have found that activation-induced up-regulation of IL-2 mRNA in Jurkat T cells is blocked by cycloheximide,[3] suggesting that regulation of the IL-2 promoter in Jurkat cells, unlike normal resting T cells, depends on newly synthesized proteins. In addition, an N-terminal deletion mutant GR, similar to that used by Northrop *et al.* (54) has been reported to transactivate a GRE in a Dex-dependent manner in yeast (56). Interestingly, when a GR mutant in the C-terminal zinc finger that was defective in transactivation and yet capable of mediating suppression (20), another transactivation-defective mutant, and the dimerization-deficient mutant GR were stably expressed in SAOS2 osteosarcoma cells, treatment with Dex caused growth-arrest and induction of one or both of the cyclin-dependent kinase inhibitors $p21^{Cip1}$ and $p27^{Kip1}$ to a degree similar to that of the wild type GR (56). These observations are consistent with the possibility that a Dex-induced gene product could participate in blocking transcriptional induction of the IL-2 promoter.

Among the genes reported to be induced by glucocorticoids, the requirement for a newly synthesized protein to block IL-2 induction appeared to be best satisfied by GILZ, whose leucine zipper is characteristic of transcriptional regulators and whose expression is limited to lymphoid cells (34). Expression of GILZ inhibited activation-induced up-regulation of the FasL promoter, as well as the promoters of two transcription factors involved in FasL regulation, Egr-2 and Egr-3. Up-regulation of Egr-2 and Egr-3 requires the participation of NFAT, and indeed a critical NFAT site has been identified in the promoters of each gene (38, 39). With rare exceptions, NFAT elements are composite elements that contain an AP-1-binding site and are thus more accurately termed NFAT/AP-1 elements (51). In fact, GILZ potently inhibited gene expression driven by an AP-1-responsive element. The activity of the intact IL-2 promoter depends on the proximal TRE (57), thus accounting for the increased inhibition of the IL-2 promoter over that of the NFAT/AP-1 element.

The observation that the GILZ leucine zipper does not interact with c-Fos or c-Jun but is capable of mediating homodimerization implies that GILZ can form a homodimer *in vivo*. However, the near-wild type repressive activity of a GILZ mutant lacking the leucine zipper suggests that GILZ can function as a monomer. The leucine zipper could also enable dimerization with other partners, such as the closely related protein TSC-22, which also has a leucine zipper and can bind GILZ *in vitro*. TSC-22 was identified on the basis of the up-regulation of its mRNA by transforming growth factor-β, can also be induced by Dex, and exhibited transcriptional repressive activity when tethered to a promoter through fusion to the DNA-binding domain of the Gal4 protein (58). Although homologous to and capable of heterodimerization with GILZ, TSC-22 appears to have distinct biological activities. For example, we have found that in Jurkat cells TSC-22 behaved as a transcriptional activator, causing a 3- to 5-fold increase in the PMA and ionomycin-stimulated activity of the IL-2 reporter when transiently expressed (data not shown). Thus, it is possible that these two leucine zipper-containing proteins can modify each other's behavior when concomitantly expressed. Interestingly, in a study using microarray analysis to identify developmentally regulated genes in B cells, GILZ was found to be expressed in resting B cells from a mouse transgenic for a HEL-specific antigen receptor and was down-regulated when the cells were activated with antigen (59). Other genes encoding known transcriptional repressors, such as *Id3, LKLF,* and *BKLF*, were also down-regulated in the activated B cells, consistent with the view that GILZ may act as a transcriptional repressor *in vivo* and indicative of a role for GILZ outside of T cells. In any case, the results presented in this report indicate that in addition to possibly causing direct interference through the GR, in T cells glucocorticoids induce at least one molecule (GILZ) that itself can bind to and interfere with AP-1.

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[3] P. R. Mittelstadt and J. D. Ashwell, unpublished observations.

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