Positive Regulation of the Glucocorticoid Receptor in Human T-cells Sensitive to the Cytolytic Effects of Glucocorticoids*

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Regulation of glucocorticoid receptor (GR) protein and mRNA were examined in the human leukemic T-cell line CEM-C7. Unlike other cells in which GR regulation has been examined, the growth of these cells is inhibited by glucocorticoids, leading to cell death. Treatment of glucocorticoid-sensitive CEM-C7 cells with 1 µM dexamethasone for 18 h resulted in an increase in both cytoplasmic and nuclear GR protein, as determined by immunoblotting with anti-human GR antisera. Analysis of GR mRNA levels by Northern blotting revealed a corresponding increase in mRNA in steroid-treated cells. An increase in GR mRNA was detectable after as little as 3 h of treatment with dexamethasone, and GR mRNA concentration continued to increase for at least 18 h, well before the onset of growth arrest or cell death. GR mRNA concentration was not altered after dexamethasone treatment of the glucocorticoid-resistant mutant cell line ICR27TK.3, which lacks functional GR. Thus, the increase in GR seen in glucocorticoid-sensitive cells is a GR-mediated response. These results are in sharp contrast to the down-regulation of GR reported in other cells and tissues, and suggest that regulation of the GR by its cognate ligand may be tissue-specific.

Glucocorticoid hormones affect the growth and development of a wide range of eukaryotic cells and tissues, eliciting a variety of catabolic and anabolic responses. In all cases the hormonal response is mediated by the glucocorticoid receptor (GR) protein. Several lines of evidence indicate that intracellular GR concentration is an important factor in determining the extent of the biological response of cells to glucocorticoids. Gehring et al. (1) have shown that there is a direct relationship between endogenous GR concentration and glucocorticoid sensitivity in mouse T-cells. Similarly, Bourgeois and Newby (2) found a correlation between GR gene dosage and dose response in mouse WEHI-7 cells. More recently, and more directly, it has been shown that, after transfection of the GR gene into cells lacking functional GR, the magnitude of the transcriptional response from either a cotransfected reporter gene (3) or the endogenous tyrosine aminotransferase gene (4) is proportional to the number of copies of the GR gene which were transfected.

There are numerous reports that glucocorticoid treatment results in a decrease in GR concentration (5–9), suggesting that the degree and duration of the biological response may be modulated by the ligand itself. Receptor concentration is decreased in part by the shortened half-life of the steroid-occupied receptor (10). In addition, it has recently been shown that, in a variety of tissues, there is a decrease in GR mRNA after steroid treatment (11, 12). This result suggests that regulation of GR activity may also occur at the level of GR transcription and/or mRNA stability. In either case, the observation that glucocorticoids can alter the concentration of their own receptor suggests that such regulation may attenuate glucocorticoid responses and thus contribute to the overall regulation of hormonally responsive genes.

In contrast to the anabolic effects of steroids seen in many cells, glucocorticoid treatment of lymphoid cells, particularly those of thymic origin, often produces a series of profound catabolic responses leading ultimately to cell lysis (13). Similar effects have been observed in several permanent lymphoid cell lines (2, 14, 15). In particular, cells of the human T-cell leukemic line CEM-C7 are irreversibly arrested in the G1 phase of the cell cycle and killed after exposure to glucocorticoids (16). This cytolytic response is dependent on the presence of functional GR (17). To determine whether regulation of the GR by its ligand plays a role in the response of T-cells to glucocorticoids, we have investigated the regulation of the GR protein and its cognate mRNA in glucocorticoid-treated CEM-C7 cells. Our findings show that, in sharp contrast to results reported for other tissues and systems, the amount of GR protein and mRNA in glucocorticoid-sensitive CEM-C7 cells increases significantly in response to steroid treatment. These results suggest that, in cells programmed to die after exposure to glucocorticoids, induction of GR may play an important role.

EXPERIMENTAL PROCEDURES

Cells and Cell Culture—The dexR line 6TG1.1 and the dexR- mutant ICR27TK.3 were derived from the dexR human leukemic cell line CEM-C7 as previously described (17, 19). Cells were grown as previously described (16).

Preparation of Cytoplasmic and Nuclear Extracts—Cytoplasmic and nuclear proteins were isolated by a modification of the method of Weinberger et al. (20). Cells were washed twice with 10 ml of phosphate-buffered saline (1.5 mM KH2PO4, 8 mM Na2HPO4, 137 mM...
NaCl, 2.7 mM KCl; pH 7.2) and lysed by freezing on dry ice followed by resuspension in lysis buffer (10 mM HEPES, 5 mM MgCl₂; pH 7.5). The lysate was subjected to centrifugation for 2 min at 750 × g. The crude nuclear pellet was washed twice in lysis buffer containing 0.1% Nonidet P-40 and the nuclear preparation was boiled with lysis buffer containing 500 mM NaCl.

Immunoblotting—SDS-PAGE was performed as described by Laemmli (21) in 8% polyacrylamide gels with a 3% polyacrylamide stacking gel. Molecular weights were determined from standard curves constructed from the mobilities of the prestained proteins: phosphorylase b (Mr = 101,000), BSA (Mr = 74,000), ovalbumin (Mr = 50,000), and carbonic anhydrase (Mr = 33,000). Following SDS-PAGE, proteins were electrophoretically transferred to nitrocellulose paper at 500 mA for 4 h using a “Transphor” electrophoresis transfer unit (Hoewler). Transfer was performed in buffer containing 0.025 M Tris, 0.192 M glycine, and 20% methanol. Following the transfer, the filter was incubated for 1 h with TBS (20 mM Tris, 0.5 mM NaCl; pH 7.5) containing 2% non-fat dry milk and incubated overnight at 4°C in 10 ml of TBS containing a 1:100 dilution of polyclonal anti-human GR antisera AC40 (prepared as previously described (Ref. 18); and 2% non-fat dry milk. The filter was washed sequentially (for 15 min each) with TBS, TBS containing first 0.1% Tween 20, then 0.05% Tween 20, and finally 0.1% BSA, and then incubated for 1 h with 32P-protein A (0.1 µCi, 30 Ci/mg) in TBS containing 0.1% BSA. Following sequential 15 min washes in TBS containing first 0.1% BSA, then 0.5% Tween 20, and finally 0.05 M NaCl, the filter was air-dried and exposed to x-ray film.

Affinity Labeling and Immunoprecipitation—Cells were washed twice in Hank’s balanced salts solution, and the final pellet was frozen on dry ice. After thawing on wet ice, the pellet was resuspended in 1.0 ml of buffer (10 mM HEPES, 1 mM EDTA; pH 6.0) per 25 × 10⁶ initial cells and centrifuged at 12,000 × g for 10 min at 4°C. The supernatant was incubated in the presence of 100 nM [3H]DM for 3 h and immunoprecipitated with anti-human GR antisera performed as previously described (22).

Isolation of Cytoplasmic RNA—Total cell RNA was isolated by a modification of the method of Nienhuis et al. (23). Briefly, cells were washed twice with phosphate-buffered saline and resuspended in 0.1 M Tris-HCl (pH 7.5) containing 0.15 M NaCl and 2 mM MgCl₂. Nonidet P-40 was added to 0.5%, and the suspension was centrifuged at 750 × g for 10 min. The supernatant was brought to 5 mM EDTA and 1% SDS, and then extracted four times with an equal volume of phenol-chloroform-isomyl alcohol (2:24:1). After the final extraction, 2.5 volumes of ethanol were added. After overnight incubation at −20°C, the RNA was isolated by centrifugation. The RNA was washed with 70% ethanol, resuspended in distilled water, and stored in 70% ethanol at −70°C.

Preparation of Labeled Probes—The 402-base pair EcoRI insert of plasmid pGR is contained in the sequence corresponding to the immunogenic domain of the hGR, was isolated in low-melting agarose and labeled using [α-32P]dCTP by the method of Feinberg and Vogelstein (24), as directed by the manufacturer (Pharmacia LKB Biotechnology Inc.). The 18 S RNA probe was labeled by nick translation of plasmid pGR (25) according to the procedure supplied by the manufacturer (Bethesda Research Laboratories), with the exception that the labeling reaction contained 0.66 µM [α-32P]dCTP and 2.5 µM unlabeled dCTP.

Northern Blotting—RNA was denatured with glyoxal and dimethyl sulfoxide and electrophoresed through 1% agarose according to standard procedures (26). The RNA was then transferred overnight to 0.01 M Tris-HCl (pH 7.5) containing 0.15 M NaCl and 2 mM MgCl₂. Nonidet P-40 was added to 0.5%, and the suspension was centrifuged at 750 × g for 10 min. The supernatant was brought to 5 mM EDTA and 1% SDS, and then extracted four times with an equal volume of phenol-chloroform-isomyl alcohol (2:24:1). After the final extraction, 2.5 volumes of ethanol were added. After overnight incubation at −20°C, the RNA was isolated by centrifugation. The RNA was washed with 70% ethanol, resuspended in distilled water, and stored in 70% ethanol at −70°C.

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RESULTS

Effects of Glucocorticoid Treatment on Immunoreactive Cytosolic and Nuclear GR—Regulation of the GR protein was examined by immunoblotting with anti-human GR antisera. Although the anti-human GR antisera used in this study were prepared against highly purified preparations of human GR, they are contaminated with antibodies against other, non-receptor, proteins (18, 20). In addition, CEM cells contain considerably less GR (15,000–20,000 sites/cell) than other human lymphoid cell lines which have been studied with these antisera (≥100,000 sites/cell) (18, 20). Consequently, in addition to the 92-kDa component characteristic of the full-sized GR, a number of other immunoreactive bands were also seen when crude cytosol was analyzed by immunoblotting (Fig. 1A, lane 1). To confirm the identity of the 92-kDa component, GR in crude cytosol was affinity labeled with [3H]DM, immunoprecipitated with anti-human GR antisera, and fractionated by SDS-PAGE. GR was then visualized by immunoblotting or fluorography. The results clearly show that the single immunoreactive [3H]DM-labeled component (Fig. 1A, lane 4) co-migrates with the 92-kDa band detected in crude cytosol by immunoblotting. In addition, after immunoprecipitation with immune serum, subsequent immunoblotting detects primarily the 92-kDa component (Fig. 1A, lane 2). Thus, the 92-kDa component seen in crude cytosol represents the intact steroid binding protein of the human GR glycoprotein.

To examine GR regulation in glucocorticoid sensitive (dex⁺) human lymphoid cells in culture, dex⁺ cells were treated for either 30 min or 18 h with 1 µM dexamethasone, and cytoplasmic and nuclear fractions were prepared. In the absence of steroid, the majority of the GR is located in the cytoplasmic fraction. However, after 30 min of steroid treatment, a significant fraction of the receptor is in the nuclear fraction. Therefore, 30 min was chosen as the control time point to allow for redistribution of occupied receptor from the cytoplasmic to the nuclear fraction, and thus provide a more valid basis of comparison to the cells treated for 18 h. Cyttoplasmic and nuclear extracts were fractionated by SDS-PAGE, and the GR was visualized by immunoblotting (Fig. 1B). The results show that after 18 h of steroid treatment there is an increase in both cytoplasmic and nuclear 92-kDa immunoreactive GR (Fig. 1B).

Effect of Glucocorticoid Treatment on GR mRNA—The results presented in Fig. 1 suggest that the increase in immunoreactive GR protein seen after prolonged steroid treatment might be the result of new GR synthesis. We therefore examined the effects of steroid treatment on GR mRNA concentration. Fig. 2 shows a Northern blot of RNA prepared from untreated cells (lanes 1 and 2) and from cells treated for 18 h with 1 µM dexamethasone (lanes 3 and 4). Filters were probed with a 32P-labeled 402-base pair GR cDNA fragment derived from the “immunoreactive” domain of the human GR cDNA, chosen because of its relative lack of homology to other steroid receptor mRNAs. There is a marked increase in the intensity of the 7.0-kilobase GR-specific band in RNA isolated from steroid treated cells, indicating that the increase in GR protein was not due simply to changes in stabilization or degradation of existing GR protein.

To examine the time course of GR mRNA induction, GR mRNA was isolated from dex⁺ cells at various times after steroid treatment, size-fractionated, and analyzed by Northern blotting. The results (Fig. 3) indicate that there is a significant increase in GR mRNA after as little as 3 h of steroid treatment and that the concentration of GR mRNA continues to increase thereafter. We have previously shown that steroid-induced inhibition of cell growth and cell death do not occur until 18 h after steroid treatment (16). Indeed, even after 18 h of steroid treatment, there is no loss in ability

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of individual cells to form colonies and no alteration in the cell cycle distribution of treated cultures (16). Therefore, the increase in GR mRNA concentration precedes the catabolic effects of steroid on these cells and cannot be attributed to steroid-induced inhibition of cell growth or cell death.

**Requirement for Functional GR**—To determine whether the increase in GR mRNA seen after steroid treatment of dex\(^+\) cells is a GR-mediated response, the ability of dexamethasone to evoke an increase in GR mRNA concentration in the “receptorless” (\(r^-\)) cell line ICR27TK.3 was examined. These cells, derived from the dex\(^+\) cell line CEM-C7 after chemical mutagenesis, are completely resistant to the cytolytic activity of glucocorticoids and contain virtually no glucocorticoid binding activity (17, 19). However, they continue to express both a 7.0-kilobase GR mRNA and a 92-kDa immunoreactive GR protein, suggesting the presence of a mutation in the steroid binding domain of the protein.\(^3\) Comparison of RNA isolated from steroid-treated and untreated \(r^-\) cells with RNA isolated from untreated and treated dex\(^+\) cells revealed that, in contrast to dex\(^+\) cells, there is no increase in GR mRNA in \(r^-\) cells after steroid treatment (Fig. 4). Thus, in dex\(^+\) cells, the presence of functional receptor is necessary for both the cytolytic response and the steroid-induced increase in GR mRNA concentration. The data in Fig. 4 also indicate that dex\(^+\) cells contain more GR mRNA than \(r^-\) cells. However, this difference appears to be the consequence of small amounts of corticosteroid present in the fetal bovine serum used in the culture medium. When cells are grown in medium from which steroids have been removed, there is no difference in the amount of GR mRNA in dex\(^+\) and \(r^-\) cells.\(^4\)

\(^{3}\) J. M. Harmon and E. B. Thompson, manuscript in preparation.

\(^{4}\) L. P. Eisen and J. M. Harmon, manuscript in preparation.
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**DISCUSSION**

Previous studies have found that in response to either in vitro or in vivo administration of glucocorticoids the GR content of a wide variety of cells and tissues decreases (5–12). These decreases have been attributed to both a decrease in the half-life of the GR protein (10) and a decrease in steady-state levels of GR mRNA (11, 12). In the present study, glucocorticoid regulation of GR protein and mRNA in a glucocorticoid-sensitive leukemic T-cell line was examined. In response to dexamethasone treatment it was found that both immunoreactive GR protein and GR mRNA increased. Thus, our results appear to be in conflict with those of previous studies. In particular, the results reported here appear to contradict those of Schlecte et al. (7) and Shipman et al. (8), who found that administration of corticosteroids to normal volunteers resulted in a decrease in GR concentration in circulating lymphocytes. There are several possible explanations for this apparent discrepancy. In both in vivo studies, GR was measured by radioligand binding rather than immunoblotting. Thus, it is possible that, whereas steroid treatment induced an increase in GR protein, only a portion was in a form capable of binding steroid. In addition, the two in vivo studies measured GR concentration in total lymphocyte populations. Analysis of lymphocyte surface antigens indicates that CEM cells express the surface antigen CD4 and are therefore immunologically related to helper T-cells (30). Consequently, it is possible that an increase in GR concentration in circulating CD4+ cells was masked by a compensating decrease of GR in other, more abundant, classes of lymphocytes. In addition, it has been reported that administration of glucocorticoids produces a specific redistribution of helper T-cells out of peripheral circulation (31). Thus, it is possible that increased levels of GR in CD4+ cells were not detected because these cells were no longer available for assay.

The mechanism(s) of glucocorticoid-induced growth arrest and lymphocyte death are poorly understood. Recent evidence suggests that the lympholytic process may be related to the induction of a specific endonuclease (32). Such induction, however, cannot explain the cell cycle-specific effects of corticosteroids on the growth of CEM-C7 cells. Exposure of CEM-C7 cells to glucocorticoids results in their irreversible arrest in the G1 phase of the cell cycle and progressive cell death after a period of 18 h (16); the earliest evidence of changes in nuclear morphology or chromatin fragmentation cannot be seen until 32–36 h after steroid treatment (33, 34). Examination of oncogene expression in both mouse and human lymphoid cell lines has shown a correlation between rapid repression of c-myc expression and inhibition of cell growth (34–36), suggesting that inhibition of cell growth may be related to the repression of genes involved in cell proliferation. However, analysis of somatic hybrids constructed between glucocorticoid-resistant cells containing functional GR and receptorless mutants derived from glucocorticoid-sensitive cells has demonstrated that the dex+ phenotype is dominant and therefore involves the induction of a putative "lysis" gene(s) (37, 38).

The time course of GR induction reported here is similar to the time course of c-myc repression in S49 and CEM-C7 cells (34–36). Given the direct relationship between GR concentration and biological response (1–4), particularly in lymphoid cells (1, 2), it is therefore possible that induction of GR is necessary for maximum repression of c-myc, or other genes involved in cell proliferation. Such a model is consistent with the dominant nature of the dex+ phenotype. It is also consistent with the observations that in some dex+ variants isolated from murine mammary tumor virus-infected S49 cells, expression of functional GR appears to be markedly reduced (39, 40).

Androgens (41, 42), estrogens (43, 44), and vitamin D (45–47) have all been shown to elicit an increase in their respective receptors. Presumably, such regulation is important in modulating the biological response to the hormone. Indeed, it has been proposed that defective up-regulation of androgen receptors is responsible for some forms of human androgen insensitivity (41). The fact that GR appears to be increased only in T-cells subject to the catabolic effects of glucocorticoids suggests that this increase may be a tissue-specific response related to the biological effects of glucocorticoids on immune function.

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