Characterization of Glucocorticoid Receptors and Glucocorticoid Receptor mRNA in Human Leukemia Cells: Stabilization of the Receptor by Diisopropylfluorophosphate

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We have shown that cytosol samples from human leukemia cells frequently contain glucocorticoid receptor fragments that have a mol wt (M₀) of ~52,000. In the present study we demonstrate that the M₀ ~ 52,000-receptor fragments are derived from intact glucocorticoid receptors (M₀ ~ 97,000) by the action of a serine protease. M₀ ~ 52,000-receptor fragments were present in cytosol from 24 of 52 leukemia cell samples. Only normal size glucocorticoid receptors were present in cytosol samples if diisopropylfluorophosphate (DFP), a potent inhibitor of serine proteases, was added to the hypotonic buffer used for cytosol preparation. Receptor proteolysis was not inhibited by hydrolyzed DFP, benzamidine, phenylmethylsulfonylfluoride, aprotinin, iodoacetamide, or mercuric chloride. The leukemia cell protease digests the receptor at a different site than chymotrypsin, which digests the intact receptor to produce a M₀ ~ 40,000 receptor fragment. Receptor messenger RNA (mRNA) in S49 mouse lymphoma cells and in human leukemia cells was analyzed by Northern hybridization with a cDNA for the normal glucocorticoid receptor. Mutant S49 mouse lymphoma cells that have abnormally small glucocorticoid receptors (M₀ ~ 48,000) make a 5.0-kilobase receptor transcript in addition to the normal size 6.5-kilobase receptor transcript. A normal size receptor transcript of 6.5 kilobases was present in all of the human leukemia cells whether or not M₀ ~ 52,000-receptor fragments were present. Therefore, abnormalities of glucocorticoid receptor mRNA, which may give rise to the synthesis of foreshortened receptors in certain mutant mouse lymphoma cells, are apparently absent from human leukemia cells.

The effects of glucocorticosteroids on cells are mediated through receptors that bind the steroid hormone molecule to form a steroid-receptor complex. The steroid-receptor complex then interacts with specific regions in the genome to regulate gene transcription. Glucocorticoids kill leukemia and lymphoma cells and, therefore, are an important therapeutic modality for leukemias and lymphomas. Although the exact mechanism of cell killing by glucocorticoids is unknown, studies of glucocorticoid-sensitive and glucocorticoid-resistant mouse lymphoma cell lines have demonstrated that the cell killing process is mediated through glucocorticoid receptors and that a variety of receptor defects can result in glucocorticoid resistance.

Because of the importance of glucocorticoids in the therapy of human leukemias and lymphomas, there is considerable interest in the structural and functional properties of glucocorticoid receptors in human leukemia and lymphoma cells. Recent studies have found that glucocorticoid receptors in many human leukemia cells are abnormal in terms of their physical properties and their ability to bind to DNA cellulose or diethylaminoethyl (DEAE) cellulose. Glucocorticoid receptors in cytosol from human leukemia cells are subject to digestion by endogenous proteases. Thus it has been suggested that previously described abnormalities of glucocorticoid receptor function could be due to the presence of receptor fragments in leukemia cell cytosol and that glucocorticoid receptors in most, if not all, human leukemia cells are normal in size prior to digestion by endogenous proteases.

The mechanisms of glucocorticoid receptor degradation in cytosol from both normal cells and leukemia cells is currently being studied by several groups of investigators. Sherman et al demonstrated proteolytic degradation of the intact glucocorticoid receptor in rat liver and kidney cytosol to the meroreceptor, which is the smallest receptor fragment (M₀ ~ 20,000 to 25,000) that retains the steroid binding site. The extent of receptor degradation appeared to correlate with the activity of "lysine-specific" endopeptidases. Both Sherman et al and Holbrook et al demonstrated the presence of meroreceptors in cytosol from human leukemia cells. The specific protease(s) responsible for receptor degradation in human leukemia cell cytosol have not been identified. However, Sherman et al found high activities of peptidases of various specificities in human leukemia cell cytosols and showed that a lysine-specific endopeptidase isolated from rat kidney cytosol is capable of converting the glucocorticoid receptor in chronic lymphocytic leukemia cells to a meroreceptor. Holbrook et al found that glucocorticoid receptors from acute nonlymphocytic leukemia cells could be stabilized by a factor from chronic lymphocytic leukemia cells. Therefore receptor degradation to the meroreceptor form is influenced not only by the presence or absence of receptor degrading proteases but also by the level of factors in leukemia cell cytosol that inhibit receptor proteolysis.

We recently found that cytosol from human leukemia cells frequently contains a M₀ ~ 52,000-receptor fragment. The
M, ~ 52,000-receptor fragment was not unique to a particular type of leukemia and was present in samples from previously untreated and previously treated patients. This receptor fragment is considerably larger than the meroreceptor fragment described previously by others (see above) and corresponds closely in size to the nuclear transfer increased (nt) mutant-glucocorticoid receptor in mouse lymphoma cells. These mutants contain a receptor mRNA that is reduced in size and therefore results in synthesis of a foreshortened receptor molecule.

The present study was undertaken to determine the mechanism responsible for the M, ~ 52,000-receptor fragment in human leukemia cell cytosol. Our studies demonstrate for the first time that human leukemia cells contain a serine protease that digests the intact glucocorticoid receptor to a M, ~ 52,000-fragment. We find that glucocorticoid receptors in over 50 different leukemia cell samples are normal in size if disopropylfluorophosphate, a potent inhibitor of serine proteases, is used to stabilize the receptor. Consistent with this observation, we find that the receptor mRNA in human leukemia cells has a normal size.

METHODS

Materials. All chemicals, including proteases, protease inhibitors, and unlabeled dexamethasone, were purchased from Sigma Chemical Co, St. Louis. Acrylamide, N,N',methylenebisacrylamide, and Coomassie blue R-250 were from Bio-Rad Laboratories, Richmond, CA. The low mol wt standards kit for gel electrophoresis was purchased from Pharmacia Fine Chemicals, Piscataway, NJ. [6,7-3H]Dexamethasone-21-mesylate was from New England Nuclear, Boston.

Buffers. Phosphate buffered saline (PBS) was prepared as described previously. The hypotonic buffer used in cytosol preparation consisted of 25 mmol/L Tris pH 8.2, 1 mmol/L EDTA, 10% glycerol, and 20 mmol/L Na2MoO4.

Cell Lines. The Victor human leukemia cell line (a B lymphoblast cell line) was given by Dr Robert Allen (American Red Cross, St. Louis). Mouse lymphoma cell lines and methods of cell culture were described previously.

Leukemia cell isolation. Human leukemia cells were isolated from either peripheral blood or bone marrow by Ficoll-Hypaque separation as described previously. Only freshly isolated cells were used for experiments.

Affinity labeling. The method of labeling receptors in intact cells with [3H]dexamethasone mesylate was described in detail previously. Briefly, cells were washed with PBS and resuspended in PBS in Eppendorf microcentrifuge tubes so that each tube contained 10 to 20 million cells. The cell suspensions were incubated for four hours at 4°C with 200 mmol/L [3H]dexamethasone mesylate. Duplicate cell suspensions contained either the radiolabeled steroid alone (labeled – in figures) or the radiolabeled steroid plus 100-fold excess unlabeled dexamethasone (labeled + in figures). At the end of the incubation period, the cells were pelleted and the cell pellets were frozen at ~ 80°C for 15 minutes and then thawed in hypotonic buffer at 4°C for 15 minutes to prepare cytosol. The cytosol was then boiled for two minutes in the sample buffer for gel electrophoresis and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as detailed previously. Gels were then boiled for two minutes in the sample buffer for gel electrophoresis (SDS-PAGE) as detailed previously. Molecular weight standards were phosphorylase b (97,400), bovine serum albumin (67,000), ovalbumin (45,000), carbonic anhydrase (30,000), trypsin inhibitor (20,100), and lactalbumin (14,400). References for the mol wt values were given previously.

Protease inhibitors. To inhibit proteolysis of the affinity-labeled receptor during cytosol preparation, protease inhibitors were added to the hypotonic buffer immediately prior to use. Disopropylfluorophosphate (DFP) was added to the hypotonic buffer to give a final concentration of 5 mmol/L. To exclude the possibility that the inhibitory effect of DFP was due to an impurity present in certain commercial preparations of DFP, samples of DFP were treated with cupric ion and imidazole to catalyze the hydrolysis of DFP. In other experiments a stock solution of phenylmethylsulfonylfluoride (PMSF) was prepared in absolute ethanol and added to the hypotonic buffer to give a final concentration of 10 mmol/L. Benzamidine was added to the hypotonic buffer to give a final concentration of 5 mmol/L. Aprotinin (2 mg/ml; 14.5 trypsin inhibitory U/mg) was added to the hypotonic buffer at a final dilution of 1:1000. A 100 mmol/L stock solution of iodoacetamide was added to the hypotonic buffer to give a final concentration of 1 mmol/L. A stock solution of 0.01 mol/L HgCl2 was added to the hypotonic buffer to give a final concentration of 0.01 mM. The hypotonic buffer was prepared without EDTA when HgCl2 was used.

Limited protease digestion. A concentrated solution of chymotrypsin in deionized water was prepared fresh for each experiment. An aliquot of the chymotrypsin solution was added to affinity-labeled cytosol and incubated at 25°C for 15 minutes. The cytosol was transferred to the gel electrophoresis sample buffer and boiled to stop the reaction.

RNA isolation and analysis. Total cellular RNA was isolated from leukemia cells by sedimentation through cesium chloride. The RNA was analyzed by Northern blot hybridization using a cDNA for the rat liver glucocorticoid receptor as described previously.

RESULTS

Affinity labeling human leukemia cell receptors. Human leukemia cells were isolated from peripheral blood or bone marrow and then incubated with [3H]dexamethasone mesylate to affinity label the glucocorticoid receptors. Cytosol was then prepared and subjected to SDS-PAGE to determine the mol wt of the affinity-labeled receptors. Multiple proteins in cells bind [3H]dexamethasone mesylate and therefore appear as bands on autoradiograms of gels. To distinguish the specific binding proteins, or receptors, from the nonspecific binding proteins, duplicate cell suspensions were incubated with [3H]dexamethasone mesylate in the presence or absence of 100-fold excess unlabeled dexamethasone. The unlabeled dexamethasone competes with the [3H]dexamethasone mesylate for binding to receptors but does not compete for binding to nonsaturable binding sites on other proteins. Therefore, on autoradiograms, band(s) that correspond to affinity-labeled receptor(s) can be distinguished from nonreceptor bands because the receptor bands are seen only in the absence of competing steroid (lane –) and are not seen in the presence of competing unlabeled steroid (lane +). Small differences in the amount of cytosol applied to the – lane and + lane on gels could give rise to apparent differences in the intensity of corresponding bands on the two lanes. Therefore, only those bands that differ markedly in intensity between – and + lanes are considered to be receptor bands. For clarity, receptor bands on autoradiograms are designated by an arrowhead.

We determined the mol wt of glucocorticoid receptors in...
Values represent the number of leukemia cell samples that contained either M, ~ 97,000 receptors or both M, ~ 97,000 and M, ~ 52,000 receptors.
ing Mr, 52,000-receptor fragments in leukemia cytosol, a variety of protease inhibitors with different protease specificities were included in the hypotonic buffer used for cytosol preparation. Inhibitors that have major specificity for serine proteases (PMSF, aprotinin, and benzamidine) and inhibitors that have major specificity for cysteine proteases (HgCl₂ and iodoacetamide) did not inhibit receptor digestion (not shown). Certain preparations of DFP are contaminated with a factor that inhibits cysteine proteases.21,22 The serine protease inhibitory activity of DFP is eliminated by hydrolysis, but the cysteine protease inhibitory activity is stable upon hydrolysis.21,22 DFP that was hydrolyzed to eliminate its serine protease inhibitory activity did not block the generation of Mr, 52,000-receptors (not shown). In addition, in studies that are not shown, DFP did not inhibit receptor digestion by the cysteine protease papain. Therefore, the protease responsible for digestion of the intact leukemia cell receptor to a Mr, 52,000-fragment appears to belong to the serine class of proteases.

Limited protease digestion. Limited digestion of normal size glucocorticoid receptors with chymotrypsin is known to separate the immunoactive domain from the steroid-binding and DNA-binding domains of the receptor molecule.24,25 To determine if the endogenous protease in human leukemia cells digests the glucocorticoid receptor at the same site as chymotrypsin, [3H]dexamethasone mesylate-labeled cytosol from human leukemia cells was incubated with a range of concentrations of chymotrypsin prior to gel electrophoresis. In cytosol that contained only Mr, 97,000-receptors, chymotryptic digestion produced a Mr, 40,000-receptor fragment (Fig 3). In cytosol that contained both Mr, 97,000- and Mr, 52,000-receptors, chymotryptic digestion eliminated both the Mr, 97,000- and the Mr, 52,000-receptors and produced a Mr, 40,000-receptor fragment (Fig 2). These results indicate that the endogenous leukemia cell protease digests the glucocorticoid receptor at a different site than does chymotrypsin and that the Mr, 52,000 receptor fragment in leukemia cytosol contains the chymotrypsin sensitive site that is present in the intact Mr, 97,000 receptor.

Mixing studies. To further substantiate that proteolysis was responsible for producing the Mr, 52,000 receptor fragment, cells of the Victor human leukemia cell line were incubated with [3H]dexamethasone mesylate and then mixed with unlabeled fresh human leukemia cells immediately prior to cytosol preparation. The human leukemia cells were preincubated with 20 μmol/L unlabeled dexamethasone to prevent their receptors from binding the [3H]dexamethasone mesylate. A typical experiment is shown in Fig 4. This experiment employed the same human leukemia cell sample that was employed in the experiment shown in Fig 2. In the absence of added human leukemia cells, cytosol from the Victor cells contained only Mr, 97,000-receptors. The addition of the unlabeled human leukemia cells to the affinity-labeled Victor cells prior to cytosol preparation generated Mr, 52,000-receptor fragments from the intact affinity-labeled receptor.

The leukemia cell samples employed in this study contained variable numbers of red blood cells and platelets. To
determine if red blood cells or platelets, rather than leukemia cells, could be the source of the protease that digests receptors to the $M_r \sim 52,000$-fragment, experiments were performed in which human red blood cells or human platelets were added to affinity-labeled leukemia cells prior to cytosol preparation. The admixture of these cells did not produce receptor degradation (data not shown).

**Receptor mRNA.** Receptor mRNA from human cell samples was compared to receptor mRNA from mouse lymphoma cell lines. Glucocorticoid receptor mRNA in six different human leukemia cell samples was analyzed by Northern blot hybridization using a receptor cDNA for the rat liver glucocorticoid receptor, and results from four of these samples are shown in Fig 5. A normal 6.5-kilobase (kb) receptor mRNA was observed in human cells that contain both normal size receptors and the $M_r \sim 52,000$-fragments. The patient samples containing $M_r \sim 52,000$-receptor fragments (lanes 5, 6, 7) have receptor mRNA that looks identical to the mRNA from the patient sample containing only intact receptors (lane 2). The faintness of the 6.5 kb bands in lanes 5 and 7 may reflect lower levels of receptor mRNA in these leukemia samples or degradation of the RNA during preparation. The wild type S49 mouse lymphoma cells, which have normal size ($M_r \sim 97,000$) glucocorticoid receptors, have a 6.5 kb receptor mRNA (lane 4). The nt' mutant mouse lymphoma cells ($S49.143r$), which have smaller than normal glucocorticoid receptors ($M_r \sim 48,000$), have a 5.0 kb receptor mRNA. We conclude that the receptor mRNA in human leukemia cells is the expected size regardless of the mol wt of receptors in cytosol preparations, unlike the highly characterized 5.0 kb receptor mRNA present in nt' mutant mouse lymphoma cells.19

**DISCUSSION**

We demonstrate that cytosol from human leukemia cells frequently contains steroid binding receptor fragments that have a $M_r$ of $\sim 52,000$ in addition to intact receptors that have a $M_r$ of $\sim 97,000$. Several samples also appeared to contain $M_r \sim 30,000$-receptor fragments. The present study provides two types of evidence that prove that the $M_r \sim 52,000$-receptor fragment arises from the intact normal size receptor by proteolytic digestion during cytosol preparation. First, formation of the $M_r \sim 52,000$-receptor fragment was blocked by inclusion of the potent protease inhibitor DFP in the hypotonic buffer used for cytosol preparation. Second, admixture of fresh unlabeled human leukemia cells to an affinity-labeled human leukemia cell line prior to cytosol preparation resulted in digestion of the intact affinity-labeled receptors to $M_r \sim 52,000$-receptor fragments. We previously reported similar mixing studies to be negative.11 This led us to the incorrect conclusion that proteolysis was not responsible for the formation of $M_r \sim 52,000$-receptor fragments in the cytosol of human leukemia cells. The previous mixing studies involved the mixture of cytosol samples that had been stored at $-80^\circ C$ rather than the mixture of fresh intact cells as in the present study. Therefore the failure of the earlier mixing studies to demonstrate receptor proteolysis suggests that the leukemia cell protease responsible for receptor digestion may not be stable upon storage at low temperatures.

Limited digestion of the normal glucocorticoid receptor by chymotrypsin is known to separate the immunoreactive domain of the receptor from the steroid-binding and DNA-binding domains.24,25 The leukemia cell protease described here must digest the intact $M_r \sim 97,000$ glucocorticoid receptor at a different site than does chymotrypsin, since chymotryptic digestion of the intact receptor produced a $M_r \sim 40,000$-receptor fragment (Fig 3). The $M_r \sim 52,000$-receptor fragment in human leukemia cell cytosol could be digested to a $M_r \sim 40,000$-receptor fragment by chymotrypsin, indicating that the $M_r \sim 52,000$-receptor fragment retains the chymotrypsin sensitive site that is present in normal receptors (Fig 2).

The $M_r \sim 52,000$-receptor fragment was detected in leukemia cell samples obtained from 24 of 52 different patients. The presence of the $M_r \sim 52,000$-receptor fragment was consistent when leukemia cell samples were obtained again from the same patient at time intervals of up to 1 year. We previously demonstrated that receptor digestion does not occur in some leukemia cell cytosol samples even when.
proteolysis is encouraged by prolonged incubation or incubation at 25°C to 37°C. The reason receptor digestion occurs in some but not all human leukemia cell samples is unknown. It is possible that the protease responsible for receptor degradation may not be present in all leukemia cells or that some leukemia cells may contain a naturally occurring protease inhibitor that prevents the receptor from proteolysis. Alternatively, the protease activity may be similar in all leukemia cells but some leukemia cells may contain glucocorticoid receptors that are abnormally sensitive to digestion by the protease. We are attempting to distinguish between these possibilities at the present time. Receptor digestion does not occur in normal human peripheral blood mononuclear cell samples under the assay conditions employed here. However, we have observed receptor digestion to a Mr ~ 52,000-fragment when normal human peripheral blood mononuclear cell cytosol is incubated for two hours at 25°C (data not shown). Receptor digestion under these conditions is inhibited by DFP. Therefore, the protease responsible for receptor digestion may not be unique to leukemia cells but appears to be present, although probably at a lesser activity, in normal peripheral blood mononuclear cells.

The Mr ~ 52,000-receptor fragment described here is similar in size to the defective receptors present in cytosol from nt' mutant mouse lymphoma cells. This observation raised the possibility that the receptor abnormality in human leukemia cells and in the nt' mutant mouse lymphoma cells may involve a common mechanism. In this regard there is some evidence that glucocorticoid receptors in intact nt' mutant cells are normal in size and undergo degradation during cytosol preparation. However, the receptor abnormalities in human leukemia cells and in nt' mutant mouse lymphoma cells differ in several respects. First, a variety of different protease inhibitors, including DFP, do not inhibit the formation of defective receptors in cytosol from nt' mutant mouse lymphoma cells (Distelhorst et al, unpublished data). Second, the Mr ~ 52,000-receptor in human leukemia cells contains a chymotrypsin-sensitive site, whereas the defective receptors in nt' mutant mouse lymphoma cells are relatively resistant to digestion by chymotrypsin (Distelhorst et al, unpublished observations). Third, the receptor mRNA in nt' mutant mouse lymphoma cells is decreased in size compared to the size of receptor mRNA in wild type cells. In the present study we show that the size of receptor mRNA in human leukemia cells appears to be normal whether or not the Mr ~ 52,000-receptor fragment is observed. Therefore abnormalities of glucocorticoid receptor mRNA, which may give rise to the synthesis of foreshortened receptors in certain mutant mouse lymphoma cells, are apparently absent from human leukemia cells.

Stevens et al showed that glucocorticoid receptors in chronic lymphocytic leukemia cells are often degraded to a form having a Stokes radius of 3.5 nm. According to the work of Sherman et al, a receptor fragment of this size would be expected to have a Mr ~ 40,000 to 60,000. Therefore it is possible that the Mr ~ 52,000-receptor fragment described by us is the same as the lower mol wt form of the receptor described previously by Stevens et al. Sherman et al and Holbrook et al found that cytosol from human leukemia cells frequently contained meroreceptors in addition to normal size receptors. The meroreceptor, described by Sherman et al in rat liver and kidney cytosol, represents a degraded receptor that has a Mr ~ 20,000 to 25,000. Although similar size receptor fragments (Mr ~ 30,000) were detected in several leukemia samples studied here, the major receptor fragment observed in the present study was significantly larger (Mr ~ 52,000). There are two potential explanations for the difference in size between receptor fragments detected in the present study and earlier studies. First, we analyzed receptors under reducing and denaturing conditions by gel electrophoresis, whereas the size of receptors in earlier studies was measured under non-denaturing conditions by gel filtration. Mendel et al demonstrated that gel electrophoresis under denaturing conditions is a more sensitive technique for detecting intermediate size receptor fragments (Mr ~ 50,000) than gel filtration under non-denaturing conditions. Second, in the present study, cytosol samples were incubated for only a brief period of time (~30 minutes) prior to gel electrophoresis, whereas the assay methods employed in earlier studies required cytosol incubations for longer periods of time, thus permitting more extensive receptor degradation.

Although we tested a variety of protease inhibitors with different specificities for serine and cysteine proteases, only DFP inhibited receptor digestion to the Mr ~ 52,000-fragment. Although the major effect of DFP is to inhibit serine proteases, commercial preparations of DFP often contain an impurity that inhibits cysteine proteases. Since the serine protease inhibitory activity of DFP is sensitive to hydrolysis, whereas the cysteine protease inhibitory activity is resistant to hydrolysis. Since hydrolyzed DFP did not inhibit generation of the Mr ~ 52,000-receptor fragment, it appears that the protease responsible for receptor digestion belongs to the serine class of proteases. The leukemia cell protease that generates the Mr ~ 52,000-receptor fragment may be different from the protease responsible for meroreceptor formation in leukemia cytosol, since the latter has been reported to be a "lysine specific" protease. Mende et al showed that intact glucocorticoid receptors in rat thymus cytosol are converted to a Mr ~ 52,000-receptor fragment when the cytosol is incubated at 3°C for two hours. Proteolysis of the rat thymus glucocorticoid receptor is prevented by calpastatin, an inhibitor of calcium activated cysteine proteases. Therefore it appears that receptor proteolysis in rat thymus cells is due to the activity of a calcium-activated cysteine protease. It seems unlikely that the same protease is responsible for digestion of the human leukemia receptor to a Mr ~ 52,000-receptor fragment for the following reasons. First, iodoacetamide, an inhibitor of calcium-activated cysteine proteases in mammalian cells, did not inhibit human leukemia cell-receptor digestion. Second, the hypotonic buffer used for cytosol preparation in the present study contained EDTA, which would be expected to inactivate calcium-activated proteases. Third, under the assay conditions employed in the present study, intact receptors in rat thymus cells are stable, even when cytosol is incubated for two hours at 4°C (Distelhorst et al, unpublished observations).
Protease inhibitors are known to affect glucocorticoid receptor binding, inactivation, degradation, and transformation in vitro. However, the effect of protease inhibitors on glucocorticoid receptor function in vivo is unknown. We are currently isolating and characterizing the serine protease from human leukemia cells that produces the $M_t \sim 52,000$-receptor fragment. Also, we are trying to determine if this protease affects the function or turnover of glucocorticoid receptors in vivo.

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Characterization of glucocorticoid receptors and glucocorticoid receptor mRNA in human leukemia cells: stabilization of the receptor by diisopropylfluorophosphate

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