Absence in Glucocorticoid-resistant Mouse Lymphoma P1798 of a Glucocorticoid Receptor Domain Responsible for Biological Effects

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ABSTRACT

Glucocorticoid-resistant (CR), in contrast to glucocorticoid-resistant (CS), mouse lymphoma P1798 was shown to lack anti-glucocorticoid receptor immunoreactivity. Antibodies raised against the purified rat liver glucocorticoid receptor (GR) cross-reacted with the GR from CS, but not with the GR from CR, P1798 lymphoma. Using highly specific antisera against the GR in an indirect competitive enzyme-linked immunosorbent assay, it was demonstrated that α-chymotrypsin digestion of the GR from CS P1798 lymphoma caused a separation of a “resistant-like” nonimmunogenic steroid and DNA-binding domain (Stokes’ radius, 3.3 nm) from an immunogenic domain (Stokes’ radius, 2.6 nm). In contrast to CS P1798 lymphoma, neither before nor after α-chymotrypsin digestion, immunoreactivity could be found in the cytosol from CR P1798 lymphoma. This assay is used after chromatography on DNA-cellulose or gel filtration on Agarose A (0.5 m). These results suggest that the domain of the CS GR containing the immunogenic determinant(s), normally removed by limited proteolysis by α-chymotrypsin, appears to be missing in CR P1798 lymphoma cytosol. It seems that this domain plays an important role in the mechanism of action of glucocorticoids. This might suggest that a mutation has occurred affecting the genome resulting in defective transcription of the receptor gene(s) in CR P1798 lymphoma.

INTRODUCTION

The presence of GR4 in the cell is the key requirement for expression of glucocorticoid effects in a variety of target tissues (1). However, this does not always guarantee CS. Sibley and Tomkins (21) isolated variant clones of mouse lymphoma S49 cells that displayed normal levels of glucocorticoid binding but were unaffected by concentrations of dexamethasone that normally caused extensive lysis of wild-type S49 cells. Several groups have later described CR cells, mainly lymphocytes, both murine and human lymphoproliferative cell lines, which contain GR but do not respond to glucocorticoids in a normal manner (2, 5, 8–10, 13, 14, 20, 26, 27, 31, 32).

Stevens et al. (24, 26) have described earlier a mutant receptor-positive CR P1798 mouse lymphoma. Characterization of GR from the P1798 lymphoma cells reveals in the CS P1798 cells a GR with a Stokes’ radius of 6 nm and a calculated molecular weight of 90,000. However, GR from CR P1798 cells has a Stokes’ radius of 2.8 nm and a calculated molecular weight of 40,000. Furthermore, GR from CR P1798 cells has an increased affinity for DNA, since higher ionic strength was needed for elution from DNA-cellulose when compared to GR from CS P1798 cells. A similar “resistant-like” form of GR could be obtained from CS P1798 cytosol by limited proteolysis with α-chymotrypsin (25). This gave rise to a receptor fragment with a Stokes’ radius of 2.8 nm and tighter DNA binding, identical to the GR from CR P1798 cells. No effect of α-chymotrypsin was seen on GR from CR cells. A similar 3-nm GR with increased DNA affinity is also obtained in rat liver cytosol after α-chymotrypsin digestion of the 6-nm GR (4, 30).

We have described earlier several antisera raised against the purified rat liver GR (16, 17). Using these antisera in an indirect competitive ELISA in studies on rat liver GR, immunoreactivity and specifically bound radioactivity corresponding to GR chromatin in various chromatographic systems (17). This indicates that the antisera are highly specific for the GR in this assay system. However, the anti-GR antibodies interact only with the 6.1-nm GR and not with the steroid-binding 3.6-nm nor the 1.9-nm proteolytic forms. This is in contrast to antibodies raised by Govindan (11) and Govindan and Sekeris (12), who observed cross-reactivity of anti-M, 90,000 GR antibodies to M, 45,000 GR. The 3.6-nm fragment contains the steroid- and DNA-binding sites, while the 1.9-nm fragment contains only the steroid-binding site (30). Treatment with α-chymotrypsin separates an immunological domain (fragment) from the steroid- and DNA-binding domain (4, 17). The immunoreactive non-steroid-binding domain of the GR is preserved after α-chymotrypsin digestion. It could be separated from the 3-nm steroid- and DNA-binding domain and characterized by gel filtration and DNA-cellulose chromatography as a mainly 2.6-nm domain with very low affinity for DNA (4). Since GR from CR P1798 lymphoma very much resembles the properties of the α-chymotrypsin-induced 3-nm steroid- and DNA-binding domain of GR from rat liver cytosol and CS P1798 lymphoma, this suggests that the GR from CR P1798 lymphoma lacks the immunoreactive domain.

The aims of this study were (a) to investigate the cross-reactivity of the anti-rat GR antibodies to GR from CS and CR P1798 mouse lymphoma and (b) to study whether, in CR P1798 lymphoma cytosol, there exists any anti-GR immunoactivity...
which is not linked to the steroid- and DNA-binding domain of the receptor protein.

MATERIALS AND METHODS

Source of Tumors and Preparation of Cytosol. The CS and CR strains of mouse lymphoma P1798 were maintained in female BALB/c mice as described previously (25). After removal of fat and necrotic, hemorrhagic, and nontumor material, cytosol was prepared by mincing the solid tumors in EPG buffer, homogenizing in EPG buffer in a glass-Teflon Potter-Elvehjem homogenizer (3 strokes), and centrifuging at 170,000 × g for 45 min at 2°C. After removal of the floating lipid layer, the resulting supernatant was used as cytosol. The cytosol was labeled by incubation with 100 nm [1,2,4-3H]TA, specific activity, 37 Ci/mmol (New England Nuclear, Boston, Mass.) for 1 hr at 0°C. In order to determine unspecific binding, a parallel aliquot of labeled cytosol was incubated in the presence of a 100-fold excess of unlabeled TA (E. R. Squibb & Sons, Inc., Princeton, N. J.). Unspecific binding was 15 to 25% of total binding. In the case of DNA-cellulose chromatography (see below), elution was performed by incubating the labeled cytosol for 30 min at 25°C, followed by a 10-min cooling period on ice. Proteolytic digestion with α-chymotrypsin was performed as described below. Prior to incubation with antisera, the labeled cytosol was treated with one-third volume of dextran-coated charcoal (Dextran T-500 (Pharmacia Fine Chemicals, Uppsala, Sweden): charcoal (Merck AG, Darmstadt, Federal Republic of Germany), 1:10 (w/v)) at a final charcoal concentration of 1% (w/v), in order to remove free steroid.

Proteolytic Digestion. Limited proteolysis of the labeled GR was carried out as described previously (30) with α-chymotrypsin (Worthington Biochemical Corp., Freehold, N. J.; 81 units/mg protein), using 0.8 µg/A280 - 310 and incubating for 30 min at 10°C. The incubation was terminated by adding a 20-fold concentration of lima bean trypsin inhibitor (Worthington).

Preparation and Specificity of Antiserum. Purified GR was prepared as described by Wränge et al. (29). After the second DNA-cellulose column, the GR was further purified to apparent homogeneity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and administered to rabbits. For further details, see Ref. 4. The antiserum was purified on protein A-Sepharose as described previously (17). For all experiments, protein A-purified antiserum (A3) was used. Normal rabbit serum and preimmune serum were purified in the same way. The protein concentration of total binding. In the case of DNA-cellulose chromatography (see below), activation was performed by incubating the labeled cytosol for 30 min at 25°C, followed by a 10-min cooling period on ice. Proteolytic digestion with α-chymotrypsin was performed as described below. Prior to incubation with antigen, the labeled cytosol was treated with one-third volume of dextran-coated charcoal (Dextran T-500 (Pharmacia Fine Chemicals, Uppsala, Sweden): charcoal (Merck AG, Darmstadt, Federal Republic of Germany), 1:10 (w/v)) at a final charcoal concentration of 1% (w/v), in order to remove free steroid.

Chromatographic Methods. Gel filtration and DNA-cellulose chromatography were performed as described (4), except that cytosol was diluted to 10 A280 - 310/ml prior to activation and applying to DNA-cellulose.

ELISA. Indirect competitive ELISA was performed as described (4, 17) with a few minor changes. Samples to be analyzed (0.2 ml) were incubated at 4°C overnight with 50 µl purified antiserum diluted 1:40 (final dilution, 1:200). After incubation, the amount of antibodies not bound to antigen in the test sample was measured on micro-ELISA plates (M129 B; Dynatech Laboratories, Ltd., Billingshurst, Sussex, United Kingdom) coated with 20 ng purified GR (29) in each well. This incubation was performed for 15 min at 37°C. After washing (see Ref. 17), the plates were incubated with 50 µl (per well) of peroxidase-conjugated swine antirabbit immunoglobulin (Dako Immunoglobulin, Ltd., Copenhagen, Denmark) at a 1:50 dilution in 0.05% (v/v) Tween 20 (Merck AG):0.1% (w/v) bovine serum albumin in phosphate-buffered saline, pH 7.4 (8 g NaCl:0.2 g K2HPO4: 2.9 g Na2HPO4: 2H2O:0.2 g KC1/liter), for 30 min at 37°C. Color development was obtained as described (17), and absorbance was measured in a Titertek Multiskan spectrophotometer (Flow Laboratories, Inc., McLean, Va.) at 492 nm. Color development in the well was inversely proportional to the amount of GR in the test sample: using normal rabbit serum compared to anti-GR serum gave less than 10% of the color reaction. The minimum detection level is 0.2 to 0.5 nm GR with a maximum sample volume of 0.2 ml.

The presence of α-chymotrypsin and/or lima bean trypsin inhibitor at the concentrations used did not interfere with the ELISA (4, 16), since a parallel analysis of only buffer in the presence of α-chymotrypsin and/or lima bean trypsin inhibitor showed no ELISA inhibition. Furthermore, no difference in ELISA reaction was observed whether the cytosol was dextran-coated charcoal treated or not.

Radioactivity Measurement. Radioactivity was measured as described earlier (17).

RESULTS

The anti-rat liver GR antibodies were tested for cross-reactivity against [3H]TA labeled GR from CS and CR P1798 lymphoma cytosol using affinity chromatography on protein A-Sepharose columns. As can be seen from Chart 1, [3H]TA:GR complex from CS P1798 lymphoma incubated with anti-GR antiserum was retained on the protein A-Sepharose column. In contrast, anti-GR antiserum did not cause retention of the [3H]TA:GR complex from CR P1798 lymphoma. α-Chymotrypsin digestion of CS P1798 cytosol prior to incubation with anti-GR antiserum resulted in loss of radioactivity retained on the column. These results indicate that GR from CR P1798 lymphoma and the steroid-binding domain of α-chymotrypsin-digested CS P1798 lymphoma cytosol are not recognized by the anti-GR antiserum, which was raised against the 6.1-nm rat liver GR. Radioactivity in the flow through volume was checked still to be protein bound as judged from gel filtration chromatography, both in the case of CR and α-chymotrypsin-digested CS P1798 cytosol. No radioactivity was retained when untreated CS P1798 was incubated with normal rabbit serum. The lack of cross-reactivity of the antibodies with the [3H]TA:GR complex from α-chymotrypsin-digested CS P1798 lymphoma is not due to an effect of α-

![Chart 1. Protein A-Sepharose chromatography of GR:antibody complexes from CS, α-chymotrypsin-treated CS, and CR P1798 lymphoma cytosol. Preparation of cytosol, labeling with [3H]TA, and treatment with α-chymotrypsin were performed as described in "Materials and Methods." Labeled cytosol (100 µl) (16.8 A280 - 310/ ml) corresponding to 185 and 90 fmol [3H]TA:GR complex from CS and CR cytosol, respectively, was after treatment with dextran-coated charcoal incubated with 50 µl purified antiserum for 90 min at 4°C. The incubation mixtures were then applied on 2 ml columns of protein A-Sepharose equilibrated in EPG buffer. After a washing with 2-column volumes of EPG buffer, 0.1 µl acetic acid was applied. Fractions (0.5 ml) were collected, and radioactivity was measured. Arrow, start of elution with 0.1 µl acetic acid.](image-url)
Immunological Studies on Corticoid-resistant Receptors

Chymotrypsin on the antibodies, since use of α-chymotrypsin covalently linked to agarose (Enzite; Miles Laboratories, Ltd., Stoke Poges, Slough, England) and separated from the proteolytically digested sample prior to incubation with antibodies gave identical results.

Since our antiserum recognizes the immunoactive non-steroid-binding domain of rat liver GR even after cleavage of this domain from the steroid- and DNA-binding domain by α-chymotrypsin digestion (4, 16), the antiserum was used to search for immunoactivity in CR P1798 lymphoma cytosol. Using an indirect competitive ELISA based on the anti-GR antiserum (4, 17), immunoactivity could be followed and compared to the radioactivity profile in different chromatograms.

When labeled CS P1798 cytosol was analyzed by gel filtration on Agarose A (0.5 m) and when the fractions were assayed for radioactivity and immunoactivity using ELISA, both the radioactivity and immunoactivity eluted together closely after the void volume, corresponding to the 6-nm GR (Chart 2A). However, α-chymotrypsin digestion of labeled CS P1798 cytosol separated an immunoactive domain from the steroid- DNA-binding GR domain (Chart 2B). The radioactivity eluted at a volume corresponding to a Stokes’ radius of about 3.3 nm, whereas the immunoactive domain eluted at a volume corresponding to a Stokes’ radius of about 2.6 to 2.8 nm. Following fractionation by gel filtration, cytosol from CR P1798 lymphoma was found not to contain any immunoactivity (Chart 2C). Radioactivity corresponding to the CR P1798 GR eluted at a Stokes’ radius of about 3.3 nm, identically to the α-chymotrypsin-induced steroid- and DNA-binding domain of GR from CS P1798 cells. α-Chymotrypsin digestion of CR P1798 cytosol did not change the radioactive or immunoactive profile compared to untreated CR P1798 cytosol. Mixing experiments between purified rat liver GR (80% purity, Ref. 29) and CR P1798 mouse lymphoma cytosol did not cause any change in Stokes’ radius of the purified rat liver GR (not shown), indicating the absence of proteolytic activity against the GR in cytosol from CR P1798.

The slightly different Stokes’ radius of GR from CR P1798 (2.8 nm) or α-chymotrypsin-digested CS P1798 mouse lymphoma cytosol (2.8 nm) described by Stevens and Stevens (25) on one hand and described in this paper (3.3 nm) on the other hand, as well as the varying Stokes’ radius of α-chymotrypsin-digested rat liver GR (3.6 and 3.3 nm, respectively) described by Wränge and Gustafsson (30) and Carlstedt-Duke et al. (3), are due to chromatography in different buffers and on different gel matrices.

In Chart 3, the immunoactivity and the radioactivity profiles of CS and CR P1798 lymphoma cytosols after chromatography on DNA-cellulose are shown. In the case of CS P1798 lymphoma cytosol, immunoactivity and radioactivity cochromatograph (Chart 3A). Activity was seen at 0.16 to 0.17 M NaCl and in the flow through volume. The latter activity corresponds to unre-
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DISCUSSION

Since our antibodies bind to a portion of the GR, which may play an important role in eliciting the glucocorticoid response (4), it was considered of value to characterize anti-GR immunoactivity in CS and CR P1798 mouse lymphoma cytosol.

In the present study, we show that the anti-rat liver GR antiserum cross-reacts with GR from CS P1798 lymphoma but not with GR from CR P1798 lymphoma. The same results were obtained by Stevens et al. (23) using an antiserum raised against partially purified rat liver GR by Eisen (7).

Using an indirect competitive ELISA based on specific antibodies against the purified rat liver GR (17), it is possible to detect unlabelled GR or immunoactive fragments of it. The ELISA was used to study whether any anti-GR immunoactivity exists in CR P1798 lymphoma cytosol but not linked up with the steroid- and DNA-binding domain of the receptor protein. This might represent a situation where endogenous proteolysis leads to division of the GR in CR P1798 cells into separate domains, similarly to the in vitro a-chymotrypsin digestion of CS P1798 lymphoma cytosol (cf. above: physicochemically identical steroid- and DNA-binding fragment in CR and CS P1798 cells, respectively). A second explanation is that different domains, synthesized separately, fail to combine with each other to form the native GR. If these situations occurred, the immunoactive fragments would presumably be detected in the ELISA (cf. below). Alternatively, the absence of anti-GR immunoactivity in CR P1798 mouse lymphoma cytosol may suggest that the GR from CR P1798 is synthesized directly as a polypeptide corresponding to the 3-nm form, due to a defect at the gene level or at later steps involved in genomic expression.

In contrast to CS P1798 lymphoma cytosol, no immunoactivity could be found in fractionated cytosol from CR P1798 lymphoma analyzed by gel filtration (Chart 2C) or DNA-cellulose chromatography (Chart 3B). a-Chymotrypsin digestion of the CS P1798 lymphoma cytosol separates the steroid- and DNA-binding fragment from the immunoactive fragment, the latter being readily detectable with the ELISA (Chart 2B).

Whereas the immunoactive fragment(s) from a-chymotrypsin-digested rat liver GR bound to DNA-cellulose with low affinity (eluted at 0.06 m NaCl, Ref. 4), no immunoactive fragment(s) from a-chymotrypsin-digested CS P1798 lymphoma cytosol binding to DNA-cellulose was observed. This might suggest that some differences exist between rat liver GR and GR from CS P1798 lymphoma or between cytosol components affecting DNA binding.

Yamamoto and coworkers (22, 31, 32) have isolated and characterized several mutant CR strains of S49 mouse lymphoma cells. The GR of the nt variant of the S49 cells resembles the GR of CR P1798 lymphoma. Nordeen et al. (15) have affinity-labeled the GR from various S49 cells using the synthetic gestagen, R5020. They found that GR from wild-type S49 cells had a molecular weight of 87,000, whereas GR from nt' S49 cells had a molecular weight of 39,000, when analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Similar results were obtained by Dellweg et al. (6) when TA was used for affinity labeling. The results of the limited proteolysis of GR from CR P1798 cells might suggest that the receptor in CR P1798 cells is synthesized as a larger precursor, which then undergoes rapid intracellular processing (i.e., proteolysis) to the 3-nm form. This, however, is not very likely as experiments with mixed CR and CS P1798 cytosols have shown no conversion of the 6-nm CS GR to a 3-nm form (24, 25). Furthermore, hybrids between wild-type CS S49 cells and nt' CR S49 cells showed a dominance of the CS properties (31). Finally, the results presented in this paper also argue against intracellular proteolysis of a 6-nm GR, since no immunoactive fragment(s) were detected in CR P1798 lymphoma cytosol. Another mechanism for modulation of receptor activity could be inhibitors of receptor-degrading enzymes; CS cells might contain such inhibitors, whereas CR cells might be devoid of them. This possibility seems to be excluded by the mixing experiment between purified rat liver GR (80% purity, cf. Ref. 29) and cytosol from CR P1798 cells which did not lead to any change in the Stokes’ radius of the rat liver GR. The evidence...
presented for the apparent lack of the immunoactive domain in CR P1798 lymphoma suggests that this domain is missing in these cells and that a mutation has occurred affecting the genome resulting in defective transcription of the receptor gene(s). However, other alternative explanations for the absence of an immunoactive domain cannot be ruled out, e.g., a change in the CR P1798 GR structure with a consequently altered sensitivity to attack by proteolytic enzymes (15), resulting in loss of immunoactivity. Failure to detect the immunoactive fragment(s) in CR P1798 cells might be due to a subassay threshold concentration of the immunoactive receptor domain or dispersion of the immunogenic site among several other fragments during chromatography. Also, the missing immunoactive domain might possibly be found in a cell compartment other than the soluble cytosolic fraction.

The apparent lack of the immunoactive domain in the CR P1798 cells appears to indicate an important function of this domain with regard to the biological activity of the receptor, possibly in connection with the specific interaction of the receptor complex with the genome. We have shown earlier that the purified rat liver GR specifically interacts with a cloned fragment of the mouse mammary tumor virus gene (18) which is regulated complex with the genome. We have shown earlier that the concentration of the immunoactive receptor domain or dispersion of immunoactivity. Failure to detect the immunoactive fragment(s) in CR P1798 cells might be due to a subassay threshold concentration of the immunoactive receptor domain or dispersion of the immunogenic site among several other fragments during chromatography. Also, the missing immunoactive domain might possibly be found in a cell compartment other than the soluble cytosolic fraction.

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