Steroid-induced Conformational Changes at Ends of the Hormone-binding Domain in the Rat Glucocorticoid Receptor Are Independent of Agonist Versus Antagonist Activity*

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The underlying molecular mechanism for the expression of agonist versus antagonist activity for a given receptor-steroid complex is still not known. One attractive hypothesis, based on data from progesterone receptors, is that agonist versus antagonist binding induces unique conformational changes at the C terminus of receptors, which can be detected by the different fragments produced by partial proteolysis. We now report that the determinants of glucocorticoid receptor (GR)-agonist complex activity are more complex. Steroid binding can cause a conformational change in the GR that was detected by partial trypsin digestion, as described previously (Simons, S. S., Jr., Sistare, F. D., and Chakraborti, P. K. (1989) J. Biol. Chem. 264, 14493–14497). However, there was no uniformity in the digestion patterns of unactivated or activated receptors bound by a series of six structurally different antagonists including the affinity labeling antiglucocorticoid dexamethasone 21-mesylate. A total of four resistant bands were observed on SDS-polyacrylamide gels in the range of 30–27 kDa. Using a series of point mutations and epitope-specific antibodies, it was determined that the 30-kDa species represented the entire C-terminal sequence of amino acids 518–795, whereas the other bands arose from additional N-terminal and/or C-terminal cleavages. Bioassays with GRs containing various point and deletion mutations failed to reveal any C-terminal alterations that could convert antagonists into biologically active agonists. Thus, the presence or absence of C-terminal amino acids of the GR did not uniquely determine either the appearance of smaller trypsin-resistant fragments or the nature of the biological response of receptor-bound antagonists. When compared with the current model of the ligand-binding domain, which is based on the x-ray structures of the comparable region of thyroid and retinoic acid receptors, the present results suggest that sequences outside of the model structure are relevant for the binding and biological activity of GRs.

Ligand binding to the cognate intracellular receptor is the obligate first step by which steroid hormones in the circulatory system regulate gene transcription in selected cells of mammals. In general, the steroid receptors contain two transactivation domains, AF-1 and AF-2, in the amino- and carboxyl-terminal portions of the molecule, respectively (1). The binding of agonists to the ligand-binding domain (LBD)1 in the carboxyl-terminal half of the receptor is thought to cause a conformational change to uncover/create the AF-2 domain that regulates the transcriptional activation of receptors bound to the appropriate hormone response element (Ref. 2; reviewed in Refs. 3 and 4). Many antisteroids can also cause the binding of receptors to hormone response elements (5, 6), but the resulting complexes appear to be transcriptionally inactive (7–9). This ability of antisteroids to block the action of agonist steroids makes them useful both as probes of the mechanism of steroid hormone action and as drugs. Antiestrogens are commonly used to treat estrogen-dependent breast cancers, and antiandrogens are prescribed to combat prostate cancer (10–12).

Despite the differences in the final biological response, the initial steps for most agonist and antagonist steroids are identical. The high affinity binding site in the LBD appears to be the same for both classes of steroid, as indicated by the affinity labeling of the same amino acid of the human estrogen receptor by an estrogen agonist (ketononestril aziridine) and antagonist (tamoxifen aziridine) (13). Both classes of steroid can cause release of hsp90 after steroid binding that is accompanied by the acquisition of high affinity DNA binding (Refs. 5 and 6; reviewed in Ref. 14). Perhaps most revealing is that virtually all antagonists have been observed to exhibit partial agonist activity under some condition. The fact that even the “best” antagonists for glucocorticoid (15–17), progesterone (18, 19), and estrogen (8, 20, 21) receptors can be made to display significant amounts of agonist activity suggests that the differences between agonist and antagonist complexes are quantitative rather than qualitative. Thus, receptors complexed with either agonists or antagonists share at least a portion of the various components required for transcriptional activation.

While the distinction in biological activity between agonist and antagonist steroids may not be absolute, there usually are major differences in the amount of activity that need to be explained. Clearly, the structure of each steroid is of primary importance. Often changes in just the substituents of the basic steroid structure are sufficient to convert an agonist into an antagonist. However, the same substituent may not be equally effective within each group of agonists (22). Thus, structure-

1 The abbreviations used are: LBD, ligand-binding domain; GR, glucocorticoid receptor; rGR, rat glucocorticoid receptor; TAPS, 3-[tris(hydroxymethyl)methyl]aminopropanesulfonic acid; TBS, Tris-buffered saline; DM, dexamethasone 21-mesylate.

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activity relationships have yet to provide a satisfactory framework for predicting the properties of a given steroid. For this reason, attention has shifted from differences in the structure of the steroid to possible modifications in receptor conformation following steroid binding.

The most commonly used method for detecting conformational changes has been site-selective proteolysis. This method was first used to study the tertiary structure of the DNA- and non-DNA-binding forms of glucocorticoid receptors (GRs) (23) and then to establish a conformational change in GRs following steroid binding (24). These studies employed affinity labeling to identify the various receptor fragments. Since then, the utility of the method has been greatly expanded both by the availability of anti-receptor antibodies and by the use of in vitro transcribed, [35S]methionine-labeled receptor in the elegant studies of O’Malley and co-workers (25–27). Thus, protease digestion studies have confirmed that steroid binding induces conformational changes in all of the members of the steroid receptor superfamily that have been examined (reviewed in Ref. 4). Even more tantalizing were the observations that antisteroids appeared to place the receptor in a conformation that rendered the carboxyl-terminal tail of the protein more susceptible to proteolytic cleavage (25). At the same time, studies with the long form of the human progesterone receptor (B form) indicated that deletion of the last 42 amino acids, which were required for the binding of progesterone but not the antiprogestin RU 486, permitted RU 486 to act as an agonist (28). Apparently similar phenomena for other receptors and additional confirmatory data have subsequently appeared (25, 29–33). Thus, it has been proposed that the transcriptional activation by antisteroids is controlled by a steroid-induced conformational change in the C terminus of the LBD that can be detected by differential proteolysis of the individual receptor-steroid complexes (25, 34).

Other studies, however, with partially proteolized complexes of steroid-bound androgen (35, 36), estrogen (37), mineralocorticoid (38), and progesterone (39) receptors and retinoic acid receptor α (31, 40) have raised questions about the generality of this hypothesis. The purpose of this study was to address several major unanswered questions concerning the importance of the C-terminal sequences of the GR LBD in the expression of antiglucocorticoid activity. Thus, we wanted to know whether steroid-induced conformational changes in the GR were specific for agonist versus antagonist steroids and where precisely in the receptor LBD the steroid-induced conformational changes occurred. To answer these questions, we have used trypsin to probe rat GRs bound by a variety of steroidal and nonsteroidal ligands. The receptor fragments were identified with affinity labeling and/or anti-receptor antibodies. Receptor mutations were used to confirm various assignments.

MATERIALS AND METHODS

Unless otherwise indicated, all operations were performed at 0 °C.

Chemicals—[1,2,4-3H]Dexamethasone was purchased from Amersham Corp. [6,7,8-3H]Dexamethasone 21-mesylate (DM), 85020, and Enlightning were obtained from NEN Life Science Products. TAPS, dextran, and lyso phosphatidylcholine were purchased from Calbiochem. Hydrofluor was from National Diagnostics, Inc. (Atlanta, GA). Tween, acrylamide, bisacrylamide, and SDS were purchased from Bio-Rad. Decylcortizol, RU 486, and ZK 98,299 were gifts from Schering AG, Berlin, respectively. Dexamethasone oxetanone (41) and DM (42) were prepared as described. All other chemicals were obtained from Sigma, including N’-p-tosyl-l-lysine chloromethyl ketone, N’-p-tosyl-l-lysine, and N’-2-phényl ethyl chloromethyl ketone-treated trypsin. The polyclonal antibody αP1, which was raised against the carboxyl-terminal region of the rGR (amino acids 440–795) (16), was a gift from Dr. Bernd Groner (Friedrich Miescher-Institut, Basel, Switzerland), and the antibody hGRα, which was raised against the C-terminal 19 amino acids of human (and rat or mouse) GR (43), was a gift from Dr. George Chrousos (NICHHD, National Institutes of Health, Bethesda, MD). The anti-GR-(45–795) antibody (44) was a gift from Dr. Heinrich Westphal (University of Marburg, Marburg, Germany).

Buffers and Solutions—TAPS buffer (25 mM TAPS, 1 mM EDTA, and 10% glycerol (pH 8.8)) was adjusted to pH 8.8 or 9.5 at 0 °C with NaOH. Tris-buffered saline (TBS) contained 20 mM Tris and 0.28 mM NaCl (pH 7.5). The 2 × TBS sample buffer contained 0.6 mM Tris (pH 8.85), 0.2 M dithiothreitol, 2% SDS, 20% glycerol, and bromphenol blue. The Western blot transfer buffer was composed of 25 mM Tris (pH 8.3), 192 mM glycine, and 20% methanol.

Preparation of Mutant Receptors—The K536A mutant GR was prepared using the Sculptor in vitro mutagenesis kit (Amersham Corp.) by annealing a synthetic oligonucleotide, with the underlined base changes to create the desired mutant (5′-AAAACTCTTAGCAAAATAGTCTCGT-3′), to the full-length single-stranded GR cloned in the pIT218U plasmid (45). After the annealed oligonucleotide was extended and ligated using Klenow polymerase and T4 DNA ligase, the remaining single-stranded template was removed with T5 exonuclease. The resultant linear strand was extended using T4 DNA polymerase and ligated into the XhoI–EcoRI site of the cosmid containing the wild-type (pSVL-GR) or mutant receptors into monolayer cultures of COS-7 cells. The Cs1, Cs2, and Cs1/Cs2 mutant GRs were gifts from Dr. Sandro Rusconi (University of Fribourg, Fribourg, Switzerland).

Preparation of Receptors—HTC cell cytosol containing steroid-free receptors was prepared as reported (23). Transfected COS-7 cell cytosol was made by resuspending the frozen cell pellet in TAPS buffer (pH 9.5), slowly thawing the pellet on ice, followed by centrifugation at 17,000 × g. The supernatant was used as cytosol.

For purified receptors, HTC cell cytosol was first covariently labeled by incubation with [3H]DM for 2.5 h at 0 °C. A 6-ml aliquot of labeled cytosol was loaded onto a PD10 column packed with 3.7 ml of DNA-cellulose (Pharmacia Biotech Inc.) that had been equilibrated with TAPS buffer (pH 8.8) containing 50 mM NaCl. The column was washed with a 100-fold excess of the same buffer, and the receptor was eluted in 0.4 M fractions with TAPS buffer (pH 8.8) containing 500 mM NaCl. The peaks of radioactivity were stored at −80 °C until needed (47).

Steroid Binding Assays and Proteolytic Digestion of Mutant Receptors—For Scatchard analysis, duplicate aliquots of HTC cell or transiently transfected COS-7 cell cytosol were incubated in TAPS buffer (pH 8.8) plus 20 mM sodium molybdate with 0.625–50 nM [3H]dexamethasone for 2.5 h at 0–4 °C. Free steroid was removed by adding an aliquot of 10% dextran-coated charcoal solution. Specific binding was determined by subtracting the nonspecific binding seen in the presence of unlabeled dexamethasone from the total binding. The affinity (Kd) was determined by plotting the ratio of bound steroid/free steroid versus bound steroid.

Proteolytic digestion was performed with HTC or transfected COS-7 cell cytosol that had been incubated with ethanol or 1 μM steroid for 2.5 h at 0–4 °C. Only unactivated receptor solutions contained 20 mM sodium molybdate. Receptor-steroid complexes were activated by heating at 20 °C for 30 min. Affinity-labeled receptors were prepared by incubating cytosol with 150 nM [3H]dexamethasone for 20–30 min at 0 °C. A 6-ml aliquot of labeled cytosol was loaded onto a PD10 column packed with 3.7 ml of DNA-cellulose. After washing the column with TAPS buffer (pH 8.8) plus 0.1% SDS, the receptor was eluted in 0.4 M NaCl with 0.1% SDS and 0.1% sodium dodecyl sulfate. The receptor was then subjected to polyacrylamide gel electrophoresis and stained with Coomassie Blue R-250 for 30 min at room temperature. The gels were destained overnight in a solution of 10% methanol and 7.5% acetic acid, and 0.01% Coomassie Blue R-250 for 30 min at room temperature. The gels were dried on a Bio-Rad Model GF plates at 80 °C. After marking the positions of the molecular mass markers (Pharmacia) with a fluorescent paint, the gels were scanned against a 0 °C film.

Fluorography—Samples were diluted 1:2 in 2 × SDS sample buffer, heated for 5 min in a boiling water bath, and analyzed on 12% polyacrylamide gels run in a water-cooled (15 °C) Bio-Rad Protein II slab gel apparatus. Gels were fixed and stained in a solution of 50% ethanol, 7.5% trichloracetic acid, and 0.01% bromphenol blue in room temperature. The gels were destained overnight in a solution of 10% methanol and 7.5% acetic acid and then incubated with constant shaking in Enlightning for 1 h, followed by a 10% polyethylene glycol 8000 solution for 30 min at room temperature. The gels were dried on a Bio-Rad Model 443 slab gel drier at 80 °C for 2 h and exposed to Kodak X-Omat XAR-5 film at −80 °C for at least 2 weeks after marking the positions of the molecular mass markers (Pharmacia) with a fluorescent paint.
Western Blotting—Samples were diluted with 2 × SDS sample buffer and analyzed on polyacrylamide gels as described above. The gels were equilibrated in transfer buffer for 30 min at room temperature prior to electrophoretic transfer of receptor to nitrocellulose membranes in a Bio-Rad Transblot apparatus (100 mA overnight followed by 250 mA for 2 h). The nitrocellulose was stained in Ponceau S (0.02% Ponceau S and 0.04% glacial acetic acid in water) to localize molecular mass markers, incubated with 10% Carnation nonfat dry milk in TBS for 45 min, and washed three times with TBS containing 0.1% Tween (0.1TTBS) for 5 min. Primary antibody was diluted in 0.1TTBS (1:20,000 for aP1, 1:5000 for hGRα, or 1:5 for anti-GR-(788–795)) and added to the nitrocellulose for a 2-h incubation at room temperature. Biotinylated anti-rabbit or anti-mouse (for the antibody provided by Dr. Heinrich Westphal) secondary antibody and ABC reagents (each diluted 1:1000; Vector Laboratories, Inc., Burlingame, CA) were each added for sequential 30-min incubations at room temperature. After the incubation periods with primary antibody, secondary antibody, and ABC reagents, the nitrocellulose was washed three times for 5 min each with 0.1TTBS and an additional three washes with TBS containing 0.3% Tween immediately after incubation with the ABC reagents. Detection of signal was performed by enhanced chemiluminescence using the recommended protocol of the supplier (Amersham Corp.). The positions of the molecular mass markers were indicated by overlaying with a fluorescent paint marker.

RESULTS

Protease Digestion of Unactivated Complexes—A total of six antiglucocorticoids and two glucocorticoids (22, 49–51) of relatively different structures were selected (Fig. 1). HTC cell receptors were prebound by each steroid in the presence of 20 mM sodium molybdate to afford unactivated complexes that do not bind DNA. A series of fragments in the range of 30–27 kDa were visualized by Western blotting after digestion with chymotrypsin and trypsin (Fig. 2) or lysyl endopeptidase C (data not shown). With all three proteases, one or more intense bands at ≈29 kDa were seen for receptors prebound by RU 486 as opposed to dexamethasone. The smaller band(s) was also seen with ZK 98,299 but not with any other antiglucocorticoid examined (Fig. 2 and data not shown). Thus, a unique digestion pattern with lysyl endopeptidase C, chymotrypsin, or trypsin appears to be a function of receptors bound by steroids containing a bulky 11β-substituent (Fig. 1) as opposed to diagnostic property of unactivated GRs bound by antiglucocorticoids in general. Furthermore, receptors bound by the most potent agonist, deacetylcoritavazol (52), gave the same lower band after lysyl endopeptidase C digestion as seen for RU 486 (data not shown).
shown). Therefore, no obvious correlation with steroid activity existed among the digestion patterns of unactivated receptor-steroid complexes for any of the three proteases.

The axiom that one steroid molecule is bound per receptor monomer has only recently received direct support from biochemical (reviewed in Ref. 4) and x-ray crystallographic (53, 54) studies. However, earlier reports of a second site, especially with high concentrations of steroid (55), have lately attracted considerable attention (56) with the description of synergistic responses among weak estrogens (57). We were unable to detect such a second site. The chymotrypsin or trypsin digestion pattern of receptors bound by 150 nM DM ± 15 μM dexamethasone (Fig. 2) and the chymotrypsin cleavage patterns of receptors bound by different concentrations of RU 486 (30 nM, 1 μM, or 15 μM plus 150 nM DM) were unaffected by the different steroid concentrations (data not shown). Therefore, the conclusion that the binding of these steroids to a possible second site does not dramatically alter the tertiary structure of the LBD. By the same criteria, the binding of the agonist dexamethasone and that of the antagonist DM were indistinguishable (Fig. 2).

Protease Digestion of Activated Complexes—The operational difference between glucocorticoid and antiglucocorticoid steroids arises from the ability of the respective activated receptor-steroid complexes to transactivate responsive genes. Consequently, any general conformational differences might also be visible only at the level of activated complexes. To examine this possibility, we concentrated on trypsin digestion since the greater specificity of trypsin versus chymotrypsin (58, 59) would facilitate the identification of the observed cleavage sites. As had been reported with the unactivated complexes (24), the activated complexes were also much more resistant to proteolysis than were the steroid-free receptors (Fig. 3A). Furthermore, the four species at 30–27 kDa observed upon digestion of the various activated complexes were apparently identical to those seen in Fig. 2 with the unactivated complexes, with the 30-kDa species being a precursor of the 28-kDa fragment (Fig. 3B).

The same 30-kDa band was formed by the digestion of steroid-free, dexamethasone-bound, and DM-labeled receptors (Fig. 3 and data not shown). Likewise, the 28-kDa product was the same whether the receptors were bound by dexamethasone or affinity-labeled by DM. Thus, agonists and antagonists bind to the same general region of activated GRs. In fact, both agonists and all five antagonists examined gave the same 30- and 28-kDa tryptic fragments. No difference in the size of these fragments was observed when using the endogenous receptor of HTC cells versus overexpressed receptors from transiently transfected COS-7 cells (data not shown).

A 27-kDa band was obtained at higher trypsin concentrations with antiglucocorticoids containing a bulky 11β-substituent. However, as for the unactivated complexes (Fig. 2), the production of this species was not associated with the binding of any other antagonists (Fig. 3). A weak band at 29 kDa was often produced, but with no relationship to the type of steroid bound (Figs. 2 and 3A; see also below). Therefore, no tryptic digestion pattern of activated glucocorticoid-steroid complexes was characteristic of either agonist or antagonist steroids.

Amino Termini of Tryptic Fragments—It had already been established that the 30-kDa tryptic fragment begins at amino acid 518 of the rat receptor (60). As was previously reported (23, 24) and confirmed in Fig. 3B, the 28-kDa material appears to derive from the 30-kDa fragment. Thus, C- or N-terminal cleavage of the 30-kDa species could generate the 28-kDa band. N-terminal cleavage was examined by construction of a mutant (K536A) at the first available trypsin site after amino acid 518. The K536A mutant bound dexamethasone with wild-type affinity, as determined by Scatchard plots ($K_d = 2.29 \pm 0.70$ nM ($n = 2$) versus $2.57 \pm 0.01$ nM ($n = 2$) for wild-type receptors). Trypsin digestion of K536A gave no 28-kDa fragment under conditions where all of the wild-type receptor was converted to 28 kDa (Fig. 4A). In fact, no 28-kDa band was observed upon

**Fig. 3.** Digestion of activated complexes with trypsin. A, aliquots of COS-7 cell cytosol that had been transiently transfected with wild-type cDNA plasmids were incubated as described in the legend to Fig. 2, except that no sodium molybdate was present and all steroids were at 1 μM. Receptor-steroid complexes were then activated (30 min at 20 °C) before proceeding with digestion with the indicated concentrations of trypsin for 1 h at 0 °C. Proteolytic fragments were identified as described in the legend to Fig. 2. See Fig. 1 for definitions of abbreviations used. B, the 30-kDa tryptic fragment of GR complexes was converted to a 28-kDa fragment with increasing trypsin digestion. Aliquots of activated receptors bound by 1 μM dexamethasone (D) or RU 486 (R) were digested with increasing concentrations of trypsin for 1 h at 0 °C and then identified as described in the legend to Fig. 2.
trypsin digestion of K536A receptors complexed with any of the steroids of this study (data not shown). Fluorography of DM-labeled receptors confirmed both that the 30-kDa fragment of the K536A mutant was receptor and that no 28-kDa receptor species was generated (data not shown). This establishes that the 28-kDa fragment is formed by a cleavage at Lys-536, just amino-terminal to the steroid-binding domain of amino acids 550–795 (61).

The 27-kDa fragment was seen only after trypsin digestion of wild-type receptors complexed with RU 486 and, to a much smaller extent, with ZK 98,299 (Figs. 2 and 3). Digestion of the K536A mutant bound by a variety of steroids yielded no 27-kDa fragment (Fig. 4A and data not shown). Instead, the above-mentioned 29-kDa band was prominent in the presence of RU 486 and less intense with ZK 98,299. Therefore, both the 27- and 28-kDa bands require cleavage at Lys-536 for their formation. The fact that the K536A mutation appears to shift the 28- and 27-kDa trypsin digest bands to the same 30- and 29-kDa fragments that were obtained with wild-type receptors (Fig. 4A) argues that there are two sets of proteolytic fragments. The first set (30 and 29 kDa) would have Gly-518 as their N terminus; the second set (28 and 27 kDa) would begin at Thr-537.

Identification of the C Terminus of Fragments—Given the observed molecular mass of the 30-kDa fragment and its known amino terminus of Gly-518 (60), the predicted C terminus would be one of the five basic amino acids between Lys-761 and the end of the receptor. Two antibodies were helpful in precisely determining the cleavage point: hGRa, which recognizes the C-terminal amino acids 777–795 of the rGR (43), and an antibody raised against the rat sequence of amino acids 788–795 (44). Both the 30- and 28-kDa fragments were recognized by each antibody, thereby establishing that no C-terminal cleavage had occurred (Fig. 4, B and C). Therefore, the 30-kDa fragment is composed of amino acids 518–795, whereas the 28-kDa species corresponds to amino acids 537–795.

It is noteworthy in Fig. 4C that the antibody to the C-terminal eight amino acids did not recognize the 42-kDa trypsin fragment (23, 24). Thus, this species does not extend to amino acid 795 and represents an intermediate in a different branch in the digestion of receptors by trypsin.

Neither the 29- nor the 27-kDa trypsin digest product was visualized by the antibody against amino acids 788–795 (Fig. 4, A versus B). This, in combination with their above-established

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Protease Digestions Cannot Predict Receptor Complex Activity

A

| Trypsin (µg/ml): | 50 | RU486 | 250 | RU486 |
|-----------------|----|-------|----|-------|
| Receptor:       |    |       |    |       |
| W               | 97.4 | 66.3 | 45 | 30.6 |
| CD              | 97.4 | 66.3 | 45 | 30.6 |
| CS1             | 97.4 | 66.3 | 45 | 30.6 |
| CD/CS1          | 97.4 | 66.3 | 45 | 30.6 |
| CS1/CD          | 97.4 | 66.3 | 45 | 30.6 |
| W               | 21.5 | 15.0 | 10.5 | 6.0 |
| CD              | 21.5 | 15.0 | 10.5 | 6.0 |
| CS1             | 21.5 | 15.0 | 10.5 | 6.0 |
| CD/CS1          | 21.5 | 15.0 | 10.5 | 6.0 |
| CS1/CD          | 21.5 | 15.0 | 10.5 | 6.0 |

B

| Trypsin (µg/ml): | 50 | RU486 | 250 | RU486 |
|-----------------|----|-------|----|-------|
| Receptor:       |    |       |    |       |
| W               | 21.5 | 15.0 | 10.5 | 6.0 |
| CD              | 21.5 | 15.0 | 10.5 | 6.0 |
| CS1             | 21.5 | 15.0 | 10.5 | 6.0 |
| CD/CS1          | 21.5 | 15.0 | 10.5 | 6.0 |
| CS1/CD          | 21.5 | 15.0 | 10.5 | 6.0 |

Fig. 5. Trypsin digestion fragments of wild-type and mutant receptors as detected by region-specific anti-receptor antibodies. Aliquots of COS-7 cell cytosol that had been transiently transfected with wild-type (wt) or mutant receptor (see “Results”) cDNA plasmids were digested with trypsin at the indicated concentrations for 30 min at room temperature and identified by Western blotting. The lower intensity of the species derived from CD and CS1/CD and detected by the anti-GR-(777–795) antibody (Fig. 5B) versus aP1 (Fig. 5A) is probably due to the disruption of one or more antigenic sites by the deletion of amino acids 780 and 781.

The fact that added dexamethasone was able to protect the CS1 and CS1/CD receptors from digestion by low concentrations of trypsin (Fig. 5A) was unexpected in view of the lack of dexamethasone binding affinity in Scatchard plots. This protease resistance appears to result from a combination of the mutation itself (see below) plus some low affinity binding of dexamethasone that probably is removed by the charcoal used during work-up of the Scatchard assays.

Relationships between the Protease Digestion Patterns and the Biological Activity of Mutant Receptor-Steroid Complexes—Although there was no qualitative or quantitative correlation between the biological activity and the protease digestion pattern of steroid bound to the wild-type receptor, such a relationship might exist among mutant receptors. The CS1 mutant was described to be transcriptionally active in intact cells when complexed with RU 486, but not with dexamethasone or DM (51, 62). In CV-1 cells, the transiently transfected CS1 receptors displayed no activity with the antagonist ZK 98,299, significant activity with RU 486, and the most activity with aP1 (Fig. 5A) was unexpected in view of the lack of dexamethasone binding affinity in Scatchard plots. This protease resistance appears to result from a combination of the mutation itself (see below) plus some low affinity binding of dexamethasone that probably is removed by the charcoal used during work-up of the Scatchard assays.

As might be expected, the missing two amino acids of the CD and CS1/CD mutants gave rise to slightly smaller 30- and 28-kDa species than those seen for the wild-type or CS1 mutant receptors (Fig. 5). This effect is more clearly visible in Fig. 5B, where only those fragments containing amino acids within the epitope of positions 777–795 (i.e. 30 and 28 kDa) were detected by Western blotting. The lower intensity of the species derived from CD and CS1/CD and detected by the anti-GR-(777–795) antibody (Fig. 5B) versus aP1 (Fig. 5A) is probably due to the disruption of one or more antigenic sites by the deletion of amino acids 780 and 781.

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DISCUSSION

This study identifies the protease digestion fragments of unactivated and activated GRs complexed with a series of structurally varied agonists and antagonists. A series of two to four protease-resistant species of 30–27 kDa were observed with trypsin, chymotrypsin, and lysyl endopeptidase C. No difference was seen between unactivated and activated complexes. Thus, while the association of non-receptor proteins such as hsp90 (14, 63) restricted the accessibility of some antibodies to unactivated GR (64), the conversion of unactivated GR to activated complexes caused no obvious change in the trypsin digestion patterns (Figs. 2 and 3) (23, 25).

No relationship was noted between the digestion patterns with three different proteases and the transcriptional activity of the various receptor-steroid complexes. Most of our studies utilized trypsin, which afforded 30- and 28-kDa species from complexes of wild-type receptors with all steroids. A weak 29-kDa band was obtained in the presence of dexamethasone and two of the antagonists, RU 486 and ZK 98,299 (Figs. 2, 3B, and 6B). A 27-kDa fragment was observed with RU 486 and ZK 98,299, but not with any other antiglucocorticoid (Figs. 2 and 3A). These observations did not simply reflect unequal amounts of agonist activity with the various antisteroids. RU 486, ZK 98,299, progesterone, and DM each elicited ≤15% agonist activity in CV-1 (data not shown) and HeLa (Ref. 51 and data not shown) cells, but yielded very different amounts of 27-kDa fragment. In other situations, some of the antagonists of Fig. 1 produced substantially more agonist activity, but were still unable to produce any of the 27-kDa fragment that would be expected on the basis of the steroid’s partial antagonist activity. This lack of correlation might have been anticipated since the amount of agonist activity displayed by a given receptor-antiglucocorticoid complex is usually not constant, but varies with parameters such as the gene examined (17, 46, 65), the presence of cis-acting elements (66–69), and the total amount of GR present in the cell (51).

We are aware of only one previous protease digestion study of the GR with multiple ligands (17). Those results were similar to ours, although fewer ligands were examined and the cleavage sites were not identified. Again, no antagonist-specific pattern from trypsin digestion was observed. No resistant bands were obtained with receptors bound by the antagonist ZK 98,299, possibly because the ZK 98,299-bound receptors assumed a protease-sensitive conformation at room temperature (17). However, these trypsin digestions were run at a higher temperature (room temperature for 20 min versus 0 °C for 60 min).

FIG. 6. Effect of different steroids on the trypsin digestion patterns of CS1 and GR-(1–766) mutant receptors. A, aliquots of COS-7 cell cytosol that had been transiently transfected with wild-type (wt) or mutant (CS1) receptor cDNA plasmids were treated with vehicle (final concentration = 1% EtOH) or a 1 μM concentration of the listed steroids, activated, digested with the indicated concentrations of trypsin, and identified by Western blotting with the aP1 antibody as described in the legend to Fig. 2. B, truncated GR-(1–766) in the cytosol of transiently transfected COS-7 cells was incubated with 1 μM steroid, activated, digested with the indicated concentrations of trypsin, and identified by Western blotting with the aP1 antibody as described in the legend to Fig. 2. Note the somewhat more abundant 29-kDa band in 50 μg/ml trypsin digests of wild-type receptors bound by agonist (dexamethasone) and antagonist (RU 486). See Fig. 1 for definitions of abbreviations used.
Protease Digestions Cannot Predict Receptor Complex Activity

Our inability to observe antagonist-specific protease digestion patterns and the presence of protease cleavage at both ends of the LBD in the current data also support the conclusion that agonists and antagonists bind to a common site in the GR. Therefore, our earlier conclusions regarding ligand-induced conformational changes (23, 24, 59, 70, 71) are not unique for the affinity labeling antiglucocorticoid DM, but are also seen with noncovalently binding agonists and antagonists (see Figs. 2 and 3).

Another property of progesterone receptors is that deletion of carboxyl-terminal sequences causes selective binding of anti-progestins to give complexes with agonist activity (28). This has received additional convincing support (33), leading to the hypothesis that the protease digestion patterns specifically associated with antiprogestin activity are due to a conformational change in the most C-terminal sequence of the receptor. However, there has been some difficulty in establishing the generality of this hypothesis, even for progesterone receptors. Human B form progesterone receptors lacking the C-terminal 12 and 32 amino acids were found to be completely inactive with the antiprogestin RU 486 (62). Human androgen receptor lacking the C-terminal 12 residues, which contain most of the highly homologous antagonist-specific sequence of human progestosterone receptors (33), did not bind either androgens or most antiandrogens (36) and was inactive with the one antiandrogen that would still bind (62). C-terminal deletions of 28, 33, or 55 residues from the rGR (62) and mouse and rat GRs lacking 14 or 42 amino acids (72) were inert. Finally, the GR(1-766) lacking the C-terminal 29 residues bound RU 486 (Fig. 6B), but still did not display any activity.

The positions of the trypsin cleavage sites in the rGR are interesting in relationship to the recently proposed three-dimensional structure of steroid receptor LBDs based on the x-ray structures of the steroid-bound LBDs of the retinoic acid receptor and the retinoid X receptor (2). The two amino-terminal cleavage sites, positions 517 and 536 of the rGR (Fig. 7), are before the start of both the LBD at Leu-550 (61) and the α-helical sandwich structure of the LBD, which extends from residue 544 to 776 (2). Carboxyl-terminal trypsin cleavage occurred at Lys-781, which again is beyond the sequences of the general LBD model (2). For this reason, it is not obvious how the binding of steroid would affect this position. However, for GRs, this region is definitely important as point mutations among these C-terminal 18 amino acids can have major effects on dexamethasone binding (reviewed in Refs. 3 and 73). Furthermore, deletion of the last five amino acids reduced steroid binding by a factor of 20 (74), whereas removal of all residues

Fig. 7. Summary of the trypsin digestion pattern of the wild-type GR. The solid bar is a schematic of the C-terminal ~290 amino acids. The positions of lysine and arginine residues are indicated by solid and broken vertical lines, respectively. Relevant basic amino acids discussed in the text are numbered. The arrowhead corresponds to the position of the cysteine that is covalently labeled by DM. Arrows show the locations of the mutations for the CS1 and CS1/CD mutants. Antigenic sites for hGRs (solid) and anti-GR (768-795) (broken) are shown under the C-terminal receptor sequence. The sequences assigned to the 30-27-kDa fragments discussed in the text are given below, with the numbers under each bar designating the terminal amino acids.

30 kDa

29 kDa

28 kDa

27 kDa

The precise trypsin cleavage sites of each of the fragments in the range of 30–27 kDa have been determined to occur at both ends of the LBD (Fig. 7). The 30- and 28-kDa species were now found to be slightly larger than originally predicted from their migration on SDS-polyacrylamide gels (24). While there were major differences in the rate of trypsin digestion of steroid-free and DM-bound receptors (23, 24), there was no apparent distinction in the size of the fragments (Fig. 3A). The current data also support the conclusion that agonists and antagonists bind to a common site in the GR. Therefore, our earlier conclusions regarding ligand-induced conformational changes (23, 24, 59, 70, 71) are not unique for the affinity labeling antiglucocorticoid DM, but are also seen with noncovalently binding agonists and antagonists (see Figs. 2 and 3).

Our inability to observe antagonist-specific protease digestion patterns and the presence of protease cleavage at both ends of the LBD are very similar to what has been reported for several other steroid receptors. Digestion of human androgen receptor complexes with subtilisin gave smaller fragments with some (but not all) antiandrogens (35). Subsequent investigations of the trypsin digestion patterns established that the binding of the antiandrogen RU 486 permitted a C-terminal cut. However, similar digestion was not observed with any of the other four antagonists examined, and cleavage sites were found at both ends of the RU 486-bound LBD (36). A detailed study of trypsin digestion of human retinoic acid receptor α complexes uncovered an antagonist-specific digestion pattern for trypsin, elastase, and chymotrypsin. However, all cleavages were at either end of the LBD, with the size differences deriving from alternative N-terminal cleavages (31). With trypsin, the smaller antagonist-specific fragment probably resulted from digestion at Arg-217, which is in the Ω-loop (40). C-terminal digestion of retinoic acid receptor α occurred within the F domain distal to the LBD, so the C terminus of the LBD was intact in all fragments (31). Finally, consistent antagonist-specific protease digestion patterns could not be obtained with human estrogen receptors (37) or human mineralocorticoid receptors (Ref. 38; reviewed in Ref. 4).

A close correspondence between the protease digestion patterns and the transcriptional activity of receptor-steroid complexes has been described with progesterone receptors. Following the initial observation that the carboxyl terminus of antagonist-bound human progesterone receptors was preferentially cleaved by proteases (25), numerous additional studies have provided strong supporting evidence (29, 32, 34). However, a recent study with six progesterone receptor ligands and 23 different proteases, including trypsin, noted a variety of cleavage patterns as opposed to any strict correlation between fragment size and agonist versus antagonist activity of the complexes (39). We do obtain C-terminal cutting by trypsin at Lys-781 of the rGR bound by the antiglucocorticoids RU 486 and ZK 98,299. However, while this trypsin-detected conformation may contribute to the antagonist activity of RU 486 and ZK 98,299, it clearly is not required for the antagonist activity of several other antiglucocorticoids that do not engage C-terminal trypsin cuts (Figs. 2 and 3).

Another property of progesterone receptors is that deletion of carboxyl-terminal sequences caused selective binding of anti-progestins to give complexes with agonist activity (28). This has received additional convincing support (33), leading to the hypothesis that the protease digestion patterns specifically associated with antiprogestin activity are due to a conformational change in the most C-terminal sequence of the receptor. However, there has been some difficulty in establishing the generality of this hypothesis, even for progesterone receptors. Human B form progesterone receptors lacking the C-terminal 12 and 32 amino acids were found to be completely inactive with the antiprogestin RU 486 (62). Human androgen receptor lacking the C-terminal 12 residues, which contain most of the highly homologous antagonist-specific sequence of human progestosterone receptors (33), did not bind either androgens or most antiandrogens (36) and was inactive with the one antiandrogen that would still bind (62). C-terminal deletions of 28, 33, or 55 residues from the rGR (62) and mouse and rat GRs lacking 14 or 42 amino acids (72) were inert. Finally, the GR(1-766) lacking the C-terminal 29 residues bound RU 486 (Fig. 6B), but still did not display any activity.
after Lys-781 eliminated both the binding of triaminolone acetonide and the biological activity of agonists and antagonists (72). Therefore, this region of the GR seems to be required for binding in a manner that cannot be predicted from the current model.

In conclusion, this study suggests that the protease digestion pattern of ligand-bound glucocorticoid receptors is not of general predictive value regarding the biological activity of the final receptor-steroid complex. A similar difficulty in predicting the activity from protease digestion patterns has been reported for the steroid-bound complexes of several other steroid receptors. Thus, the role of C-terminal sequences in the expression of antagonist action seems to vary among steroid receptors. Whether this is due to receptor-specific associations of transcriptional coactivators and corepressors, to interactions with proteins bound to cis-acting DNA elements of the regulated genes, to subtle differences in the tertiary structures of the LBD, or to yet other factors remains to be established.

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Steroid-induced Conformational Changes at Ends of the Hormone-binding Domain in the Rat Glucocorticoid Receptor Are Independent of Agonist Versus Antagonist Activity

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