Steroid Binding Activity Is Retained in a 16-kDa Fragment of the Steroid Binding Domain of Rat Glucocorticoid Receptors*

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The steroid binding domain of the rat glucocorticoid receptor is considered as extending from amino acids 550 to 795. However, such a synthetic protein (i.e. amino acids 547-795; Mₚ = 31,000) has been reported to show very little affinity for the potent synthetic glucocorticoid dexamethasone. We now disclose that digestion of steroid-free rat glucocorticoid receptors with low concentrations of trypsin yields a single species, of Mₚ = 16,000, that is specifically labeled by dexamethasone 21-mesylate. This 16-kDa fragment retains high affinity binding for [³H]dexamethasone that is only ~23-fold lower than that seen with the intact 98-kDa receptor. Analysis of the protease digestion patterns obtained both with trypsin and with lysylendopeptidase C allowed us to deduce the proteolytic cleavage maps of the receptor with these enzymes. From these protease maps, the sequence of the 16-kDa fragment was identified as being threonine 537 to arginine 673. These results show that glucocorticoid receptor fragments smaller than 34 kDa do bind steroids and that the amino acids Thr⁵³⁷-Arg⁶⁷⁷ constitute a core sequence for ligand binding within the larger steroid binding domain. The much slower kinetics in generating the 16-kDa fragment from affinity-labeled receptors suggests that steroid binding causes a conformation change in the receptor near the cleavage sites.

The steroid binding domain of glucocorticoid receptors has been found by molecular biology techniques to comprise the carboxyl-terminal ~250 amino acids of the protein (1-3). Nevertheless, the receptor fragment spanning this region (i.e. residues 547-795) has a very low affinity for the synthetic glucocorticoid dexamethasone (9a-fluoro-11β,17,21-trihydroxy-16α-methylpregna-1,4-diene-3,20-dione); the Kᵣ of this fragment for dexamethasone is ~350 times less than that of the intact receptor (3). The shortest reported segment possessing high affinity for dexamethasone is the 34-kDa fragment of 497-795, which binds dexamethasone with a Kᵣ that is five times less than that of the 98-kDa receptor (3). Units smaller than 547-795 (3), including the mero-receptor (4), have no observable affinity for dexamethasone. Furthermore, deletion of the carboxyl-terminal 29 amino acids appears to reduce the Kᵣ for dexamethasone by a factor of ≥1000 (3). All portions of the steroid binding domain appear to be necessary, since deletions and point mutations throughout this region have been found to eliminate steroid binding (1-3, 5). The identification of methionine 622, cysteine 656, and cysteine 754 by electrophilic (6) or photoaffinity (7) labeling of the rat receptor afforded additional evidence for the involvement of this large region in steroid binding. However, in spite of these data, it seems unlikely that all of the carboxyl-terminal ~250 amino acids are intimately involved in the construction of the steroid binding cavity, since other activities are also encoded in this domain. Thus, sequences involved in transcriptional activation (8, 9) and the association of hsp90 (10) plus regions of homology with the other steroid receptors (10-12) are also found in this region. As part of the current efforts to localize the various activities in the carboxyl-terminal third of the receptor, we have used proteolytic enzymes as a means of peeling away the outer layers of the receptor to expose the core unit of the steroid binding domain. We now report the preparation of such a core unit that contains only half of the canonical steroid binding domain. Additional data argue that this region is defined by the sequence of threonine 537 to arginine 673 in rat binding receptors.

Materials and Methods

Chemicals—[¹⁴C]Dexamethasone (Sigma), [³H]dexamethasone (40 or 47 Ci/μmol, Amersham Corp.), and [³H]dexamethasone 21-mesylate (37.3 or 49.9 Ci/μmol, Du Pont-New England Nuclear) were commercially available. TAPS (Ultron grade) was purchased from Behring Diagnostics. Methyl methanethiolosulfonate was used as received from Aldrich and stored at 0 °C. Reagents for SDS-polyacrylamide gel electrophoresis including Coomassie Blue R-250 were from Sigma and Staphylococcus aureus V8 protease was from Miles. The molecular weight standards (obtained from Pharmacia LKB Biotechnology Inc.) were: P, phosphorylase b, Mₛ = 97,400; B, albumin, Mₛ = 66,300; O, ovalbumin, Mₛ = 45,000; C, carbonic anhydrase, Mₛ = 30,600; S, soybean trypsin inhibitor, Mₛ = 21,500; L, α-lactalbumin, Mₛ = 14,400; 1, myoglobin, Mₛ = 17,200; 2-6, cytochrome b₅₆₃ fragments of myoglobin with the following sizes (daltons, 14,600, 8,240, 6,380, 2,560), and 1,695 (note: standards 3 and 4 often appeared as an unresolved broad band with an average Mₛ of 7,310); SP, substance P, Mₛ = 1,347, was from Peninsula Laboratories, Inc. Florosphere Ul-Emit autoradiography marker was from Du Pont-New England Nuclear. All ¹⁴C-labeled samples were counted in Hydrofluor (National Diagnostics) at 40–55% counting efficiency in a Beckman 5801 liquid scintillation counter with automatic counts/min-to-disintegrations/min conversion.

Buffers and Solutions—TAPS buffer was composed of 25 mM TAPS, 1 mM EDTA, and 10% glycerol. The pH of the TAPS buffer was adjusted to 8.2, 8.8, or 9.5 at 0 °C with sodium hydroxide. Two-fold concentrated SDS sample buffer contained 0.6 M Tris (pH 8.85), 2% SDS, 0.2 M dithiothreitol, 20% glycerol, and bromphenol blue.

1 Unless indicated otherwise, all incubations were at 0 °C.
2 The abbreviations used are: TAPS, 3-[tris(hydroxymethyl)methyl]aminopropanesulfonic acid; HTF cells, rat hepatoma tissue culture cells; SDS, sodium dodecyl sulfate; STI, soybean trypsin inhibitor.

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Steroid Binding Activity

Cells and Preparation of Receptors—The growth of HTC cells in spinner and monolayer cultures of S77 medium supplemented with 5% fetal and 5% newborn bovine serum (Biofluids) and 0.03% glutamine has been described (13). HTC cell cytosol containing the steroid-free receptors was prepared, stored in liquid N2, and labeled at 0°C with [3H]dexamethasone or [3H]dexamethasone 21-mesyate in excess [3H]dexamethasone ± 20 mM Na2MoO4 (14, 15). Free steroid was removed by adding a 10% dextran-coated charcoal suspension in pH 8.8 TAPS buffer (added volume = 20% of reaction volume for intact receptors; for 16-kDa fragments, added volume = 30% of reaction volume in order to reduce nonspecific binding). Nonspecific binding/labeling equaled that seen with excess [3H]dexamethasone. Covalent [3H]dexamethasone 21-mesyate-labeled receptors were quantitated on SDS-polyacrylamide gels (14, 15).

Partial Purification of Activated [3H]Dexamethasone 21-Mesyate-labeled Receptors—Activated [3H]dexamethasone 21-mesyate-labeled receptors were purified by DNA-cellulose chromatography in 7.5% acetic acid containing 0.01% Coomassie Blue R-250, destained with 10% methanol, 7.5% acetic acid, incubated for 1 h in Enhance (Du Pont-New England Nuclear) and 30-60 min in 10% Carbowax PEG 6000 (formerly PEG 6000; Fisher) with constant shaking at room temperature, dried on a Bio-Rad model 443 slab gel drier at 60°C with a sheet of dialysis membrane backing (Bio-Rad) directly over the gel to prevent cracking, and fluorographed to yield the 16,100 M, ratio of bisacrylamide to acrylamide) were run in a water-cooled constant percentage acrylamide gels (10.5-11 or 15% with a 1:40 w/w of soybean trypsin inhibitor (STI), which has no effect on [3H]dexamethasone binding or [3H]dexamethasone 21-mesyate labeling of the 98 kDa receptor or any of its trypsin digest fragments (data not shown).

RESULTS AND DISCUSSION

Trypsin digestion of glucocorticoid receptors that were affinity labeled with [3H]dexamethasone 21-mesyate (18) at cysteine 656 (6) gives the normal pattern of labeled fragments on SDS gels (14, 17, 19), which includes the relatively trypsin-resistant mero-receptors at 30.4 and 28.3 (obscured) kDa that yield the 16,100 ± 150 (S.D., n = 7)-Da fragment at higher trypsin concentrations (Fig. 1A). Trypsin digestion of steroid-free receptors occurred more rapidly and demonstrated that mero-receptors can bind steroid and be covalently labeled (Fig. 1B). However, the predominant steroid-free fragment that was formed, even at low trypsin concentrations, was not mero-receptor but a species of M, = 16,000 ± 200 (n = 10) that appears to be the same as the 16.1-kDa species of Fig. 1A. It should be noted that the 16-kDa fragment was the only specifically labeled material that was generated from steroid-free receptors with 14-28 μg/ml of trypsin (Fig. 1B). Digestions of steroid-free receptor were usually conducted in the presence of 20 mM Na2MoO4, which stabilizes the 16-kDa species, and were stopped by the addition of a 10-fold excess (w/w) of soybean trypsin inhibitor (STI), which has no effect on [3H]dexamethasone binding or [3H]dexamethasone 21-mesyate labeling of the 98 kDa receptor or any of its trypsin digest fragments (data not shown).

The 16-kDa species formed in trypsin digests of crude, steroid-free receptors was identified as being a fragment of the rat glucocorticoid receptor as follows. First, the covalent labeling by [3H]dexamethasone 21-mesyate is totally inhibited by a 100-fold excess of nonradioactive dexamethasone (Fig. 1). Second, under conditions where only the 16-kDa species is generated and specifically labeled by [3H]dexamethasone 21-mesyate, specific binding of [3H]dexamethasone with high affinity is observed (Fig. 2) with an average Kd of 23 ± 8 (n = 4) times that of the intact 98-kDa receptor (2.7 ± 0.6 × 10−9 M). Third, [3H]dexamethasone 21-mesyate totally inhibits the binding of [3H]dexamethasone to preparations of the 16-kDa species (data not shown). Fourth, at the concentrations of [3H]dexamethasone and [3H]dexamethasone 21-mesyate used, the labeling efficiency by dexamethasone 21-mesyate of total 16-kDa complexes (as determined by [3H]dexamethasone binding) is the same as that seen for intact 98-kDa receptors, i.e. 70.0 ± 14.3% (S.D., n = 8) and 77 ± 16% (S.D., n = 16) (20), respectively. Furthermore, the decreased stability of the 16-kDa fragment in the absence of 20 mM Na2MoO4 was reflected by identical reductions in [3H]dexamethasone binding (49.7 ± 8.1% (S.D., n = 3)) and [3H]dexamethasone 21-mesyate labeling (47.9 ± 8.2% (S.D., n = 3)). Finally, the dose-response curves for methyl methanesulfonate inhibition of [3H]dexamethasone binding and [3H]dexamethasone 21-mesyate labeling (15) of preparations of the 16-kDa species were identical.3 These combined data

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indicate that the dexamethasone binding of a trypsin-generated fragment of the rat glucocorticoid receptor is to the same 16-kDa species that is affinity labeled by dexamethasone 21-mesyolate.

Scatchard analysis indicates that the amount of 16-kDa receptor fragment in the trypsin digest solutions is 49 ± 18% (S.D., n = 4) of the initial 98-kDa receptors (Fig. 2 and data not shown). This less than quantitative yield and the position in the receptor protein of the 16-kDa fragment both follow from the trypsin cleavage map of Fig. 3. This map was deduced from a comparison of the $M_r$ values of the observed trypsin fragments of [3H]dexamethasone 21-mesyolate-labeled receptors (14, 17) with the species predicted from the primary sequence (21). This map is supported by the report that glycine 518 is the amino terminus of the 27-kDa mero-receptor prepared from partially purified receptors (22). The observation of an ~50% yield of the [3H]dexamethasone 21-mesyolate-labeled, 1,600-Da trypsin limit digest fragment (amino acids 652-673) (17) indicates that digestion of the 18.4-kDa fragment is equally divided between the two paths of Fig. 3. The lack of fragments between 16 and 1.6 kDa (17) argues that the 16.1-kDa fragment is digested directly to the sequence of 652-673. Thus, a 50% yield of the 16.1-kDa fragment is the expected result.

The cleavage map of Fig. 3 was further supported by the fragments obtained from digestion of [3H]dexamethasone 21-mesyolate-labeled receptors with lysylendopeptidase C, which cleaves only after lysine and can cut at just four positions between amino acids 518 and 684. Thus, the predicted fragments at ~30.7, 28.2, and 17.2 kDa plus new species at 17.8 ± 0.3 kDa (S.D., n = 4; corresponding to 500-662) and 14.2 ± 0.6 kDa (S.D., n = 5; corresponding to 537-662) were observed; the trypsin digest fragments at 18.4 and 16.1 kDa, resulting from cleavage at an arginine residue, were missing (Figs. 3 and 4 and data not shown). Furthermore, lysylendopeptidase C limit digests are predicted to yield only two fragments of less than 10 kDa, i.e. 595-662 (= 8009 Da) and 598-662 (= 7624 Da), which are not resolved on our SDS gels (17). We observe just one lysylendopeptidase C limit digest band at 5.4 ± 0.6 kDa (S.D., n = 10) with no decrease in recovered $^3$H-labeled disintegrations/min after 24 versus 1 h of digestion at 20°C (data not shown). This is lower than the predicted $M_r$ as has usually been observed for [3H]dexamethasone 21-mesyolate-labeled receptor fragments in this molecular weight range (6, 17). In conclusion, the cleavage map of Fig. 3 was the only map that was internally consistent with the digestion patterns of affinity-labeled receptors that were obtained both with trypsin and with lysylendopeptidase C.

Using these protease digestion maps, we determined that trypsin digestion of both steroid-free and affinity-labeled receptors yields the same fragment of Thr537-Arg673 since the

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**FIG. 2. Scatchard analysis of [3H]dexamethasone binding to intact 98-kDa receptors and the 16-kDa fragment.** Solutions of crude receptors (30% in pH 8.8 TAPS buffer containing 20 mM Na2MoO4) were treated with buffer ± trypsin (final concentration = 15 μg/ml) for 1 h and then a 10-fold excess (w/w) of ST1 to block further digestion. Each preparation was then incubated with various concentrations of [3H]dexamethasone ± 133-fold excess of [3H]dexamethasone for 2.5 h (and with [3H]dexamethasone 21-mesyolate ± excess [3H]dexamethasone to ascertain the size of the receptor protein in each preparation). The specifically bound [3H]dexamethasone was determined by first adding a 10% dextran-coated charcoal solution to remove free steroid (see "Materials and Methods") and then subtracting the nonspecific binding seen in the presence of excess [3H]dexamethasone.

**FIG. 3. Proposed cleavage pattern for trypsin and lysylendopeptidase C digestion of rat glucocorticoid receptors.** A schematic of the carboxy-terminal half of the rat glucocorticoid receptor and the various deduced fragments are shown on the left. The numbers below each fragment correspond to the amino acid of the indicated boundary; the arrow at 566 indicates the position of that cysteine which is labeled by [3H]dexamethasone 21-mesyolate and is the radioactive label of all of the listed fragments. The amino acid responsible for the predicted cleavage is given above each individual fragment (K = lysine; blank = arginine). The calculated molecular weight of each fragment is given in the first column of numbers. The observed values ± S.D. (n = number of observations) of the fragments obtained with trypsin and lysylendopeptidase C are given in the other columns in the order of their formation. The values of the trypsin fragments marked with an asterisk are all about 900 Da larger than those seen in our previous study (17).
dexamethasone is greatly stabilized by the presence of molybdate (see above).

The inhibition of trypsin cleavage of the 30/28-kDa mero-receptors to the 16-kDa fragment by bound steroid (Fig. 1) suggests a steroid-induced conformational change in the receptor. We cannot yet exclude the possibility that the covalent labeling of cysteine 656 sterically prevents the required cleavage at both lysine 536 and arginine 673 to give the 16-kDa fragment (Fig. 3). Restricted cleavage at both sites must occur, because no fragments corresponding to proteolysis at just one site (at lysine 536 to give a 25.9-kDa species or at arginine 673 to give an 18.4-kDa species) were observed (Fig. 1 and Ref. 14) unless the affinity-labeled receptors were first denatured (data not shown and Ref. 17). Although inhibition of cleavage at arginine 673 might be expected, due to the possible proximity of the covalently labeled cysteine 656, simultaneous steric hinderance of cleavage at the more remote lysine 536 by the covalently bound steroid is less palatable. We therefore believe that this inhibition is due to a conformational change which renders the steroid-bound receptor less susceptible to proteolysis with trypsin. A conformational change following ligand binding has been proposed previously for glucocorticoid receptors (4) and has been observed for estrogen (23-25) and cAMP (26) receptors. The more rapid digestion of steroid-free receptors may also explain why previous preparations of steroid-free mero-receptors did not bind dexamethasone (4) i.e. steroid-free receptors are further digested to nonbinding fragments at the higher trypsin concentrations needed to produce mero-receptors from pre-bound receptors (cf. 56 pg/ml trypsin in Fig. 1). We do not see any difference in the chymotryptic digestion of steroid-free versus steroid-labeled receptors (data not shown).

Previous attempts to delineate the minimum glucocorticoid receptor sequence for steroid binding by construction of receptor point mutants and deletion mutants have been thwarted by the apparent absolute requirement of numerous amino acids throughout the carboxyl-terminal 250 amino acids (1-3, 5). Furthermore, this approach cannot discriminate between direct effects of mutations (or deletions) on steroid binding and indirect effects on the tertiary structure. Proteolytic enzymes offer an attractive complementary approach, since only those portions of the receptor that are exposed to solvent and accessible to macromolecules could be attacked and the hydrophobic core should remain undisturbed. Using this approach, we have now found a steroid binding core, located at 537-673, which is about half the size of the steroid binding domain but which possesses a binding affinity for dexamethasone that is ≥15 times that of similarly located

**TABLE I**

Variation in relative $K_a$ for dexamethasone with rat glucocorticoid receptor size

| Size | Amino acid sequence | Relative affinity | Reference |
|------|---------------------|------------------|-----------|
| 98kDa | 1-795 (?) | 100 | This work |
| 42kDa | 413-795 | 84 | (Simons, unpublished data) |
| 34kDa | 497-795 | 20 | 3 |
| 31kDa | 547-795 | 0.29 | 3 |
| 28kDa | 547-766 | <0.07 | 3 |
| 16kDa | 537-673 | 4.3 | This work |

The sizes of the 98-, 42-, and 16-kDa receptor species were determined from SDS-polyacrylamide gels. The sizes of the other species were calculated from their amino acid sequence. The sequence of the 42-kDa species is based on the fact that cleavage of the 98-kDa receptor by chymotrypsin to give the 42-kDa species is reported to occur at positions 409 and 413 (22) and the sequence 413-795 is closest to 42 kDa. The sizes of the 98-, 42-, and 16-kDa receptor species were determined from SDS-polyacrylamide gels. The sizes of the other species were calculated from their amino acid sequence. The sequence of the 42-kDa species is based on the fact that cleavage of the 98-kDa receptor by chymotrypsin to give the 42-kDa species is reported to occur at positions 409 and 413 (22) and the sequence 413-795 is closest to 42 kDa.

16-kDa species derived from steroid-free receptors co-migrates with the major band seen in this region after trypsin digestion of partially purified [3H]dexamethasone 21-mesylate labeled receptors after treatment with buffer for 1 h at 22°C (lane 3), 27 μg/ml of lysylendopeptidase C for 20 min at 10°C (lane 4), or 150 μg/ml of trypsin for 1 h at 22°C (lane 5) were analyzed by SDS-polyacrylamide gel electrophoresis along with trypsin digests (14 μg/ml) of steroid-free receptors that were subsequently labeled with [3H]dexamethasone 21-mesylate ≥ excess [3H]dexamethasone (Dex) (lanes 6 and 7). An equal volume of the pre-labeled trypsin digest of partially purified complexes was mixed with each of the post-labeled digests of steroid-free receptors in lanes 10 and 11. The fluorography of the gel and the identity and size of the molecular weight markers (P, B, O, C, S, L, and 1-6) is given under “Materials and Methods.”
fragments that are almost twice as large (Table I). It should be noted that the 11 amino acids 663–673 may be very important for steroid binding, since no dexamethasone binding to, or dexamethasone 21-mesylate labeling of, either the 511/518–662 fragment of trypsin digests or any of the lysylendopeptidase C fragments smaller than 28 kDa (all of which end at 662) was seen (data not shown and Figs. 1B and 3). It has been proposed recently that a 17-amino acid sequence (683–706 of the rat receptor) is required for the tight binding of steroids to receptors (27). The present results show that this 17-amino acid sequence is not absolutely required for steroid binding to the glucocorticoid receptor. This and other sequences undoubtedly do contribute some to the overall binding affinity of the intact receptor, since the 16-kDa core sequence does not retain all of the affinity of the 98-kDa holoreceptor. In this respect, the reduced affinity of the core steroid binding unit is like the reduced transcriptional activity of the core unit for enhancer activation (i.e. amino acids 407–556 of the rat) (28).

With the identification of a small portion of the steroid binding domain that retains high affinity for dexamethasone, it is now possible to systematically examine the rest of this domain for other activities (8–12). Given the general similarity in the ~250 amino acid steroid binding domains of the other steroid receptors, it will also be interesting to see if a similar ~135 amino acid sequence represents the core steroid binding unit of the other receptors.

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