Communication

Creation of “Super” Glucocorticoid Receptors by Point Mutations in the Steroid Binding Domain*

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Almost all modifications of the steroid binding domain of glucocorticoid receptors are known to cause a reduction or loss of steroid binding activity. Nonetheless, we now report that mutations of cysteine 656 of the rat receptor, which was previously suspected to be a crucial amino acid for the binding process, have produced “super” receptors. These receptors displayed an increased affinity for glucocorticoid steroids and a decreased relative affinity for cross-reacting steroids such as progesterone and aldosterone. The increased in vitro affinity of the super receptors was maintained in a whole cell bioassay. These results indicate that additional modifications of the glucocorticoid receptor, and probably the other steroid receptors, may further increase the binding affinity and/or specificity.

Steroid binding is the first step in a series of events that translate the structural information of the steroid into the observed biological response. Molecular biology experiments have defined the 250 carboxyl-terminal amino acids as being the steroid binding domain of glucocorticoid receptors. In this region, >96% of the amino acid sequence in the human, mouse, and rat receptors is identical. The homology between the binding domains of all of the steroid receptors (androgen, estrogen, glucocorticoid, mineralocorticoid, and progesterone) is much less but still extensive. This homology offers a reasonable explanation for the fact that virtually every steroid appears to interact with more than one class of receptors. Thus it has proved difficult to selectively recognize the biologically active form of the various receptors on the basis of steroid binding. The consequences of such cross-reactivity are manifold. It complicates the identification of the steroid binding form of receptors and causes unwanted side effects in in vitro experiments with cells containing the offending receptors. In clinical settings, the side effects can be severe, such as to limit long-term glucocorticoid therapy to only those cases that are not easily remedied by other protocols.

One solution to this problem is to modify the steroid binding domain to cause increased specificity of steroid binding. Increased binding affinity would also be desirable since lowering the concentrations of steroid needed for full glucocorticoid response would also decrease the binding (and lower the biological response) with other receptors. Unfortunately, all published reports indicate that this will be very difficult to accomplish. For the glucocorticoid receptor, terminal deletions to give species smaller than amino acids 497–795 (all numbering is for the rat receptor sequence) resulted in more than a 300-fold reduction in affinity. The only exception involves a 16-kDa fragment of the rat receptor which was obtained by partial trypsin digestion of steroid-free receptors. The affinity of this 16-kDa fragment is 23-fold lower than that of the intact receptor, but it maintains all of the steroid binding specificity of the intact receptor and still binds heat shock protein 90. Most internal deletions or substitutions and point mutations of the glucocorticoid receptor steroid binding domain either eliminate or greatly decrease steroid binding. It thus appears that, aside from the few changes that are seen in rat vs human vs mouse receptors, the native sequence may be optimal for binding glucocorticoid steroids with high affinity and specificity and that many amino acids are crucial for steroid binding.

There have been numerous efforts to identify the crucial amino acids involved in steroid binding to the glucocorticoid receptor. The initial candidates were cysteine and lysine and arginine. In fact, it has long been known that intact thiols are involved in the steroid binding of all receptors. Almost all modifications of the steroid binding domain of glucocorticoid receptors are known to cause a reduction or loss of steroid binding activity. Nonetheless, we now report that mutations of cysteine 656 of the rat receptor, which was previously suspected to be a crucial amino acid for the binding process, have produced “super” receptors. These receptors displayed an increased affinity for glucocorticoid steroids and a decreased relative affinity for cross-reacting steroids such as progesterone and aldosterone. The increased in vitro affinity of the super receptors was maintained in a whole cell bioassay. These results indicate that additional modifications of the glucocorticoid receptor, and probably the other steroid receptors, may further increase the binding affinity and/or specificity.

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1. P. K. Chakraborti, M. J. Garabedian, K. R. Yamamoto, and S. S. Simons, Jr., manuscript in preparation.
2. The abbreviations used are: Dex-Mes, dexamethasone mesylate; Dex, dexamethasone; 5α-DHT, 5α-dihydrotestosterone; TAPS, 3-[tris(hydroxymethyl)methyl]aminopropanesulfonic acid; SDS, sodium dodecyl sulfate; DMEM, Dulbecco's minimal Eagle's medium; PBS, fetal bovine serum; PBS, phosphate-buffered saline.
Unles otherwise indicated, all operations were performed at 0 C. Reagents—Nonradioactive dexamethasone (Dex), cortisone, adrenosterone, and 5-dihydrotestosterone (5a-DHT) (all from Sigma), 17β-estradiol (Calbiochem), [3H]Dex (40 and 46 Ci/mmol, Amersham Corp.), and [3H]Dex-Mes (44.7 Ci/mmol, Du Pont-New England Nuclear) were commercially available. RU 486 was a generous gift contains 0.6 of the rat glucocorticoid receptor (24) were gifts from Dr. Robert Harrison (University of Arkansas for Medical Science) and Dr. Bernd Groner (Friedrich Miescher-Institut), respectively. Biotinylated anti-mouse and anti-rabbit second antibodies for Western blotting were from Vector Laboratories.

Buffers and Solutions—TAPS buffer (25 mM TAPS, 1 mM EDTA, and 10% glycerol) was adjusted to pH 8.8 or 9.5 at 0 °C with sodium hydroxide. Two-fold concentrated SDS sample buffer (2 X SDS) contains 0.6 M Tris (pH 8.85), 12% SDS, 0.2 M dithiothreitol, 2% glycerol, and bromphenol blue. Transfer buffer for Western blotting contains 25 mM Tris, 192 mM glycine, 20% methanol in water (pH 8.3 at room temperature). Transfer buffer was 20 mM Tris and 0.28 M NaCl in water (pH = 7.5 at room temperature).

Construction and Identification of Mutant cDNAs—The various cDNAs corresponding to point mutations at Cys-640, -565, and -661 have been described. Briefly, the mutant cDNAs were prepared by oligonucleotide-directed point mutagenesis and transiently expressed from an SV40-driven expression vector in COS-7 cells.

Growth and Transfection of Cells—Monolayer cultures of COS-7 cells were grown in DMEM ( Gibco) with 5% FBS (Biofluids). Wild type and mutant receptor expression vector (pSVL) plasmids (10 μg) were introduced into COS-7 cells (100-1000 mm2 dish) by standard calcium phosphate transfection methods. After ~16 h at 37 °C in a 5% CO2 incubator, excess calcium phosphate and precipitate were removed by washing with PBS. The cells were incubated for another ~48 h in DMEM plus 5% FBS, harvested by trypsinization followed by centrifugation (for 10 min at 1500 x g) and washing 3 times with PBS, and stored at -80 °C until used.

Steroid Binding Assays—COS-7 cell cytosol containing the steroid-free receptors was obtained by the lysis of cells at -80 °C and centrifugation at 15,000 x g (25). For competition binding assays, duplicate aliquots (72 μl) of COS-7 cell cytosol (30.5% in pH 8.8 TAPS, 27 mM Na2MoO4, buffer) were treated with 4 μl each of [3H]Dex (in pH 8.8 TAPS buffer) and various concentrations of nonradioactive competing steroid (in 20% EtOH in pH 8.8 TAPS buffer; final concentration of [3H]Dex ≈ 3 X 10-6 M). The average specific binding, determined after 2.5 or 24 h of incubation by first adding a 10% dextran-coated charcoal solution (added volume = 20% of reaction solution volume) to remove free steroid and then subtracting the nonspecific binding seen in the presence of excess nonradioactive Dex, was expressed as a percentage of the noncompeted control and plotted versus the log concentration of the competing steroid. The Rodbard-corrected (26) Kd, where the Kd of dexamethasone = 1, was determined from the concentration of nonradioactive steroid that caused 50% inhibition of [3H]Dex binding.

For Scatchard analysis, duplicate aliquots (76 μl) of COS-7 cell cytosol (31.6% in pH 8.8 TAPS, 21 mM Na2MoO4, buffer) were incubated with 4 μl of [3H] ± 500 X nonradioactive Dex in pH 8.8 TAPS (final [3H]Dex concentrations were 0.3-50 X 10-8 M) for 24 h before determining the average specific binding to receptors as described above.

Determination of Biological Activity of Mutant Receptors—Subconfluent cultures of CV-1 cells were co-transfected, using standard calcium phosphate procedures, with 0.2 μg of VARO receptor expression vector (mutant receptor driven by the SV40 enhancer and the γ-globulin promoter (Ref. 27)) and 1 μg of G418 TCO reporter vector (chloramphenicol acetyltransferase gene driven by the thymidine kinase promoter (to -10b base pairs) and a 46-base pair synthetic glucocorticoid response element derived from the murine mammary tumor virus long terminal repeat (Ref. 28)) for each 60-mm dish. Cells were incubated overnight with the DNA precipitates, after which they were washed twice with PBS and treated with fresh medium (DMEM supplemented with 5% PBS) containing steroids. After an additional 24 h, extracts were prepared by four freeze-thaw cycles (~75 °C, 65 °C) and centrifuged for 5 min at 15,000 x g. Heat-treated extracts (5 min, 65 °C) were normalized for protein content and the amount of expressed chloramphenicol acetyltransferase enzyme activity, in terms of 1°C-acetylated chloramphenicol, was determined by a nonchromatographic assay (29).

Polyacylamide Gel Electrophoresis—Samples diluted 1:2 in 2 X SDS buffer were fractionated on constant percentage acrylamide gels (between 9 and 15%) and subjected to Tris-glycine gel (23) run in a water-cooled (15 °C) Protein II slab gel apparatus (Bio-Rad) at 30 mA/gel (25 mA/gel for 15% gels; 20 mA/gel while in the stacking gel for all gels). Gels were fixed, stained, and fluorographed as described (16).

Western Blotting—Electrophoretic transfer from SDS-polyacrylamide gels to nitrocellulose, conducted in a Transblot (Bio-Rad) apparatus (~15 h at 100 mA, then 250 mA for 90 min), followed by incubation with primary and secondary antibodies, and staining was conducted as described elsewhere (21). The primary antibodies were diluted 1:1000 (aP1) or 1:20 (BUGR-2 tissue culture medium) in 0.1% Tween in Tris-buffered saline.

Expression of Receptors with Point Mutations at Cys-640, -565, and -661—Rat glucocorticoid receptors with four different point mutations were examined: cysteine-to-serine at positions 640, 656, and 661 and cysteine-to-glycine at position 656. Cell-free studies were conducted with extracts of COS-7 cells that had been transiently transfected with the corresponding cDNAs. The expression of the wild type and mutant receptors was identical, as determined by Western blotting (Fig. 1A). The presence of the lower M, bands in Fig. 1A for all receptors is probably due to alternative translational starts (30). This conclusion was strengthened by the observation that chymotrypsin digestion of both authentic 98-kDa receptor and the transiently expressed wild type receptor gave, after removal of the amino-terminal half of the receptors, an identical 42-kDa fragment (Fig. 1B), which has the same binding affinity as the 98-kDa receptor (2, 8).

![Fig. 1](western blot analysis of transiently expressed mutant receptors. A, cytosols from COS-7 cells transiently transfected with wild type and mutant receptor cDNAs, along with cytosols from pUC19 transiently transfected COS-7 cells and from HTC cells, were separated on a 9% SDS-polyacrylamide gel and, after transfer to nitrocellulose, blotted with BUGR-2 anti-receptor antibody. Molecular weight markers (P = phosphorylase B, 97,400 Da; B = bovine albumin, 66,300 Da; O = ovalbumin, 45,000 Da) ≥ prestaining are in lanes 1 and 9. B, cytosols from HTC cells (lanes 10 and 11) and COS-7 cells without (lane 12) and with (lane 13) transiently transfected wild type receptor cDNA were treated with 14 μg of chymotrypsin for 1 h at 0 °C as indicated, analyzed as in A, and Western-blotted with aP1 anti-receptor antibody. The intact 98-kDa receptor (*) and 42- kDa fragment (○) are indicated.)
Further evidence that the desired point mutations had been effected was obtained by affinity labeling with \(^{[3]H}\)Dex-Mes (31). After separation on SDS-polyacrylamide gels and visualization by fluorography, a specifically labeled band was seen for the 640 and 661 mutant receptors at the same molecular weight as for the wild type, 98-kDa receptor. In contrast, no specifically labeled species was seen for either of the 656 mutant receptors. This is the expected result since Dex-Mes is known to affinity-label only Cys-656 in the rat receptor (17).

**Steroid Binding Specificity of the Mutant Receptors**—The specificity of steroid binding was determined by Rodbard correction (26) of the data from 2.5-h competition binding assays (20, 32). The results (Table IA) show that there was almost no change in specificity after the mutation of Cys-640. Mutation of Cys-661 had little effect on the binding of RU 486 or cortisol but caused a 6-fold decrease in the binding by 5α-DHT and an approximately 10-fold decrease for progesterone, aldosterone, and 17β-estradiol. The effect of mutating Cys-656 depended on the amino acid which was introduced. Replacement with glycine (to give C656G) produced much the same change in specificity as seen for C661S, except that there was less of an effect on aldosterone binding and no effect on cortisol binding. Replacement of Cys-656 with serine (to give C656S) caused a 6-fold decrease in relative affinity for progesterone, aldosterone, and 5α-DHT and a major reduction (±10-fold) only for 17β-estradiol.

Competition assays of short duration (e.g. 2.5 h) usually give the correct relative affinity values. However, since such short assays do not allow the binding of \(^{[3]H}\)Dex to reach equilibrium, inaccurate values can be obtained for slowly dissociating sterids (32, 33). Interestingly, in 24-h assays that are approximately at equilibrium, the binding selectivity was found to increase. Thus the specificity for cortisol vs aldosterone binding to the C656G receptor (defined as the ratio of affinities relative to Dex) was raised from 20-fold in the 24-h assay to 83-fold in the 24-h assay; this ratio was 4.2-fold at both time points with the wild type receptor (Table IB). Similarly, the specificity of C660G for cortisol versus progesterone increased from 16-fold in the 2.5-h assay to 44-fold in the 24-h assay, while the ratio was always ~1 for the wild type receptor (Table IB).

**Steroid Binding Affinity of the Mutant Receptors**—We were surprised that none of the cysteine mutations had eliminated steroid binding (Table I). Scatchard analysis (24 h) of each of the receptors revealed that the mutations of Cys-640 and -661 did produce a 3-4-fold decrease in affinity for \(^{[3]H}\)Dex (Table II). Unexpectedly, however, the two mutations of Cys-656 resulted in a 3- and almost 9-fold increase in affinity. With regard to C660G, it should be noted that this increased affinity does not entirely compensate for the decreased affinity of aldosterone and progesterone seen in Table IB. Thus the absolute affinity of progesterone, and probably aldosterone, for the glucocorticoid receptor has decreased as a result of this mutation.

**Biological Activity of the Mutant Receptors**—It is well known that the steroid binding of receptors can be dissociated from the ability to produce a biological response (1, 2). In order to determine if either of the receptors that had been mutated at position 656 were still biologically active, CV-1 cells were transiently transfected with both a mutant receptor expression vector and a vector containing a glucocorticoid-responsive reporter gene (G,6tk/chloramphenicol acetyltransferase). Each mutant receptor was found to be fully active (Fig. 2 and data not shown). As seen in Fig. 2, Dex induction of chloramphenicol acetyltransferase activity with the C660G receptor occurred at >6-fold lower concentrations than with the wild type receptor. The close correlation between the cell-free affinity of Dex for receptors and the concentration of Dex required to induce the biological response argues that the mutation of an amino acid which is intimately involved in steroid binding (i.e. Cys-656) can give novel receptor molecules that are more selective and more responsive than the wild type receptor.

**DISCUSSION**

Molecular biology offers the prospect of constructing new proteins that have more desirable properties than the naturally occurring proteins. Unfortunately, all reported modifications of the glucocorticoid receptor steroid binding domain result in little or no steroid binding activity (1, 2, 10-12). It thus appeared that the activity and/or the proper tertiary

| Steroid   | Relative Kᵦ of steroid binding to receptor (Mean ± S.D. (n)) |
|-----------|-----------------------------------------------------------|
|           | Wild type | C640S | C656G | C656S | C660S |
| RU 486    | 3.4 ± 1.2 (3) | 6.8 ± 2.2 (3) | 2.5 ± 0.7 (3) | 3.4 (2) | 3.84 ± 1.36 (3) |
| Progesterone | 1.15 ± 0.32 (4) | 1.21 ± 0.18 (3) | 0.075 ± 0.014 (4) | 0.72 (2) | 0.090 ± 0.032 (3) |
| Cortisol  | 0.92 ± 0.099 (4) | 0.69 ± 0.08 (3) | 1.17 ± 0.49 (4) | 1.05 (2) | 0.32 ± 0.03 (3) |
| Aldosterone | 0.22 ± 0.05 (4) | 0.18 ± 0.02 (3) | 0.060 ± 0.044 (4) | 0.068 (2) | 0.016 ± 0.007 (3) |
| 5α-DHT   | 0.032 ± 0.003 (3) | 0.020 ± 0.005 (3) | 0.004 ± 0.001 (3) | 0.0128 (2) | 0.005 ± 0.001 (3) |
| 17β-Estradiol | 0.029 ± 0.006 (3) | 0.019 ± 0.007 (3) | 0.0017 ± 0.0006 (3) | 0.0015 (2) | 0.0017 ± 0.0006 (3) |

| Steroid   | Relative Kᵦ of steroid binding to receptor in |
|-----------|---------------------------------------------|
|           | 24-h assays | 2.5-h assays |
| progesterone | 0.14 | 0.0075 | 1.15 | 0.075 |
| Cortisol   | 0.18 | 0.33 | 0.92 | 1.17 |
| Aldosterone | 0.043 | 0.0040 | 0.22 | 0.060 |
wild type receptors and can be considered as "super" glucocorticoid receptors for three reasons. First, Cys-656 is date many changes in amino acid sequence. We now show, enzyme activity produced by Dex induction of wild type receptor, is also transcriptionally active at 6 times lower Dex concentration than is the wild type receptor (Fig. 2). Thus C656G and C656S added to each plate. thus can be very close to noncovalently bound steroids (17). that Cys-656 is not an essential amino acid for steroid binding. The data further imply that Cys-656 actually decreases the affinity and specificity of glucocorticoid receptor binding. Since no other steroid receptor contains a cysteine at the comparable position (6), it is likely that Cys-656 has some essential function. It remains to be elucidated what that function is. Similarly, the effect of substitutions of Cys-640 and -661, both of which have been found to be intimately involved in steroid binding are relatively minor. This suggests that, while numerous amino acids may be required for the proper tertiary folding of the binding cavity, relatively few amino acids are absolutely essential for binding.

In conclusion, a receptor that has higher affinity and specificity than the natural receptors would be advantageous in several instances. Most importantly, it would permit the use of lower doses of steroid to affect full, receptor-mediated activity. This, in turn, would cause less binding of the steroid to other receptors. The current studies with glucocorticoid receptors show, for the first time, that such improved receptors are indeed feasible. Further modifications may yield even more useful receptors for all of the steroid hormones.

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| TABLE II | Affinity of \[^3H\]dex binding to mutant receptors |
|----------------|-----------------------------------------------|
| Receptor | \(K_d \times 10^{11} M\) | S.D. |
| Wild type | 4.73 | 2.04 |
| C640S | 13.1 |
| C656G | 0.55 | 0.16 |
| C656S | 1.38 | 0.37 |
| C661S | 19.6 |

Scatchard analyses were performed in duplicate as described under "Materials and Methods." The average \(K_d\) values are listed. Values with S.D. are the result of three experiments; all other values are for two experiments.

Fig. 2. Dose-response curve for Dex induction of reporter cholorphanolino acetytransferase gene by wild type and mutant receptors. The amount of cholorphanolino acetytransferase enzyme activity produced by Dex induction of wild type (C) or C656G mutant (○) receptors that had been transiently transfected into CV-1 cells was determined as described under "Materials and Methods" and plotted as a function of the concentration of dexamethasone added to each plate.

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