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

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Pradip K. Chakraborti‡§, Michael J. Garabedian§¶, Keith R. Yamamoto¶, and S. Stoney Simons, Jr.‡‖

From the ‡Steroid Hormones Section, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892 and the ¶Department of Biochemistry, University of California, San Francisco, California 94143

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 (1, 2). In this region, >96% of the amino acid sequence in the human, mouse, and rat receptors is identical. The homology between the steroid binding domains of all of the steroid receptors (androgen, estrogen, glucocorticoid, mineralocorticoid, and progesterone) is much less but still extensive (3). This homology offers a reasonable explanation for the fact that virtually every steroid appears to interact with more than one class of receptors (4, 5). Thus it has proved difficult to selectively recognize the biologically active form of the various receptors on the basis of steroid binding (4, 5). The consequences of such cross-reactivity are manifold. It complicates the identification of the steroid binding form of receptors (6) 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 (7).

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 responses) 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 (2). 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 (8), but it maintains all of the steroid binding specificity of the intact receptor[1] and still binds heat shock protein 90 (9). Most internal deletions or substitutions and point mutations of the glucocorticoid receptor steroid binding domain either eliminate or greatly decrease steroid binding (1, 10–12). 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 (13) and lysine and arginine (14). In fact, it has long been known that intact thiols are involved in the steroid binding of all receptors (15). Direct support for this conclusion was obtained when Dex-Mes,[2] a thiol-specific (16) affinity label for glucocorticoid receptors, was shown to covalently label just one thiol in the rat receptor, *i.e.* cysteine 656 (17). As expected (17), the identical cysteine in the mouse (18) and human (19) glucocorticoid receptor is also labeled by Dex-Mes. However, recent data indicate that a vicinal dithiol group is involved in steroid binding by virtue of its ability to form an intramolecular disulfide (20, 21) and to react with arsenite (6, 21, 22), to give modified receptors which no longer bind steroid. Recently we have identified the vicinal dithiols (Cys-656 and -661) and found that yet a third thiol (Cys-640) is involved in steroid binding to glucocorticoid receptors.[1]

Based on the above data, it would be predicted that mutations of Cys-640, -656, and -661 would both reduce (or eliminate) the affinity of steroid binding to glucocorticoid receptors and decrease binding specificity. We now report that cysteine-to-serine point mutations of Cys-640 and Cys-661 did cause a reduced affinity. Surprisingly, however, mutations of Cys-656 caused an *increase* in both affinity and specificity. To the best of our knowledge, this is the first report of a steroid receptor mutation causing a higher affinity and suggests that further increases may be possible.

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§ Both authors contributed equally to this paper and should be considered as first authors.

‖ To whom correspondence should be addressed: Bldg. 8, Rm. B2A-07, NIH, Bethesda, MD 20892.

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[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; FBS, fetal bovine serum; PBS, phosphate-buffered saline.

MATERIALS AND METHODS

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

Chemicals—Nonradioactive dexamethasone (Dex), cortisol, aldosterone, and 5-dihydrotestosterone (5α-DHT) (all from Sigma), 17β-estradiol (Calbiochem), [^{3}H]Dex (40 and 46 Ci/mmol, Amersham Corp.), and [^{3}H]Dex-Mes (44.7 Ci/mmol, Du Pont-New England Nuclear) were commercially available. RU 486 was a generous gift from Dr. Etienne-Emile Baulieu (Universite Paris-Sud); progesterone was from the NIDDK Steroid Reference Collection. Other purchased reagents were TAPS (Ultrol grade, Behring Diagnostics), 4-chloro-1-naphthol (Sigma), reagents for SDS-polyacrylamide gel electrophoresis including Coomassie Blue R-250 and EIA grade Tween 20 (Bio-Rad), fluorescent Ult-Emit autoradiography marker (Du Pont-New England Nuclear), and ABC reagent for immunoperoxidase staining of Western blots (Vector Laboratories). All ^{3}H-labeled samples were counted in Hydrofluor (National Diagnostics) at 40–55% counting efficiency in a Beckman 5801 liquid scintillation counter with automatic cpm-to-dpm conversion.

Antibodies—A monoclonal anti-receptor antibody BUGR-2 (23) and a polyclonal antibody (aP1) against the carboxyl-terminal region 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 × SDS) contains 0.6 M Tris (pH 8.85), 12% SDS, 0.2 M dithiothreitol, 20% 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). Tris-buffered saline 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, -656, and -661 have been described.[1] 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 (10^6/100-mm dish) by standard calcium phosphate transfection methods. After ~16 h at 37 °C in a 5% CO_2 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 1570 × *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 × *g* (25). For competition binding assays, duplicate aliquots (72 μl) of COS-7 cell cytosol (33.5% in pH 8.8 TAPS, 27 mM Na_2MoO_4 buffer) were treated with 4 μl each of [^{3}H]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 [^{3}H]Dex ≈ 3×10^{-8} 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 percentage of the noncompeted control and plotted *versus* the $\log_{10}$ of the concentration of the competing steroid. The Rodbard-corrected (26) K_a, where the K_a of dexamethasone = 1, was determined from the concentration of nonradioactive steroid that caused 50% inhibition of [^{3}H]Dex binding.

For Scatchard analysis, duplicate aliquots (76 μl) of COS-7 cell cytosol (31.6% in pH 8.8 TAPS, 21 mM Na_2MoO_4 buffer) were incubated with 4 μl of [^{3}H] ± 500 × nonradioactive Dex in pH 8.8 TAPS (final [^{3}H]Dex concentrations were $0.3–50 \times 10^{-9}$ 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 α-globulin promoter (Ref. 27)) and 1 μg of G_{46}TCO reporter vector (chloramphenicol acetyltransferase gene driven by the thymidine kinase promoter (to −109 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 H-16 supplemented with 5% FBS) 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 × *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 ^{14}C-acetylated chloramphenicol, was determined by a nonchromatographic assay (29).

Polyacrylamide Gel Electrophoresis—Samples diluted 1:2 in 2 × SDS buffer were analyzed on constant percentage acrylamide gels (between 9 and 15% with a 1:40 ratio of bisacrylamide to acrylamide) run in a water-cooled (15 °C) Protean 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.

RESULTS

Expression of Receptors with Point Mutations at Cys-640, -656, 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. 1*A*). The presence of the lower M_r bands in Fig. 1*A* 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. 1*B*), which has the same binding affinity as the 98-kDa receptor (2, 8).

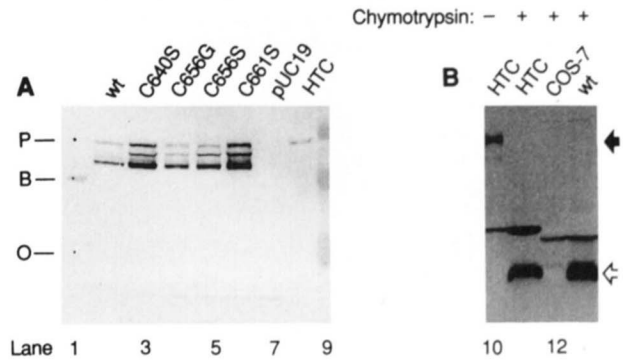

FIG. 1. **Western blot analysis of transiently expressed mutant receptors.** *A*, cytosols from COS-7 cells transiently transfected with wild type and mutant receptor cDNAs, along with cytosols from pUC19 transiently transfected COS-7 cells and from HTC cells, were separated on a 9% SDS-polyacrylamide 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.[1] 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 $\leq$3-fold reduction in relative affinity for progesterone, aldosterone, and 5α-DHT and a major reduction ($\geq$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 steroids (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 2.5-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 C656G 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 C656G, 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_{46}tk/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 C656G 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

TABLE I

Specificity of steroid binding to mutant receptors

Competition binding experiments were performed in duplicate and analyzed as described under Materials and Methods. The average values are listed along with the standard deviation (S.D.) when more than two experiments were performed. The number of experiments in section A are given in parentheses; all of the 24 hr experiments in section B were performed twice. Specific [^{3}H]Dex binding was usually between 15,000 and 58,000 dpm.

A. 2.5-h competition assays

Steroid	Relative K_a of steroid binding to receptor (Mean ± S.D. (n))				
	Wild type	C640S	C656G	C656S	C661S
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)	0.010 ± 0.007 (3)	0.0017 ± 0.0006 (3)	0.0015 (2)	0.0017 ± 0.0006 (3)

B. 24 *versus* 2.5-h competition assays

Steroid	Relative K_a of steroid binding to receptor in			
	24-h assays		2.5-h assays	
	Wild type	C656G	Wild type	C656G
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

TABLE II
Affinity of [^{3}H]dex binding to mutant receptors

Receptor	$K_d \times 10^{-9}$ 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.

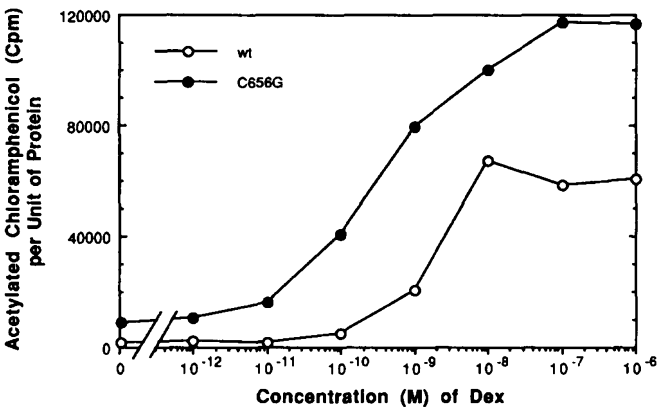

FIG. 2. **Dose-response curve for Dex induction of reporter chloramphenicol acetyltransferase gene by wild type and mutant receptors.** The amount of chloramphenicol acetyltransferase enzyme activity produced by Dex induction of wild type (○) or C656G mutant (●) receptors that had been transiently transfected 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.

folding of the steroid binding domain is unable to accommodate many changes in amino acid sequence. We now show, however, that substitution of Cys-656 with either serine or glycine yields mutant receptors that not only have higher affinity for glucocorticoids such as Dex (Table II) and cortisol (Table I) but also have a higher absolute (for C656G) and/or relative (for C656S) binding specificity for glucocorticoids (Tables I and II). The C656G receptor, which has an affinity 9-fold higher than that of the 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 are the first mutant receptors with higher affinities than the wild type receptors and can be considered as "super" glucocorticoid receptors.

These super receptors were created by the mutation of Cys-656, which has been considered a crucial amino acid in the steroid binding process for three reasons. First, Cys-656 is covalently labeled by Dex-Mes to give an adduct in which the thiol group of Cys-656 is attached to the C-21 of Dex and thus can be very close to noncovalently bound steroids (17). Second, methyl methanethiolsulfonate reacts with Cys-656 (and Cys-661) to block steroid binding (20, 21).[1] Third, sodium arsenite selectively reacts with Cys-656 and Cys-661[1] to block steroid binding (22) in a reaction that is specific for glucocorticoid receptors (6). The current results clearly demonstrate 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[1] 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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