THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 267, No. 16, Issue of June 5, pp. 11366-11373, 1992
Printed in U.S.A.

Role of Cysteines 640, 656, and 661 in Steroid Binding to Rat Glucocorticoid Receptors*

(Received for publication, November 20, 1991)

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-Laboratory of Molecular and Cellular Biology, National Institutes of Health, Bethesda, Maryland 20892 and the §Department of Biochemistry, University of California, San Francisco, California 94143

The involvement of a vicinally spaced dithiol group in steroid binding to the glucocorticoid receptor has been deduced from experiments with the thiol-specific reagent methyl methanethiolsulfonate and the vicinal dithiol-specific reagent sodium arsenite. The vicinally spaced dithiol appears to reside in the 16-kDa trypsin fragment of the receptor, which is thought to contain 3 cysteines (Cys-640, -656, and -661 of the rat receptor) and binds hormone with an ~23-fold lower affinity than does the intact 98-kDa receptor. We now report that the steroid binding specificity of preparations of this 16-kDa fragment and the intact receptor are virtually identical. This finding supports our designation of the 16-kDa fragment as a steroid-binding core domain and validates our continued use of this tryptic fragment in studies of steroid binding. To identify the cysteines which comprise the vicinally spaced dithiol group, and to examine further the role of cysteines in steroid binding, a total of five point mutant receptors were prepared: cysteine-to-serine for each suspected cysteine, cysteine-to-glycine for Cys-656, and the C656,661S double mutant. Unexpectedly, each receptor with a single point mutation still bound steroid. Even the double mutant (C656,661S) bound steroid with wild type affinity. These results suggest that none of these cysteines are directly required either for steroid binding to the glucocorticoid receptor or for heat shock protein 90 association with the receptor. However, the presence of Cys-656 was obligatory for covalent labeling of the receptor by [^{3}H]dexamethasone 21-mesylate. Studies with preparations of the 98 and 16 kDa forms of these mutant receptors revealed both that Cys-656 and -661 comprise the vicinally spaced dithiols reacting with arsenite and that any two of the three thiols could form an intramolecular disulfide after treatment with low concentrations of methyl methanethiolsulfonate. These data, in conjunction with those from experiments on the effects of steric bulk on various receptor functions, support a model for the ligand binding cavity of the receptor that involves all three thiols in a flexible cleft but where thiol-steroid interactions are not essential for binding.

Few molecular details are known about steroid binding to cognate receptors despite the pivotal role of this interaction in steroid hormone action. The ligand-binding domain for all steroid receptors is encoded by an approximately 250-amino-acid sequence at, or near, the carboxyl terminus of the receptor (1). Recent experiments with the glucocorticoid receptor argue that trypsin digestion of steroid-free receptors yields a 16-kDa fragment which is located in the amino-terminal half of the steroid-binding domain (Fig. 1) and contains many of the amino acids required for high affinity binding (8, 16). Attempts to use DNA sequence comparisons of the steroid-binding domain of various receptors to further identify the crucial amino acids in steroid binding (17) have been hindered by relatively high levels of homology (1), some of which may reflect the presence of other activities in the steroid-binding domain (Fig. 1). In view of these many activities, it is not surprising that those receptor sequences which have evolved appear to be intolerant of further modifications. In particular, almost all changes to the steroid-binding domain of glucocorticoid receptors have resulted in the reduction or elimination of steroid binding capacity (7, 12, 18–21). Therefore, one might predict that almost any amino acid change from a naturally occurring sequence would cause a loss of steroid binding activity. Thus, it has not been a simple matter to identify those amino acids which are directly involved in steroid binding.

Several early biochemical studies with the glucocorticoid receptor implicated the importance of lysine and arginine (22–24) in steroid binding. However, the first functionality reported to be required for binding was an intact -SH group of 1 or more cysteines (25–28). This conclusion was supported by the finding that a single cysteine is covalently labeled (29) by the thiol-specific electrophilic affinity label dexamethasone 21-mesylate (Dex-Mes)[1] (30, 31) (Fig. 1). A second thiol (Cys-754) has been photoaffinity labeled by triamcinolone acetonide (32), suggesting that more than one thiol group may be involved in steroid binding. In fact, several of our experiments directly supported the involvement of two thiols. Specifically, low concentrations (~0.3 mM) of the thiol-specific reagent methyl methanethiolsulfonate (MMTS) (31) prevent steroid binding, apparently by reacting with two, closely spaced thiols to yield an intramolecular disulfide (33, 34). The presence of these thiols, and their vicinal-like spacing, was confirmed by the ability of a known vicinal dithiol-specific reagent, sodium arsenite (35, 36), to block steroid binding and affinity labeling (34, 37) in a reaction that is specific for the glucocorticoid receptor (17). Since arsenite and MMTS similarly inhibited steroid binding to preparations of both the 16-kDa trypsin fragment of the receptor and the 98-kDa receptor, it appeared

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed: Bldg. 8, Rm. B2A-07, NIDDK/LMCB, NIH, Bethesda, MD 20892. Tel.: 301-496-6796; Fax: 301-402-0589.

[1] The abbreviations used are: Dex-Mes, dexamethasone 21-mesylate; MMTS, methyl methanethiolsulfonate; hsp90, heat shock protein 90; TAPS, 3-([2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino-1-propanesulfonic acid; SDS, sodium dodecyl sulfate.

that only those cysteines between Thr-537 and Arg-673 of the rat receptor could constitute the vicinally spaced dithiol functionality (34).

Several unresolved issues remained, however, concerning these vicinally spaced dithiols. First, it was conceivable that two different sets of vicinally spaced dithiols were reacting with MMTS or arsenite in the 98- and 16-kDa receptor preparations since the steroid binding affinity of the two forms of receptor was not the same (8). However, if the specificity of steroid binding to both size receptors was found to be equal, then we could justify our conclusion that the same vicinally spaced dithiol group was affected. Second, the lack of intermolecularly cross-linked receptors under conditions of MMTS-induced disulfide formation did argue that both thiols of the vicinally spaced dithiol were on the same protein but left open the possibility that both thiols were on an associated protein (34), such as heat shock protein 90 (hsp90) which is also associated with the 16-kDa fragment (16). Finally, 2 of the 3 cysteines that are candidates for the vicinally spaced dithiol group reside in an apparent hsp90 binding region (Fig. 1) and may regulate hsp90 binding (9), which suggests one mechanism for how the vicinally spaced dithiol could affect steroid binding.

In this study, we sought to determine whether the specificity of steroid binding to preparations of the 16-kDa fragment was similar to that of the intact 98-kDa receptor. In addition, we mutated each of the 3 cysteines inferred to reside in the 16-kDa fragment to try to identify which cysteines constituted the vicinally spaced dithiol group. These results, coupled with additional experiments on the tolerance of steric bulk in the steroid binding process, on trypsin digestion of the receptor, and on the association of hsp90 with receptors, suggested that the steroid-binding cavity can be viewed as a flexible cleft.

MATERIALS AND METHODS

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

Chemicals—Nonradioactive Dex, cortisol, aldosterone, and 5α-dihydrotestosterone (all from Sigma), 17β-estradiol (Behring Diagnostics), [^{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. Deacylcortivazol and RU 486 were generous gifts from Roussel-UCLAF (Paris) and Dr. Etienne-Emile Baulieu (Paris), respectively; progesterone was from the NIDDK Steroid Reference Collection; dexamethasone oxetanone was prepared as described (38). Other purchased reagents were TAPS (Ultrol grade, Behring Diagnostics), 4-chloro-1-napthol (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 counts/minute-to-disintegrations/minute conversion.

Antibodies—A monoclonal anti-receptor antibody BUGR-2 (39), a polyclonal antibody (aP1) against the carboxyl-terminal region of the rat glucocorticoid receptor (40), and a polyclonal anti-hsp90/70 antibody (41) were gifts from Dr. Robert Harrison (University of Arkansas for Medical Science), Dr. Bernd Groner (Friedrich Miescher-Institut), and Dr. Ettore Appella (National Cancer Institute), respectively. Biotinylated anti-mouse and anti-rabbit second antibodies for Western blotting were from Vector Laboratories.

Buffers and Solutions—TAPS buffer is composed of 25 mM TAPS, 1 mM EDTA, and 10% glycerol and 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, 2% 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 (TBS) was 20 mM Tris and 0.28 M NaCl in water (pH = 7.5 at room temperature).

16-kDa Tryptic Fragment—The 16-kDa fragment was generated by trypsin digestion (14 μg/ml trypsin for 1 h at 0 °C) of crude cell receptors as described (8). The sequence identification of this fragment was based on an analysis of the digestion patterns with two different proteases of receptors that had been affinity labeled at Cys-656 by Dex-Mes (8). Immunoadsorption of the 16 kDa, and the chymotryptic 42 kDa, fragments with BUGR-2 and protein A-Sepharose was conducted as before (42).

Construction and Identification of Mutants—Single-stranded DNA clones of the rat glucocorticoid receptor steroid-binding domain fragment (*Sph*I/*Eco*RI corresponding to amino acids 495–766) in M13 mp18 and 19 (Bethesda Research Laboratories), or the full-length receptor cDNA in pTZ18U (Bio-Rad), were annealed to each of the following synthetic oligonucleotides (CCTGCTCTCCTTTGCTCCTG, CTCTACCCTCCATGTATGAC, CTCTACCCGGCATGTATGAC, and GTATGACCAATCTAAACAC; underlined base is different from the wild type sequence) prepared by Synthecell (Rockville, MD). The starting point for the C656,661S double mutant was the *Sph*I/*Eco*RI fragment of the C656S receptor. Double-stranded DNA was synthesized according to the Amersham Corp. site-directed mutagenesis kit by nicking with *Nci*I or *Pvu*I, digestion with *Exo*III, and repolymerization. After transformation in JM101 or MV1190, plaques or colonies were picked and analyzed for the desired mutation. Direct DNA sequencing and/or restriction enzyme analysis confirmed the presence of the desired base change. Double-stranded DNA was prepared of these mutants and the *Sph*I/*Eco*RI fragment was used to replace the wild type sequence in the yeast expression vector pGN795 (43). The full-length mutant receptors were excised from the yeast expression vector with *Bam*HI, or from pTZ18U with *Bam*HI/*Pvu*I, and subcloned into the mammalian expression vector (VARO) (5) for experiments on the transient expression of biological activity or into the mammalian expression vector (pSVL) (44) for the transient expression of the receptor protein.

Growth and Transfection of Cells—Suspension cultures of HTC cells were grown in Swim's S77 medium with 5% fetal bovine serum, 5% bovine serum (Biofluids) plus 0.03% glutamine. Monolayer cultures of COS-7 cells were grown in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 5% fetal bovine serum. 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 of incubation at 37 °C in a 5% CO_2 incubator, the excess calcium phosphate and precipitate were removed by washing with phosphate-buffered saline. The cells were incubated for another ~48 h in Dulbecco's modified Eagle's medium, 5% fetal bovine serum and harvested by trypsinization followed by centrifugation (for 10 min at 1570 × *g*) and washing three times with phosphate-buffered saline. The washed cells were stored at −80 °C until used.

Steroid Binding Assays—HTC or 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* (45). 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 ± 1.5×10^{-5} M [^{1}H]Dex in 1% EtOH). 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; 30% for solutions of 16-kDa fragment) 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 (46) 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.

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 (31).

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 (34). The primary antibodies were

diluted 1:1000 (aP1), 1:20 (BUGR-2 tissue culture medium), or 1:6 (anti-hsp90/70) in 0.1% Tween in TBS.

RESULTS

Steroid Binding Specificity of the 16-kDa Trypsin Fragment—Steroid binding to ligand-free rat glucocorticoid receptors is retained after trypsin digestion to yield a 16-kDa fragment with the deduced sequence of Thr-537 to Arg-673 (8). Although this fragment has not yet been purified, there is no evidence that the binding activity of such proteolyzed receptors involves other associated receptor fragments. The hydrodynamic molecular mass of the 42-kDa receptor fragment resulting from chymotrypsin digestion indicates the loss of the amino-terminal half of the receptor (47) and the ~20-fold decrease in affinity for Dex accompanying further receptor digestion to the 16-kDa fragment (8) suggests the loss of additional segments. Finally, BUGR-2 anti-receptor antibody specifically recognizes both the 42-kDa fragment and the 15-kDa DNA-binding domain that is cleaved from the 42-kDa fragment by trypsin (48) in generating the 16-kDa fragment (Opoku and Simons, data not shown). Under conditions where BUGR-2 immunoadsorbs 21% of the [^{3}H]Dex-bound 42-kDa complexes, only 0.33% (average, $n = 2$) of the [^{3}H]Dex-bound 16-kDa complexes are retained (data not shown). Thus, the 16-kDa fragment, devoid of associated receptor fragments, is a strong candidate for a steroid-binding core (Fig. 1).

The specificity of steroid binding to the presumed 16-kDa fragment was examined in a cell-free competition binding assay with a total of nine steroids (Table I). These steroids were chosen for their known agonist or antagonist activity, or inactivity, for the induction of tyrosine aminotransferase (TAT) in HTC cells (49–51). The rank order, by decreasing binding affinity to the 16 kDa preparations, was very similar to that seen for cell-free binding assays with intact 98-kDa receptors (49–51). Furthermore, the affinity of each steroid (relative to that of dexamethasone) was almost the same for solutions of the 16-kDa fragment and the intact 98-kDa receptor (Table I). This argues that most, if not all, of the elements necessary for specific steroid binding to the glucocorticoid receptor are contained within the 16-kDa fragment even though the absolute affinity of Dex is ~23-fold less than that for the 98-kDa receptor (8). These results further support the view of the 16-kDa fragment as a steroid-binding core and a model for the binding of the intact 98-kDa receptor.

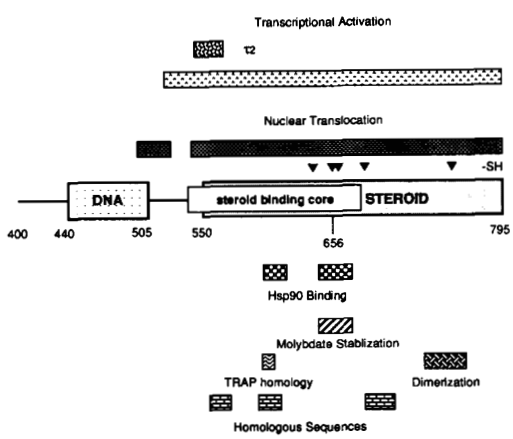

FIG. 1. **Possible activities encoded in the steroid-binding domain of the glucocorticoid receptor.** The homologies/activities which have been identified to date are transcriptional activation (2) including *τ2* (3, 4), nuclear translocation (5), steroid-binding domain (6, 7), steroid-binding core (8), hsp90 binding (9), molybdate stabilization (9), receptor dimerization sequence (10), TRAP homology sequence (11), and homologous sequences (12–15). The *filled triangles* (▼) indicate the positions of cysteines.

TABLE I

Specificity of steroid binding to 16- and 98-kDa glucocorticoid receptors

The relative binding affinity of the indicated steroids (Dex = 1) for solutions of 16- and 98-kDa receptors from HTC cells was determined for duplicate samples in separate 2.5 h competition binding assays as described under "Materials and Methods." All values are corrected for the use of subsaturating concentrations of [^{3}H]Dex as described by Rodbard (46). The average values ± range (for two experiments) or ± S.D. (for n experiments in parentheses) are listed. The relative affinity for 17β-estradiol binding to the 16-kDa receptors was determined once in duplicate.

Steroid	Relative affinity of binding to	
	16-kDa receptors	98-kDa receptors
Deacylcortivazol	9.4 ± 1.2	
RU 486	5.4 ± 0.7	3.4 ± 1.2
RU 28,362	1.56 ± 0.25	
Dexamethasone oxetanone	0.88 ± 0.06	1.27 ± 0.13
Progesterone	0.60 ± 0.33 (3)	1.15 ± 0.32 (4)
Cortisol	0.16 ± 0.02	0.80 ± 0.22 (6)
Aldosterone	0.11 ± 0.02	0.22 ± 0.05 (4)
5α-Dihydrotestosterone	0.017 ± 0.001	0.032 ± 0.003 (3)
17β-Estradiol	0.009	0.029 ± 0.006 (3)

Given the nearly identical dose-response curves for both MMTS and arsenite inhibition of steroid binding to solutions of the 16-kDa core fragment and the 98-kDa receptor (34), we suspected that these reagents affect the same vicinally spaced dithiol group in each receptor species. Because the deduced 16-kDa sequence contains only 3 cysteines (*i.e.* Cys-640, -656, and -661; see Fig. 1), we sequentially mutated each cysteine in order to determine which 2 cysteines comprise the vicinally spaced dithiol group.

Point Mutations of Cysteines within the 16-kDa Core Fragment—Mutant receptors were prepared containing either a cysteine-to-serine substitution for each of the cysteines or a cysteine-to-glycine substitution for Cys-656. A double cysteine-to-serine substitution (for Cys-656 and -661) was also constructed. The receptors were expressed by transient transfection of COS-7 cells. Surprisingly, in view of the earlier reports that many mutations eliminated steroid binding (7, 12, 18–21), each mutant receptor bound steroid to about the same extent as did the wild type receptor in control transfections and with similar affinities (52). For example, the double mutant (Cys-656 and -661) bound Dex with an affinity (4.7 ± 0.9 nM ($n = 2$)) that was indistinguishable from that of wild type receptors (4.7 ± 2.0 nM ($n = 3$)). Furthermore, approximately equal amounts of receptor protein were detected by Western blotting (52 and data not shown).

Affinity labeling of the various mutant receptors by [^{3}H] Dex-Mes was achieved only with those receptors containing substitutions at Cys-640 or -661 and not with any of the Cys-656 mutants (Fig. 2). These data corroborate our earlier conclusion (29) that Cys-656 is the only amino acid in the glucocorticoid receptor which is covalently labeled by Dex-Mes. The breadth of the specifically labeled, ~98-kDa bands in Fig. 2 appears due to alternative translational start sites to give somewhat smaller receptors (44, 52).

Binding Properties of Arsenite-pretreated Mutant Receptors—The involvement of Cys-640, -656, or -661 in the vicinally spaced dithiol group was assessed from the ability of micromolar concentrations of arsenite to block steroid binding of the various mutant receptors. It is known that the specificity of arsenite reaction with vicinal dithiols is ≥100-fold higher than with monothiols (17, 35, 36). The dose-response curves for the wild type and C640S receptors were identical (Fig. 3*A*). In contrast, mutation of Cys-661 and both muta-

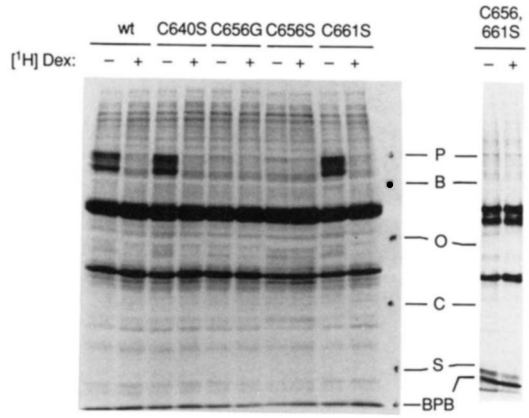

FIG. 2. **Affinity labeling of transiently transfected wild type (*wt*) and mutant receptors with [3H]Dex-Mes.** Cytosolic solutions of the wild type and mutant receptors from transiently transfected cells were labeled for 2.5 h at 0 °C with 1.5×10^{-7} M [3H] Dex-Mes ± 100-fold excess [1H]Dex. The samples were then analyzed on 10.8% SDS-polyacrylamide gels which were dried and fluorographed at −80 °C for 2 weeks as described under "Materials and Methods." The better resolution of nonspecifically labeled species at ~50 kDa in the C656,661S receptor preparation is probably because half as much protein was loaded onto the gel. The positions of the molecular weight standards (marked by Ultemit on the gel and detected on the fluorograph as a spot) are indicated by the *letters* corresponding to each protein (molecular mass (Da) is in *brackets*: *P*, phosphorylase *b* (97,400); *B*, bovine serum albumin (66,300); *O*, ovalbumin (45,000); *C*, carbonic anhydrase (30,600); *S*, soybean trypsin inhibitor (21,500); *L*, α-lactalbumin (14,400)). *BPB*, bromphenol blue.

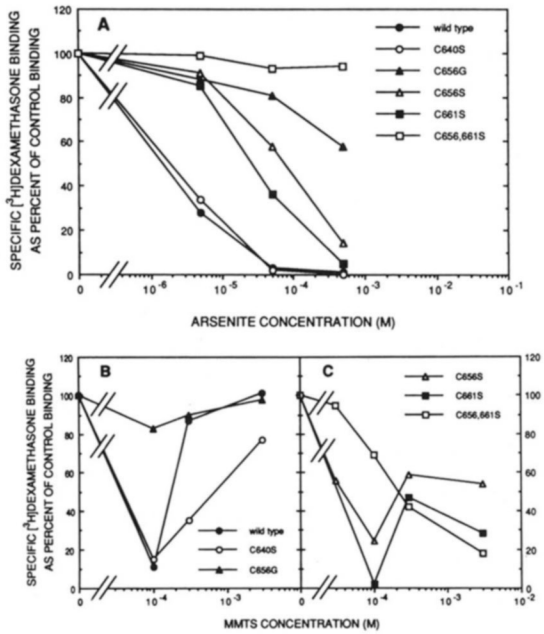

FIG. 3. **Effect of arsenite and MMTS preincubation on subsequent [3H]Dex binding of wild type and mutant 98-kDa receptors.** Cytosolic solutions of the wild type and mutant receptors from transiently transfected cells were preincubated with the indicated concentrations of arsenite for 30 min at 0 °C (*A*) or MMTS for 2.5 h at 0 °C (*B* and *C*). The total specific [3H]Dex binding capacity of each sample was then determined in duplicate, expressed as percent of the uncompeted control, and the average of two to four experiments was plotted as described under "Materials and Methods."

tions of Cys-656 caused a ≥10-fold decrease in sensitivity to arsenite. Mutation of both Cys-656 and Cys-661 gave a receptor (C656,661S) which was totally resistant to 5×10^{-4} M arsenite (Fig. 3*A*). Thus Cys-656 and -661 appear to be the vicinally spaced dithiols.

We have previously shown that the dose-response curve for MMTS inhibition of steroid binding to wild type receptors depends on the length of MMTS preincubation (33). However, the different dose-response curves in Fig. 3*A* were not a result of different kinetics of arsenite reaction with the mutant receptors. Thus, preincubation of C661S receptors with arsenite for 30 and 90 min gave identical results (data not shown). The ability of arsenite concentrations above 10^{-4} M to inhibit the binding of the Cys-656 and -661 mutants was probably due to reactions of the remaining cysteine of the vicinally spaced dithiol group (*e.g.* Cys-661 in C656S) with one of the other 18 cysteines of the receptor. This conclusion is supported by the insensitivity of the double mutant C656,661S to arsenite (see next section and "Discussion"). Collectively these results firmly establish that Cys-656 and -661 are the cysteines of the vicinally spaced dithiol group which reacts with low concentrations of sodium arsenite.

Binding Properties of MMTS-pretreated Mutant Receptors—The ability of MMTS to block steroid binding at concentrations which are lower than the thiol content of the cytosols is diagnostic for MMTS reaction with two closely spaced thiols to yield an intramolecular disulfide (33). In view of the above results with arsenite, we expected that mutations of C656 or C661 would eliminate the ability of low concentrations of MMTS to form a disulfide and inhibit steroid binding while mutation of C640 would be without effect. Such behavior was observed for C640S and C656G (Fig. 3*B*). The dose-response curve for C656G was not simply shifted to lower concentrations because preincubations with 10 and 30 μM MMTS were without effect (data not shown). The double mutant C656,661S was relatively unaffected by 0.1 mM MMTS, concentrations which dramatically reduced steroid binding to the wild type and C640S receptors (Figs. 3, *B* and *C*). These results argue that the same vicinally spaced dithiols which react with arsenite (Fig. 3*A*) also react with 0.1 mM MMTS.

Surprisingly, C656S and C661S afforded bimodal dose-response curves suggestive of wild type receptor behavior (Fig. 3*C*). Two explanations for the inhibition by 0.1 mM MMTS were considered. First, the remaining Cys-661, or Cys-656, of the above identified vicinally spaced dithiol group could react with one of the other 17 thiols outside of the 16-kDa core fragment. Alternatively, the remaining thiol might now be able to react with Cys-640. The relative insensitivity of C656,661S to 0.1 mM MMTS supports the second explanation. However, to resolve this issue, we examined the behavior of the 16-kDa core fragments of these mutant receptors, which should not contain the other 17 cysteines.

Effects of Arsenite and MMTS on Steroid Binding of Mutant Receptor 16-kDa Core Fragments—The presence of a point mutation near the trypsin cleavage site used to produce the 16-kDa fragment (*i.e.* Arg-673 (8)) could potentially alter the digestion pattern of steroid-free receptors. Fortunately, each mutant receptor was found, by [3H]Dex binding and by [3H] Dex-Mes labeling or Western blotting (Fig. 4), to give the appropriate 16-kDa core fragment. However, the C640S mutation did decrease the yield of 16-kDa core fragments by ~80% (Fig. 4 and data not shown), presumably by increasing the yield of the non-steroid binding 17-kDa fragment from the common ~18-kDa precursor (8).

Arsenite preincubation of solutions of the 16-kDa core fragments of the C640S, C656S, and C661S receptors yielded dose-response curves for [3H]Dex binding (Fig. 5*A*) that were similar to those with the intact 98-kDa receptors (*cf.* Fig. 3*A*). C640S gave wild type results while the curves for C656S and C661S were shifted to ≥10-fold higher arsenite concentra-

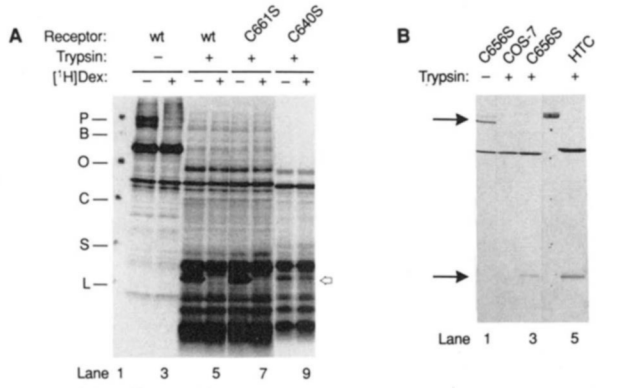

FIG. 4. **Production of 16-kDa receptors by trypsin digestion of wild type and mutant receptors.** *A*, fluorograph of [^{3}H]Dex-Mes affinity labeled 98- and 16-kDa receptors. Cytosolic solutions of the wild type and mutant receptors from transiently transfected cells were treated with buffer (*lanes 2* and *3*) or 14 μg/ml trypsin (*lanes 4–9*) for 1 h at 0 °C, followed by a 10-fold excess (w/w) of soybean trypsin inhibitor, before being labeled for 2.5 h at 0 °C with 1.5 × 10^{-7} M [^{3}H]Dex-Mes ± 100-fold excess [^{1}H]Dex. The samples were then analyzed on 15% SDS-polyacrylamide gels and fluorographed as described under "Materials and Methods." The molecular weight standards are the same as in Fig. 2. The *arrow* (◊) indicates the position of the 16-kDa core fragment. *B*, Western blot of mutant and wild type receptors ± trypsin digestion. Cytosolic solutions of HTC cells (*lane 5*) and COS-7 cells (*lanes 1–3*) before or after transient transfection with the cDNA for C656S receptors were treated for 1 h at 0 °C with buffer (*lane 1*) or 14 μg/ml trypsin (*lanes 2, 3,* and *5*) followed by a 10-fold excess (w/w) of soybean trypsin inhibitor. The samples were analyzed on 15% SDS-polyacrylamide gels and Western blotted with aP1 anti-receptor antibody as described under "Materials and Methods." *Lane 4* contains the molecular weight standards (*P* (which is detected by aP1), *B*, *O*, *C*, *S*, *L*) which were visualized with Ponceau S and marked with India ink. *Arrows* (→) indicate the positions of 98- and 16-kDa receptors.

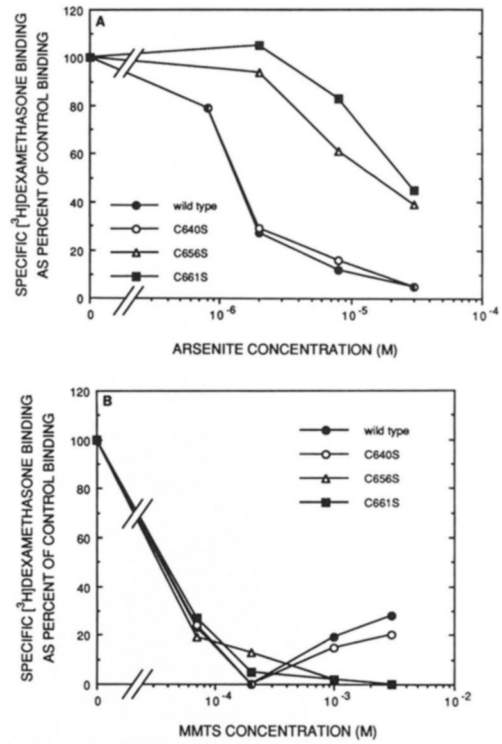

FIG. 5. **Effect of arsenite and MMTS preincubation on subsequent [^{3}H]Dex binding of 16-kDa trypsin fragments of wild type and mutant receptors.** The 16-kDa core fragments of receptors from transiently transfected cells were prepared by trypsin digestion as described in Fig. 4*A* and then preincubated with the indicated concentrations of arsenite (*A*) or MMTS (*B*) for 30 min at 0 °C. The total specific [^{3}H]Dex binding capacity of each sample was then determined in duplicate, expressed as percent of the noncompeted control, and the average of two experiments was plotted as described under "Materials and Methods."

tions. These results confirmed the above conclusion that the vicinally spaced dithiols are Cys-656 and -661 and support the notion that only the 3 cysteines of the 16-kDa core are required for the inhibition of steroid binding by arsenite.

The data of Fig. 5*B* show that steroid binding of each core receptor was eliminated by preincubation with the same low concentrations of MMTS as was seen for the intact receptors (*cf.* Fig. 3*B*). Since the inhibition by low concentrations of MMTS is characteristic of the formation of an intramolecular disulfide (33, 34), we conclude that the disulfide formation in C656S and C661S receptors does not require any thiols other than Cys-640. This argues that the receptor protein assumes a tertiary structure in which Cys-640, in addition to Cys-656 and -661, is exposed to solvent and that, under the appropriate conditions, all 3 cysteines can form disulfides with each other. The low levels of binding in Fig. 5*B* after ≥1 mM MMTS treatment were expected from previous results (34).

Effect of Steric Modifications on Receptor Properties—Millimolar concentrations of iodoacetamide, another thiol reagent, are known to block steroid binding to the glucocorticoid receptor (26, 33). This inhibition could be due either to increased steric bulk in the steroid binding cavity after reaction with Cys-640, -656, and -661 or to perturbations resulting from the reaction of other cysteines. In a test of the steric hindrance hypothesis, we found that the dose-response curves for iodoacetamide inhibition of [^{3}H]Dex binding to wild type and mutant receptors lacking one (C656G) or two (C656,661S) of the 3 cysteines were identical (data not shown). This argues that eliminating the *S*-acetamido groups either at positions 661 and/or 656 is not sufficient to restore steroid binding to iodoacetamide-treated receptors and that the modification of other residues can also eliminate steroid binding.

The yield of 16-kDa fragments from trypsin digestion of steroid-free receptors is only about 50%, presumably due to competing digestion at Lys-662 (8). Because arsenite has been shown to react with the adjacent amino acid, *i.e.* Cys-661 (see Figs. 3*A* and 5*A*), we reasoned that the steric bulk of arsenite complexed with Cys-661 might hinder digestion at Lys-662. However, not only was the total [^{3}H]Dex binding of the 16 kDa preparations the same ± arsenite preincubation but also the yield of [^{3}H]Dex-Mes-labeled 16-kDa fragment was unaltered by arsenite (data not shown). These results are consistent with the presence of a hairpin turn after Cys-661 such that arsenite binding would not hinder trypsin digestion at Lys-662. Such tight turns have, in fact, often been observed by x-ray crystallography at the ends of disulfides (53).

Effect of Arsenite on Dissociation of hsp90 from Receptors—Steroid binding to glucocorticoid receptors requires associated hsp90 (9, 54, 55), and we have recently shown that hsp90 is associated with the 16-kDa core fragment (16). The 2 cysteines which react with arsenite to block steroid binding are contained in the same region of the receptor that appears to be necessary for the binding of hsp90 (see Fig. 1). Furthermore, it has been proposed that 1 of these cysteines is an important determinant of hsp90 binding (9) and that arsenite-induced chemical stress causes the dissociation of hsp90 in intact cells (56). Thus, the arsenite-induced loss of steroid binding capacity could theoretically result from the dissociation of hsp90 as opposed to a simple steric blockage of the steroid-binding cavity. This scenario was investigated directly by treating unactivated receptors with arsenite, or buffer, immunoadsorbing the receptors and any associated hsp90

with the anti-receptor antibody BUGR2, and then Western blotting with another anti-receptor antibody (aP1) or an anti-hsp90/70 antibody to detect the presence of receptor and hsp90. As is shown in Fig. 6, arsenite preincubation of unactivated receptors did not cause the dissociation of hsp 90. Thus, arsenite inhibition of steroid binding is not due to an arsenite-induced dissociation of hsp90. Rather steric hinderance (34) still appears to be the most likely mechanism for arsenite blockage of steroid binding.

DISCUSSION

The presence of a vicinally spaced dithiol group in the steroid-binding domain of the glucocorticoid receptor has been proposed, based on the ability of low concentrations of sodium arsenite (37) and MMTS (33) to block steroid binding. We have now used point mutagenesis to identify the vicinally spaced dithiol group that reacts with arsenite as being Cys-656 and -661 of the rat receptor (Fig. 3*A*). These results confirmed our earlier conclusion that both of the vicinally spaced dithiols were on the receptor protein, as opposed to on an associated non-receptor protein (17, 34). At the same time, these point mutation studies revealed that a third cysteine (Cys-640) could become involved (Fig. 3). Thus, at least 3 cysteines are capable of influencing the steroid binding process.

Further support for the identification of these 3 cysteines came from experiments with the 16-kDa tryptic fragment of the glucocorticoid receptor (Figs. 4 and 5), which has been assigned the sequence of Thr-537 to Arg-673 in the rat receptor (8) and does not appear to be associated with any other fragments of the receptor. In fact, the affinity of the 16-kDa fragment for Dex is approximately 15-fold higher than that of the larger 31-kDa steroid binding domain (7, 8 and Fig. 1). All of the other examined properties of the 16- and 98-kDa receptors were the same: the relative affinity for a wide range of steroids (Table I), the interaction with both hsp90 (9, 16) and molybdate (8, 14), and the involvement of the same 3 cysteines in steroid binding (Figs. 3 and 5). Thus, this 16-kDa fragment appears to contain most of the "crucial" amino acids for steroid binding activity and can be considered as a steroid-binding core element.

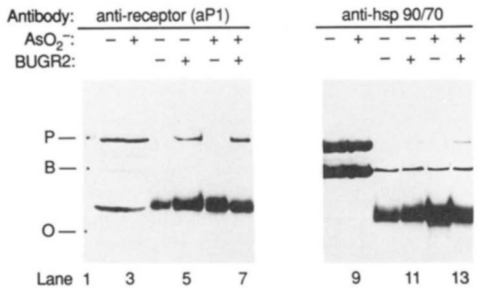

FIG. 6. **Effect of arsenite preincubation on the association of hsp90 with glucocorticoid receptors.** Cytosolic solutions of the wild type receptors from HTC cells were preincubated with buffer ± 1×10^{-4} M arsenite for 30 min followed by buffer ± BUGR-2 anti-receptor antibody for 2 h, all at 0 °C. The complexed receptors were immunoadsorbed with protein A-Sepharose CL-4B, separated on SDS-polyacrylamide (9%) gels, transferred to nitrocellulose filters, and Western blotted with aP1 anti-receptor antibodies (*lanes 2–7*) or anti-hsp90/70 antibodies (*lanes 8–13*). As controls, the samples that had not been immunoadsorbed were analyzed in *lanes 2* and *3* and *8* and *9*. The positions of receptor and hsp90 are approximately equal to that of phosphorylase *b* (*P*).

The precise function of the 3 cysteines in the steroid binding process is not yet clear since point mutations of each cysteine generated 98-kDa receptors (Fig. 3) and 16-kDa core fragments (Fig. 5) which still bound steroid. Even the intact double mutant (C656,661S) still bound Dex with wild type affinity. Furthermore, while some of the mutants displayed the expected decreased affinity, two mutations (both at Cys-656) produced "super" receptors that had higher affinities and specificities, and were transcriptionally active at lower steroid concentrations, than the wild type receptor (52). Thus, Cys-656 is not required for either steroid binding or biological activity. Therefore, the role of each of these cysteines in steroid binding appears to be indirect and some thiol-steroid interactions may actually attenuate receptor functions.

One indirect activity of Cys-640, -656, and -661 is to form a disulfide which blocks steroid binding (Figs. 3, *B* and *C*) and may be biologically relevant for preserving receptors in cells exposed to oxidizing conditions, especially in lung and skin, until the native receptor is regenerated by reducing conditions (33, 34, 57). The similarity with uteroglobin, which binds progesterone only when a disulfide bridge is reduced (58), is intriguing. It has been proposed (9) that the proximity of Cys-656 and -661 to a dipole could make one of the thiols more acidic, which favors the formation of disulfides under oxidizing conditions (59). In fact, the apparent pK_a for the affinity labeled Cys-656 is about 7.5 (60) *versus* 9–10 for simple thiols (61). The possible increased acidity of Cys-656 and -661 has also been proposed to facilitate protein-protein interactions, such as with hsp90 (9). However, any essential role of these thiols in the binding of hsp90 is now dubious. It has been established that hsp90 must be associated with the glucocorticoid receptor in order for steroid binding to occur

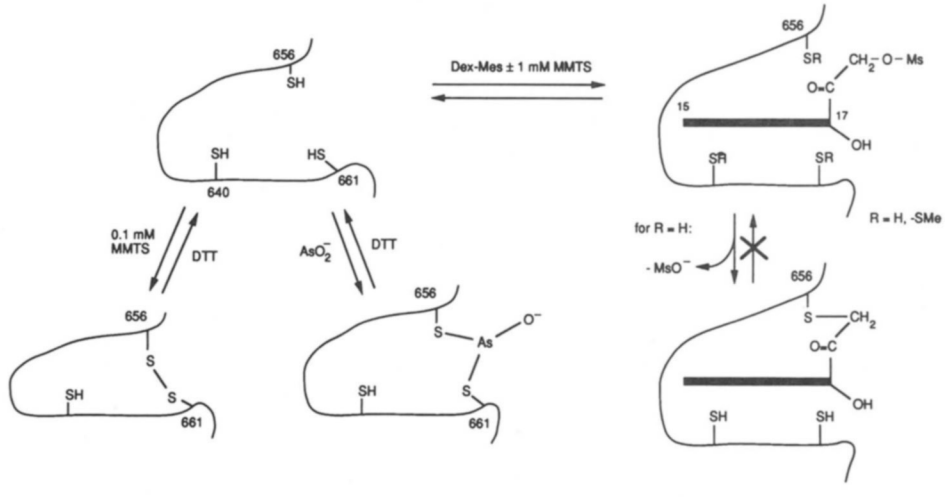

FIG. 7. **Model of glucocorticoid receptor steroid-binding cavity in region around C-21 of bound Dex.** A cross-section through a portion of the proposed steroid-binding cavity is shown (see text for details). The *solid bar* represents the steroid ring with the C-16 position of the D-ring being closest to the viewer (Ms = $-SO_2CH_3$). *DTT*, dithiothreitol.

(9, 16, 54, 55). Thus, the wild type steroid binding affinity of the double mutant lacking both Cys-656 and -661 implies that the association of hsp90 is still possible. Similarly, since C640S, C656S, and C661S all bind steroid with high affinity (52), we conclude that none of these cysteines is required for hsp90 binding. Furthermore, arsenite complexation with Cys-656 and -661 does not cause hsp90 dissociation (Fig. 6).

We have used the above data to generate a model of the steroid binding cavity around the C-21 of Dex-Mes (Fig. 7). Cys-656 and -661 have been positioned at the opening of the cleft to accommodate the facts that (*a*) trypsin appears to cleave at Lys-662 (8), (*b*) arsenite complexation of Cys-656 and -661 does not block trypsin cleavage, and (*c*) the C-21 mesylate derivative of Dex covalently labels Cys-656 while bulky substituents at the C-21 position do not prevent receptor binding (62), indicating that the region around Cys-656 is quite accessible to the bulk solvent. Cys-640 is shown as being inside the cleft, as opposed to being buried in the interior of the receptor. However, an equally plausible location would be anywhere on the same side of the cleft such that the distance between Cys-640 and Cys-656 is greater than or equal to that between Cys-640 and Cys-661 and greater than that between Cys-656 and Cys-661. This positioning of Cys-640 is required because, when either Cys-656 or -661 is missing, Cys-640 can be recruited by low concentrations of MMTS (except in C656G) to form a new disulfide with the remaining cysteine of the vicinally spaced disulfide (Fig. 5*B*). However, this recruitment of Cys-640 is energetically less favorable, at least in reactions with arsenite, as witnessed by the higher concentrations of arsenite that are required to block steroid binding (*cf.* Figs. 3*A* and 5*B*).

We propose that the steroid binding cavity is conformationally flexible. Receptor flexibility has previously been advanced (63) and might have been expected in order to accommodate steroids of different structures. However, more direct evidence for conformational flexibility includes 1) the differences in trypsin digestion of steroid-free *versus* steroid-bound receptors (8); 2) the lower affinity of Cd^{2+} *versus* arsenite, which is seen when complexation requires a spatial reorganization of a vicinally spaced dithiol (36, 37); and 3) the slow rate of intramolecular disulfide formation, which is very rapid for appropriately spaced thiols (64). However, a narrowing of the cleft, by bringing Cys-656 and -661 closer together with arsenite or the MMTS-induced disulfide, to help prevent steroid binding is not without some resistance, as reflected by the slow formation of the MMTS-induced intramolecular disulfide and the relatively poor ability of the vicinal dithiol reagent Cd^{2+} to block steroid binding. A similar effect of disulfide bond formation on cleft flexibility and access has recently been reported for lysozyme (65) and the periplasmic sulfate receptor of *Escherichia coli* (64). Higher concentrations of MMTS do introduce more steric bulk into the cleft (in the form of three -S-Me groups), but some steroid binding persists, presumably because the cleft can open up enough to accommodate the steroid. Upon entering the flexible binding cavity of the native receptor, the plane of the steroid separates Cys-656 from Cys-640 and -661 (Fig. 7). Cys-640 and -661 are now far enough away from the C-21 of Dex-Mes so that they are not affinity labeled but close enough so that they can form disulfides in the absence of bound steroid. The energy necessary for the above conformational changes could come from both the hydrophobic van der Waals interactions of protein and bound steroid and the increased entropy of released water molecules.

Our model only applies to the region around the C-21 of bound steroids. However, in order to accommodate the bulky A-ring substituents at positions 2 and 3 of deacylcortivazol, which has high affinity for the receptor (Table I) and is the most potent glucocorticoid yet described (49), it appears likely that the entire steroid binding cavity has a similar structure. The simplest cavity, with maximum hydrophobic interactions with the steroid (66), would be a groove where both one edge of the steroid and the 2 and 3 positions of the A-ring face out of the groove. Such a groove model of steroid binding, first proposed by Wolff *et al.* (66), and conformational changes upon ligand binding have been seen in the x-ray crystallographic analyses of various ligand-protein interactions (64, 65; for review, see 67). From the positioning of the C-21 group in our model (Fig. 7), we predict that the upper edge of the steroid (positions 1, 2, 9–13, and 17) would face out of the cleft. This positioning of bound steroids would easily accommodate the *p*-dimethylaminophenyl side chain of RU 486. We anticipate that bulky substituents at positions 4–8 and 14–16 will reduce affinity. Unfortunately, very few derivatives of this type have been reported (51, 68, 69) so that it is not yet possible to rigorously test this prediction.

In conclusion, studies aimed at elucidating some of the amino acids participating in steroid binding to the glucocorticoid receptor have not only identified the constituents of the previously described vicinally spaced dithiol group as being Cys-656 and -661 but have also uncovered the involvement a third thiol (Cys-640). These data have also strengthened the assignment of the region from Thr-537 to Arg-673 in the rat receptor as the core of the steroid-binding domain and have permitted us to advance a model for the steroid-binding cavity. Further studies with this model and the 16-kDa core fragment should be very helpful in determining the function of the 3 cysteines and in uncovering additional molecular details of the steroid binding process.

Acknowledgments—We thank Drs. Ettore Appella, Bernd Groner, and Robert Harrison for their gifts of antibodies, Kristen Legg for assistance in the preparation of the C656,661S double mutant, Dr. Nancy Miller for help with the competition binding experiments, Hung Luu for general technical assistance, and Dr. Alice Cavanaugh for critical review of the manuscript.

REFERENCES

1. Evans, R. M. (1988) *Science* **240,** 889–895
2. Tasset, D., Tora, L., Fromental, C., Scheer, E., and Chambon, P. (1990) *Cell* **62,** 1177–1187
3. Hollenberg, S. M., and Evans, R. M. (1988) *Cell* **55,** 899–906
4. Schule, R., Rangarajan, P., Kliewer, S., Ransone, L. J., Bolado, J., Yang, N., Verma, I. M., and Evans, R. M. (1990) *Cell* **62,** 1217–1226
5. Picard, D., and Yamamoto, K. R. (1987) *EMBO J.* **6,** 3333–3340
6. Godowski, P. J., Rusconi, S., Miesfeld, R., and Yamamoto, K. R. (1987) *Nature* **325,** 365–368
7. Rusconi, S., and Yamamoto, K. R. (1987) *EMBO J.* **6,** 1309–1315
8. Simons, S. S., Jr., Sistare, F. D., and Chakraborti, P. K. (1989) *J. Biol. Chem.* **264,** 14493–14497
9. Dalman, F. C., Scherrer, L. C., Taylor, L. P., Akil, H., and Pratt, W. B. (1991) *J. Biol. Chem.* **266,** 3482–3490
10. Fawell, S. E., Lees, J. A., White, R., and Parker, M. G. (1990) *Cell* **60,** 953–962
11. O'Donnell, A. L., Rosen, E. D., Darling, D. S., and Koenig, R. J. (1991) *Mol. Endocrinol.* **5,** 94–99
12. Danielsen, M., Northrop, J. P., Jonklaas, J., and Ringold, G. M. (1987) *Mol. Endocrinol.* **1,** 816–822
13. Picado-Leonard, J., and Miller, W. L. (1988) *Mol. Endocrinol.* **2,** 1145–1150
14. Pratt, W. B., Jolly, D. J., Pratt, D. V., Hollenberg, S. M., Giguere, V., Cadepond, F. M., Schweizer-Groyer, G., Catelli, M.-G., Evans, R. M., and Baulieu, E.-E. (1988) *J. Biol. Chem.* **263,** 267–273
15. Wang, L.-H., Tsai, S. Y., Cook, R. G., Beattie, W. G., Tsai, M.-J., and O'Malley, B. W. (1989) *Nature* **340,** 163–166
16. Chakraborti, P. K., and Simons, S. S., Jr. (1991) *Biochem. Bio-*

phys. Res. Commun. **176,** 1338–1344
17. Lopez, S., Miyashita, Y., and Simons, S. S., Jr. (1990) *J. Biol. Chem.* **265,** 16039–16042
18. Giguere, V., Hollenberg, S. M., Rosenfeld, M. G., and Evans, R. M. (1986) *Cell* **46,** 645–652
19. Byravan, S., Milhon, J., Rabindran, S. K., Olinger, B., Garabedian, M. J., Danielson, M., and Stallcup, M. R. (1991) *Mol. Endocrinol.* **5,** 752–758
20. Cadepond, F., Schweizer-Groyer, G., Segard-Maurel, I., Jibard, N., Hollenberg, S. M., Giguere, V., Evans, R. M., and Baulieu, E.-E. (1991) *J. Biol. Chem.* **266,** 5834–5841
21. Hurley, D. M., Accili, D., Stratakis, C. A., Karl, M., Vamvakopoulos, N., Rorer, E., Constantine, K., Taylor, S. I., and Chrousos, G. P. (1991) *J. Clin. Invest.* **87,** 680–686
22. DiSorbo, D. M., Phelps, D. S., and Litwack, G. (1980) *Endocrinology* **106,** 922–929
23. O'Brien, J. M., and Cidlowski, J. A. (1981) *J. Steroid Biochem.* **14,** 9–18
24. Naray, A., and Bathori, G. (1982) *J. Steroid Biochem.* **16,** 199–205
25. Baxter, J. D., and Tomkins, G. M. (1971) *Proc. Natl. Acad. Sci. U. S. A.* **68,** 932–937
26. Koblinsky, M., Beato, M., Kalimi, M., and Feigelson, P. (1972) *J. Biol. Chem.* **247,** 7897–7904
27. King, R. J. B., and Mainwaring, W. I. P. (1974) *Steroid-Cell Interactions*, pp. 109–161, University Park Press, Baltimore
28. Rees, A. M., and Bell, P. A. (1975) *Biochim. Biophys. Acta* **411,** 121–132
29. Simons, S. S. Jr., Pumphrey, J. G., Rudikoff, S., and Eisen, H. J. (1987) *J. Biol. Chem.* **262,** 9676–9680
30. Simons, S. S., Jr., and Thompson, E. B. (1981) *Proc. Natl. Acad. Sci. U. S. A.* **78,** 3541–3545
31. Simons, S. S. Jr. (1987) *J. Biol. Chem.* **262,** 9669–9675
32. Carlstedt-Duke, J., Stromstedt, P.-E., Persson, B., Cederlund, E., Gustafsson, J.-A., and Jornvall, H. (1988) *J. Biol. Chem.* **263,** 6842–6846
33. Miller, N. R., and Simons, S. S., Jr. (1988) *J. Biol. Chem.* **263,** 15217–15225
34. Chakraborti, P. K., Hoeck, W., Groner, B., and Simons, S. S., Jr. (1990) *Endocrinology* **127,** 2530–2539
35. Zahler, W. L., and Cleland, W. W. (1968) *J. Biol. Chem.* **243,** 716–719
36. Gaber, B. P., and Fluharty, A. L. (1972) *Bioinorg. Chem.* **2,** 135–148
37. Simons, S. S., Jr., Chakraborti, P. K., and Cavanaugh, A. H. (1990) *J. Biol. Chem.* **265,** 1938–1945
38. Pons, M., and Simons, S. S., Jr. (1981) *J. Org. Chem.* **46,** 3262–3264
39. Gametchu, B., and Harrison, R. W. (1984) *Endocrinology* **114,** 274–279
40. Hoeck, W., Rusconi, S., and Groner, B. (1989) *J. Biol. Chem.* **264,** 14396–14402
41. Ehrhart, J. C., Duthu, A., Ullrich, S., Appella, E., and May, P. (1988) *Oncogene* **3,** 595–603
42. Yen, P. M., and Simons, S. S., Jr. (1991) *Receptor* **1,** 191–205
43. Schena, M., and Yamamoto, K. R. (1988) *Science* **241,** 965–967
44. Miesfeld, R. Godowski, PJ, Maler, BA, and Yamamoto, KR (1986) *Cell* **46,** 389–399
45. Simons, S. S. Jr., and Miller, P. A. (1984) *Biochemistry* **23,** 6876–6882
46. Rodbard, D. (1973) in *Receptors for Reproductive Hormones* (O'Malley, B. W., and Means, A. R., eds) pp. 289–326, Plenum Press, New York
47. Gehring, U. (1988) in *Affinity Labelling and Cloning of Steroid and Thyroid Hormone Receptors* (Gronemeyer, H., ed) pp. 144–166, Ellis Horwood Limited, United Kingdom
48. Eisen, L. P., Reichman, M. E., Thompson, E. B., Gametchu, B., Harrison, R. W., and Eisen, H. J. (1985) *J. Biol. Chem.* **260,** 11805–11810
49. Simons, S. S., Jr., Thompson, E. B., and Johnson, D. F. (1979) *Biochem. Biophys. Res. Commun.* **86,** 793–800
50. Lamontagne, N., Mercier, L., Pons, M., Thompson, E. B., and Simons, S. S., Jr. (1984) *Endocrinology* **114,** 2252–2263
51. Ojasoo, T., Dore, J.-C., Gilbert, J., and Raynaud, J.-P. (1988) *J. Med. Chem.* **31,** 1160–1169
52. Chakraborti, P. K., Garabedian, M. J., Yamamoto, K. R., and Simons, S. S., Jr. (1991) *J. Biol. Chem.* **266,** 22075–22078
53. Richardson, J. S. (1981) in *Advances in Protein Chemistry* (Anfinsen, C. B., Edsell, J. T., Richards, F. M., eds) Vol. 34, pp. 223–230, Academic Press, New York
54. Nemoto, T., Ohara-Nemoto, Y., Denis, M., and Gustafsson, J. (1990) *Biochemistry* **29,** 1880–1886
55. Scherrer, L. C., Dalman, F. C., Massa, E., Meshinchi, S., and Pratt, W. B. (1990) *J. Biol. Chem.* **265,** 21397–21400
56. Sanchez, E. R. (1992) *J. Biol. Chem.* **267,** 17–20
57. Granberg, J. P., and Ballard, P. L. (1977) *Endocrinology* **100,** 1160–1168
58. Mornon, J. P., Fridlansky, F., Bally, R., and Milgrom, E. (1980) *J. Mol. Biol.* **137,** 415–429
59. March, J. (1985) *Advanced Organic Chemistry: Reactions, Mechanisms, and Structure*, 3rd ed., p. 1092, J. Wiley & Sons, New York
60. Simons, S. S., Jr., Schleenbaker, R. E., and Eisen, H. J. (1983) *J. Biol. Chem.* **258,** 2229–2238
61. Singh, R., and Whitesides, G. M. (1991) *J. Org. Chem.* **56,** 2332–2337
62. Pons, M., Robinson, T. E. J., Mercier, L., Thompson, E. B., and Simons, S. S., Jr. (1985) *J. Steroid Biochem.* **23,** 267–273
63. Wrange, O., and Gustafsson, J.-A. (1978) *J. Biol. Chem.* **253,** 856–865
64. Jacobson, B. L., He, J. J., Vermersch, P. S., Lemon, D. D., and Quiocho, F. A. (1991) *J. Biol. Chem.* **266,** 5220–5225
65. Matsumura, M., and Matthews, B. W. (1989) *Science* **243,** 792–794
66. Wolff, M. E., Baxter, J. D., Kollman, P. A., Lee, D. L., Kuntz, I. D., Bloom, E., Matulich, D. T., and Morris, J. (1978) *Biochemistry* **17,** 3201–3208
67. Janin, J., and Chothia, C. (1990) *J. Biol. Chem.* **265,** 16027–16030
68. Rousseau, G. G., and Schmit, J.-P. (1977) *J. Steroid Biochem.* **8,** 911–919
69. Raynaud, J. P., Ojasoo, T., Bouton, M. M., and Philibert, D. (1979) in *Drug Design* (Ariens, E. J., ed) Vol. 8, pp. 170–214, Academic Press, New York