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Modular Structure of Glucocorticoid Receptor Domains Is Not Equivalent to Functional Independence

STABILITY AND ACTIVITY OF THE STEROID BINDING DOMAIN ARE CONTROLLED BY SEQUENCES IN SEPARATE DOMAINS*

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A long-standing conundrum of glucocorticoid receptors has been why the steroid binding domain is active in hybrid proteins but not in isolation. For this reason, the precise boundaries of the steroid binding domain have not been defined. These questions have now been systematically examined with a variety of receptor deletion constructs. Plasmids encoding amino acids 537–673 and 537–795 of the rat receptor did not yield stable proteins, while the fusion of receptor or non-receptor sequences upstream of 537–673 afforded stable proteins that did not bind steroid. Wild type steroid binding affinity could be obtained, however, when proteins such as β-galactosidase or dihydrofolate reductase were fused upstream of receptor amino acids 537–795. Studies of a series of dhfr/receptor constructs with deletions at the amino- and carboxyl-terminal ends of the receptor sequence localized the boundaries of the steroid binding domain to 550–795. The absence of steroid binding upon deletion of sequences in the carboxyl-terminal half of this domain was consistent with improperly folded receptor sequences. This conclusion was supported by analyses of the proteolysis and thermal stability of the mutant receptors. Thus, three independent regions appear to be required for the generation of the steroid binding form of receptors: 1) a protein sequence upstream of the steroid binding domain, which conveys stability to the steroid binding domain, 2) sequences of the carboxyl-terminal amino acids (674–795), which are required for the correct folding of the steroid binding domain, and 3) amino-terminal sequences (550–673), which may be sufficient for steroid binding after the entire steroid binding domain is properly folded. These results establish that the steroid binding domain of glucocorticoid receptors is not independently functional and illustrate the importance of both protein stability and protein folding when constructing mutant proteins.

The presence of separate functional domains in glucocorticoid receptors was first postulated on the basis of protease digestion studies (1). Chymotrypsin digestion removed about half of the receptor to give a 42-kDa (3.6 nm) fragment that still bound steroid and DNA. Trypsin digestion of receptor-steroid complexes liberated an even smaller, about 30-kDa (1.9 nm) steroid-containing fragment that no longer bound DNA. Further support for this domain structure came after the cloning of glucocorticoid receptors, when it was found that the receptor could be divided up into three functionally active domains: an amino-terminal activation domain, a central DNA binding domain, and a carboxyl-terminal steroid binding domain (2, 3).

An initially surprising feature of all the steroid receptors was that functional activity was retained when the domains of different receptors were interchanged for each other or for the segments of other proteins. Particularly notable examples were the swapping of DNA binding domains of two receptors (4, 5) and the fusing of a steroid binding domain either to different positions of the original receptor or to other proteins (6, 7). When the steroid binding domain of the glucocorticoid receptor was fused to other proteins, not only was steroid binding retained, but also the activity of the rest of the fusion protein was usually controlled by steroid binding (6–13). Thus, in most instances, the various domains of the glucocorticoid receptor appeared to function autonomously. In fact, a separate evolution and function of the specific domains has been proposed (14–16). These findings are consistent with reports that transcription factors generally have a modular structure (17) and that individual protein domains, containing 100–200 amino acids (18), can fold independently of the rest of the protein (19–21).

Despite the numerous examples of domain modularity, direct evidence that a given glucocorticoid receptor domain is active in isolation exists only for the DNA binding domain and perhaps the amino-terminal domain. Thus, the fragment corresponding to amino acids 440–525 of the rat receptor, which is only slightly larger than the DNA binding domain (reviewed in Ref. 22), can bind DNA (23, 24). The amino-terminal transactivation domain also possesses transcriptional activity (7, 8), but this activity could be realized only when the domain is part of some larger DNA-binding protein. Thus, deletion of the amino-terminal domain reduced the *trans*-activation and synergism of intact glucocorticoid receptors (25), while fusion of the amino-terminal domain to the DNA binding domain of GAL4 caused an increased transcriptional activation, and synergism, from tandem arrangements of a palindromic GAL4 binding sequence (26). One example of a biologically relevant activity of the amino-terminal domain in isolation may be squelching (27). However, the observation that three antibodies to amino-terminal sequences do not inhibit squelching but do prevent transactivation (28) suggest that the two activities

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involve different processes.

To date, there has been no convincing evidence that the isolated steroid binding domain of the glucocorticoid receptor retains steroid binding activity. Very poor steroid binding was seen for the "steroid binding domain" either expressed in cell-free translation assays (29) or overexpressed in *Escherichia coli* (30). A 16-kDa tryptic fragment of the preformed native receptor, which has been deduced to be amino acids 537–673 of the rat receptor (31), does display wild type specificity of steroid binding and is not associated with the DNA binding domain in a noncovalent complex (32). However, these results pose the conundrum of how steroid binding can be retained in a fragment that is smaller than the proposed steroid binding domain, which by itself does not bind steroid. For these reasons, no consensus has emerged as to the precise limits of the steroid binding domain, which has been considered to start anywhere between amino acids 546 and 574 of the rat receptor (22).

We sought in this paper to define the limits of the steroid binding domain of the glucocorticoid receptor and to determine whether the steroid binding domain, like the DNA binding domain, can function in isolation. Additionally, we wanted to understand the differences between steroid binding receptor fragments that were prepared by proteolysis from preformed receptors (31) and those expressed directly from the corresponding mRNA (29) or cDNA (30). One plausible explanation was that other regions of the receptor are required for correct folding but that, once properly folded, an intact and functional steroid binding core can be isolated. To test this hypothesis, we prepared and examined the activity of several receptor constructs containing portions of the steroid binding domain both in isolation and fused to other proteins. Surprisingly, the steroid binding domain was found not to be functionally independent and to require extra-domain sequences for its activity.

MATERIALS AND METHODS

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

Chemicals—Nonradioactive dexamethasone (Dex)[1] (Sigma), [^{3}H]Dex (37 and 39 Ci/mmol, Amersham), and [^{3}H]Dex 21-mesylate (Dex-Mes; 44.7 Ci/mmol, DuPont NEN) were commercially available. Other purchased reagents were TAPS (Ultrol grade, Calbiochem), SDS (Research Genetics), high and low molecular weight markers for SDS-polyacrylamide gels (Pharmacia Biotech Inc.), other reagents for SDS-polyacrylamide gel electrophoresis, including Coomassie Blue R-250 and EIA grade Tween 20, and 0.2-μ nitrocellulose membrane (Bio-Rad), fluorescent Ult-Emit autoradiography marker and EN^{3}HANCE or Lightning for fluorography (DuPont NEN), Protogel and Sequalgel 6 (National Diagnostics), enhanced chemiluminescence Western blotting detection kit (Amersham), ABC reagent for immunoperoxidase staining of Western blots (Vector Laboratories), β-galactosidase expression vector (pCMVβ, Clontech), Tris and glycine (ICN Biochemicals, Inc.), and sodium molybdate (Baker Chemical Co.). All other chemicals were obtained from Sigma. 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.

Enzymes—Enzymes used in cloning include *Eco*RI (Stratagene), *Bst*BI and *Sph*I (New England Biolabs), *Pst*I, *Sal*I, *Bam*HI, *Xba*I, and T4 DNA ligase (Life Technologies, Inc.), and *Taq* polymerase (Promega).

Antibodies—A polyclonal antibody (aP1) against the carboxyl-terminal region of the rat glucocorticoid receptor was a gift from Dr. Bernd Groner (Institute for Experimental Cancer Research, Germany). Biotinylated anti-rabbit second antibodies for Western blotting were from Vector Laboratories.

Buffers and Solutions—TAPS buffer was 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) contained 0.6 M Tris, pH 8.8, 2% SDS, 0.2 M dithiothreitol, 20% glycerol, and bromphenol blue. Transfer buffer for Western blotting was made from 25 mM Tris, 192 mM glycine, 20% methanol in water (pH ~8.3 at room temperature). Tris-buffered saline (TBS) had 20 mM Tris and 0.28 M NaCl in water (pH 7.5 at room temperature).

Construction of Plasmids—All enzymatic manipulations were performed according to manufacturers' recommendations. The constructs were transformed into DH5α competent cells (Life Technologies, Inc.), selected on LB plates containing 50 μg/ml ampicillin (Digene Diagnostics, Inc.) and grown in Superbroth (Quality Biologicals, Inc.). The plasmid DNAs were extracted and purified by the Qiagen Mini or Maxi Kits.

pSVL407C was prepared by removing the 407–795 sequence as a *Bam*HI fragment from VARO407-795 (33) and inserting it into the empty pSVLT vector obtained by *Bam*HI digestion of pSVLT407-556 (34). The other truncated receptor plasmids (pSVL1-673, pSVLTm-537C, and pSVLTm537-673) were made by sequential oligonucleotide-directed point mutagenesis of the original full-length wild type receptor in pSVLGR (35) using the Amersham Oligonucleotide Site-directed Mutagenesis System (version 2.1). An *Sph*I-*Eco*RI fragment, encompassing amino acids 494–768 of the receptor, was subcloned into M13mp18. Single-stranded DNA was isolated and used as template in the mutagenesis reactions. A stop codon in place of Leu-674 was introduced first by changing Val-675 to a *Bam*HI site with the oligonucleotide 5′-GATTGCAGG<u>G</u>ATCCTATG-3′ and then altering Leu-674 to a stop codon with 5′-CAAAGAT<u>A</u>GCAGGGATCC-3′ (underlined nucleotides indicate changes from wild type sequence). A two-step procedure was also used to create the 537 boundary. Lys-536 was changed to a methionine with the oligonucleotide 5′-GAAAATCCTAACA<u>TG</u>ACAAT-AGTTCC-3′, and then an *Nde*I cloning site was introduced immediately upstream with 5′-GGAAAATCCT<u>C</u>A<u>T</u>ATGACAATAGTTC-3′. pSVL1-673 was created by ligating a *Bam*HI-*Sph*I fragment (amino acids 1–494) to a *Sph*I-*Bam*HI fragment (amino acids 494–673) into the *Bam*HI-digested pSVL vector. pSVLTm537C and pSVLTm537–673 were constructed by subcloning *Nde*I-*Bam*HI fragments containing 537C, or 537–673, into a modified pSVL vector (pSVLTm) containing a 64-base pair translational leader sequence from the herpes simplex virus thymidine kinase gene (pSVLT) (34). A *Nde*I site at the initiator methionine of the thymidine kinase leader sequence was introduced by PCR using the nucleotides 5′-TTTCAGAGGTTATTCAGG-3′ and 5′-GGTACC<u>CATATG</u>CGCTTCTACAAGGC-3′ to give the vector pSVLTm.

Fusion protein constructs between β-galactosidase (β-gal) and truncated rat glucocorticoid receptors were made by substituting the appropriate new restriction fragments into pZ540C, which contains amino acids 540–795 of the rat receptor fused in-frame to carboxyl-terminal of β-gal (33). Specific primers were used to amplify the desired regions of receptor. A *Sal*I recognition site was placed in the 5′ primer, and a stop codon plus a *Bam*HI recognition site was placed in the 3′ primer. pZ540C was digested with either *Sal*I plus *Xba*I or *Xba*I plus *Bam*HI. The 5-kb fragment from double digestion with *Xba*I plus *Bam*HI and the 3-kb fragment from *Sal*I plus *Xba*I digestion were ligated to the PCR product that had been digested with *Sal*I and *Bam*HI. All constructs were confirmed by sequencing. The primers used in PCR were as follows. For pZ537C: 5′ primer, 5′-TAGTCGACAAACAATAGTTCCTG-CAGC-3′; 3′ primer, 5′-TTGTCATAGGTCTGCTTGAGGAT-3′; for pZ537–673: 5′ primer, 5′-TAGTCGACAAACAATAGTTCCTGCAGC-3′; 3′ primer, 5′-ATGGATCCTATCTTTGTAATTCAGAGG-3′.

pGST537C was constructed by modifying the plasmid pdhfr537C, which was digested with *Eco*RI plus *Ava*I to generate three fragments (a 4.7-kb vector fragment, a 0.7-kb fragment, and a 0.6-kb fragment containing amino acids 560 to 781 of the receptor). The sequence containing full-length GST plus receptor amino acids 537 to 559 was generated by PCR using a bacterial expression vector for GST537C[2] as the template. The PCR primers used were as follows: 5′ primer is 5′-GCCAGAATTCATGTCCCCTATACTAGG-3′; 3′ primer is 5′-CACT-GCTGCAATCACTTGAC-3′. The PCR product was digested with *Eco*RI and *Ava*I and then ligated with the above 4.7- and 0.6-kb fragments to generate pGST537C.

The constructs involving a fusion of dihydrofolate reductase (DHFR) and truncated rat glucocorticoid receptors were made by modifying the plasmid pMT2D/G (Israel and Kaufman (45)). pMT2D/G contains amino acids 494–795 of the rat receptor fused to the carboxyl-terminal of DHFR, at which an *Sph*I site had been generated with adaptors. pMT2D/G affords two fragments with *Eco*RI digestion. One of the

[1] The abbreviations used are: Dex, dexamethasone; Dex-Mex, [^{3}H]Dex 21-mesylate; PCR, polymerase chain reaction; kb, kilobase(s); GST, glutathione S-transferase; DHFR (dhfr), dihydrofolate reductase; PBS, phosphate-buffered saline; TAPS, 3-[tris(hydroxymethyl)methyl]-aminopropanesulfonic acid.

[2] S. Bayly and S. S. Simons, unpublished results.

fragments (4.7 kb) was used as the vector for subsequent cloning. The other fragment (1.4 kb) was further digested with *Sph*I to isolate the desired 0.6-kb DHFR sequence. Different lengths of receptor were amplified by polymerase chain reaction from pSVLGR (35). 5′ primers for PCR have an *Sph*I recognition site upstream of the receptor cDNA sequence. 3′ Primers contain receptor cDNA followed by a stop codon plus an *Eco*RI recognition site. After digestion with *Sph*I and *Eco*RI, the PCR product was ligated to the above 4.7-kb vector and 0.6-kb DHFR-containing fragments. All the constructs were confirmed by sequencing. Primers used in PCR are as follows. For pdhfr537C: 5′ primer (for this and all constructs starting at 537), 5′-GTATAGCATGCACAATAGTTC-CTGCAGC-3′; 3′ primer (for this and all constructs ending at 795), 5′-CGGAATTCAACTTTCTTTAAGGCAAC-3′; for pdhfr537–766: 3′ primer, 5′-GCGGAATTCTATTCAATACTCATGGTC-3′; for pdhfr537–710: 3′ primer, 5′-GCGGAATTCTAAGTCATTCGAATCTCA-3′; for pdhfr537–673: 3′ primer, 5′-CGGAATTCTATCTTTGTAATTCAGAGG-3′; for pdhfr547C: 5′ primer, 5′-GCGGAATTCTAGTTCCCTTCCCTT-TT-3′; for pdhfr550C: 5′ primer, 5′-GTATAGCATGCTTGGTGTCACTG-CTG-3′; for pdhfr552C: 5′ primer, 5′-GTATAGCATGCTCACTGCTGG-AGGTG-3′; for pdhfr554C: 5′ primer, 5′-GTATAGCATGCCTGGAGGT-GATTGAAC-3′; for pdhfr556C: 5′ primer, 5′-GTATAGCATGCGTGAT-TGAACCCGA-3′. For pSVLTmdhfr494C, the open reading frame of dhfr-494C fusion protein was amplified from pMT2D/G using the following primers which contain an *Nde*I site on the 5′ primer (5′-CACT-GAGCATATGGTTCGACCATT-3′) and a *Bam*HI site on the 3′ primer (5′-AGCGGATCCATTCAACTTTCTTTAAG-3′). The PCR product was digested with *Nde*I and *Bam*HI. pSVLTm vector was prepared by digestion of pSVLTm537C with *Nde*I and *Bam*HI to release the insert GR537C. The pSVLTm vector was then purified and ligated to *Nde*I and *Bam*HI-digested, above PCR-produced dhfr-494C fusion protein sequence.

T3MSTΔ9 contains amino acids 407 to 795 of the rat receptor with deletion of amino acids 690–704 (29). T3iΔ17 encompasses amino acids 407–795 with deletion of 616–695 (29). pdhfr537Δ616–695C and pdhfr537Δ690–704C were constructed by exchanging the fragment within two *Bst*BI sites (corresponding to amino acids 487 to 707) in pdhfr537C with the corresponding fragment from T3MSTΔ9 or T3iΔ17, respectively. pSVL1–766 was constructed by inserting the *Bst*BI (at amino acid 707) to *Xba*I (in the receptor 3′-untranslated region) fragment of pdhfr537–766 into pSVLGR.

Growth and Transfection of Cells—Monolayer cultures of COS-7 cells were grown in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 5% heat-inactivated fetal bovine serum. Wild type and truncated receptor expression plasmids (10 μg) were introduced into COS-7 cells (~6 × 10^5/100-mm dish) by standard calcium phosphate transfection methods (36). Briefly, 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 plus 5% heat-inactivated fetal bovine serum and harvested by trypsinization followed by centrifugation and washing three times with phosphate-buffered saline. The washed cells were stored at −80 °C until assayed.

16-kDa Tryptic Fragment—The 16-kDa fragment was generated by trypsin digestion (14–26 μg/ml trypsin for 1 h at 0 °C) of crude cell receptors, followed by the addition of a 10-fold (w/w) excess of soybean trypsin inhibitor to stop further digestion, as described (31).

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* (37). [³H]Dex binding assays and competition binding assays all contained added 20 mM sodium molybdate (32). Briefly, 30% cytosol was incubated at 0 °C for 2.5 h with 50 nM [³H]Dex in the presence or absence of a 500-fold excess of nonradioactive Dex. Dextran-coated charcoal was used to remove the unbound [³H]Dex and, after centrifugation, the supernatant was counted in Hydrofluor. Scatchard analyses were conducted at 0 °C for 18 h with various concentrations of [³H]Dex ± 100-fold excess of nonradioactive Dex. Unbound [³H]Dex was removed with dextran-coated charcoal, and the samples were processed as above.

Expression of Receptors in E. coli—Bacterial strains containing the T7 expression vectors encoding receptor sequences 537C and 537–673 were prepared by subcloning *Nde*I-*Bam*HI fragments encompassing residues 537–673 and 537–795 into *Nde*I-*Bam*HI-digested pET3a. Single colonies were used to inoculate 4-ml cultures of Luria-Bertani medium with ampicillin and grown with vigorous shaking (300 rpm) at 37 °C until visibly turbid (5 h). Receptor protein expression was induced during an additional 2.75 h with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside. Cultures were centrifuged in a Microfuge. The pellets were resuspended in 300 μl of pH 9.5 TAPS buffer and frozen until needed at −80 °C. Upon thawing, the cells were treated with 3 volumes of 2 × SDS buffer, vortexed, heated at 100 °C for 5 min, and then centrifuged. The supernatant was then analyzed on gels.

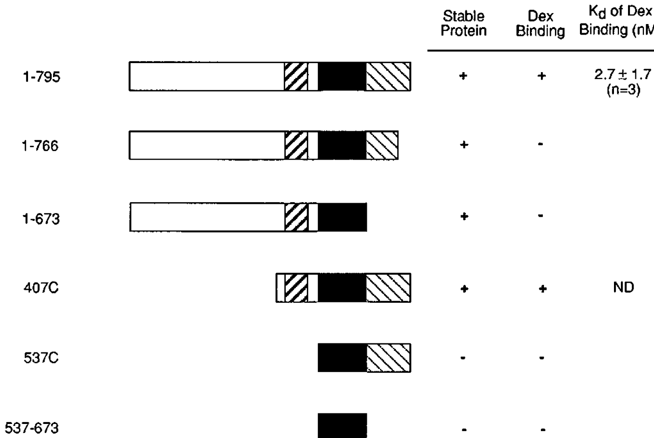

FIG. 1. **Properties of glucocorticoid receptor derivatives expressed in COS-7 cells.** cDNAs encoding the indicated regions of the rat glucocorticoid receptor in pSVL vectors were transiently expressed in COS-7 cells. The full-length receptor is 1–795. Truncated receptors are designated by their amino and carboxyl termini with C representing the carboxyl terminus so that 407C = 407–795. The presence (+) or absence (−) of each receptor protein was established by Western blotting; steroid binding (+ or −) was assessed in the presence of 5 × 10^{-8} M [³H]Dex (see "Materials and Methods"). The binding affinity was determined by Scatchard analysis and expressed as ± S.D., with the number of experiments indicated in *parentheses*. *ND* = not done. Specific regions of the receptor are as indicated: , DNA binding domain; ■, steroid binding core (amino acids 537–673); , amino acids 674–795; □, all other regions of the receptor.

Polyacrylamide Gel Electrophoresis and Fluorography—Samples diluted 1:2 in 2 × SDS buffer were analyzed on constant percentage acrylamide gels (between 8 and 14% with a 1:37.5 ratio of bisacrylamide to acrylamide) run in a water-cooled (15 °C) Protean II slab gel apparatus (Bio-Rad) at 35 mA/gel. Gels were fixed, stained, marked at the positions of the molecular weight markers with Ult-Emit, and fluorographed for 1–2 weeks at −80 °C as described (38).

Western Blotting—Electrophoretic transfer from SDS-polyacrylamide gels to nitrocellulose, conducted in a Trans-Blot (Bio-Rad) apparatus (~15 h at 100 mA, then ~250 mA for 2 h), followed by incubation with primary and secondary antibodies and staining and visualization by enhanced chemiluminescence, was performed as described (38).

RESULTS

Smallest Segment of Expressed Glucocorticoid Receptor That Retains Steroid Binding Activity—The cDNAs encoding for the intact and truncated receptors of Fig. 1 were transiently transfected into COS-7 cells, which contain very low levels of glucocorticoid receptors (see Fig. 2 below). Cytosolic extracts were then prepared to monitor the presence, and ability to bind steroid, of each receptor construct. The wild type (1–795) and amino-terminal domain truncated (407–795 = 407C) receptors both were stable proteins with very similar steroid binding and Dex-Mes affinity labeling capacity (data not shown). The affinity for dexamethasone (Dex) binding to the 407C receptors was not determined but was expected to be at least that of the smaller 42-kDa chymotryptic fragment of ≈ 413–781 (or -795), which had previously been found to possess 84% of the affinity of the intact receptor (31). By Western blotting, the expression level of wild type and 407C receptors was similar (Fig. 2). The presence of additional, lower molecular weight bands appears due to alternative translational start sites (35, 39).

Neither fragments smaller than 407C, nor larger but containing less of the carboxyl-terminal end of the receptor, displayed significant amounts of steroid binding activity (Fig. 1). Both of the carboxyl truncated receptors 1–766 and 1–673 afforded stable proteins (Fig. 2). The cell-free translated frag-

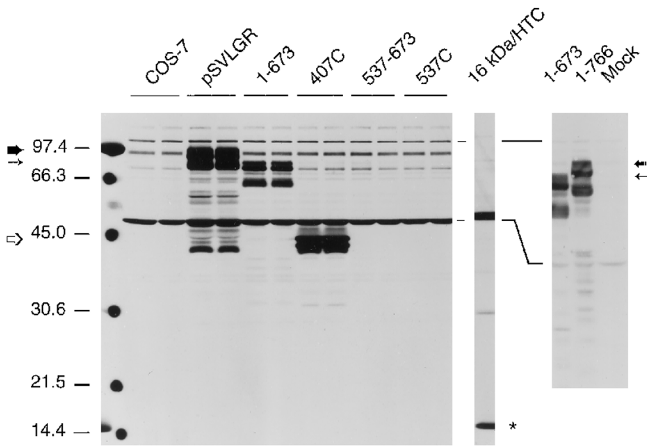

FIG. 2. **Western blot analysis of expressed glucocorticoid receptor fragments.** Aliquots of the COS-7 cell extracts of Fig. 1, along with an aliquot of mock transfected cells and 16-kDa fragment (*) prepared by trypsin digestion of steroid-free receptors from HTC cells, were separated on a 10.8%, or 9% (for 1–673, 1–766, and mock transfection), polyacrylamide gel and Western-blotted with anti-receptor antibody (aP1). The receptors were visualized by enhanced chemiluminescence, as described under "Materials and Methods" (location of the receptors is as indicated: wild type, ➡; 407C, ⇨; 1–766, ➠; 1–673, →). The positions of the molecular mass markers (phosphorylase *b* (97,400 Da), bovine serum albumin (66,300 Da), ovalbumin (45,000 Da), carbonic anhydrase (30,600 Da), soybean trypsin inhibitor (21,500 Da), and lactalbumin (14,400 Da)) were marked by Ult-Emit and visualized on film at the same time as chemiluminescence.

ment corresponding to 547–795 was reported to bind Dex with 1/350 the affinity of the intact receptor (29). Unexpectedly, the slightly larger 537–795 (predicted molecular weight = 29,924) and smaller 537–673 (predicted molecular weight = 15,546) fragments gave neither any Dex binding, or Dex-Mes labeling (Fig. 1 and data not shown), nor any detectable receptor protein in transiently transfected COS-7 cells (Fig. 2). Authentic 16-kDa fragment, corresponding to 537–673 and prepared by trypsin digestion of wild type receptors (31), was readily visualized under the same conditions (Fig. 2). The transfected, translated 537–673 fragment was also not detected when expressed in the presence of Dex, Dex-Mes, or arsenite (40, 41) to stabilize the protein (data not shown). This apparent lability did not result from COS-7 cell-specific proteases because no appropriately sized protein was seen in the cytosolic extracts of transiently transfected CV-1 or HeLa cells (data not shown). A ubiquitous protease was not digesting the expected protein fragment because authentic 16-kDa fragments were stable in cell-free extracts of COS-7 cells (data not shown). The receptor cDNA sequences were translationally viable since, in the form of the T7 expression plasmids T7537C and T7537–673, they directed the synthesis of correctly sized proteins in *E. coli*, albeit as insoluble inclusion bodies (data not shown).

Expressed 537–673 Receptor Fragments Are Rapidly Degraded in Intact Cells—The above inability to obtain receptor fragments 537–795 and 537–673 suggested either that the eukaryotic expression vector used was somehow defective or that these fragments were expressed but rapidly degraded under a variety of cellular conditions. In order to decide between these two possibilities, we developed a whole cell competitive expression assay. Briefly, the ability of 6×10^5 COS-7 cells to express steroid binding activity was found not to increase when transiently transfected with >5 μg of wild type receptor cDNA and enough pUC19 DNA to give a total of 10 μg of DNA (Fig. 3*A*). Similarly, the total amount of binding activity was relatively constant when wild type receptor cDNA was gradually replaced by cDNA for the truncated 407C receptor (data not shown). Thus, receptor cDNA was in excess under these conditions and other components, such as transcription and/or translation factors, limited the amount of steroid binding activity that could be obtained. Consistent with this saturation of the transcription/translation machinery is the fact that the total amount of receptor proteins (wild type and/or 407C) detected by Western blots was relatively constant with different ratios of transfected receptor cDNA and pUC19 DNA (Fig. 3*C*). However, a 1:1 mixture of wild type and 537–673 cDNA caused a dramatic decrease in both steroid binding activity (Fig. 3*A*) and wild type receptor protein (Fig. 3*C*). Similarly, dilution of 407C cDNA with 537–673 cDNA caused a much more extensive drop in steroid binding activity (Fig. 3*B*) and 407C receptor protein (Fig. 3*C*) than was seen with pUC19. These results are most readily interpreted as the 537–673 cDNA, but not the pUC19 DNA, competing with wild type (Fig. 3, *A* and *C*) or 407C (Fig. 3, *B* and *C*) cDNA for transcription and translation to give a protein (537–673) that is unstable. Therefore, we conclude that the inability of the 537–673 and 537–795 constructs to generate any steroid binding activity, or protein, was due to an instability of the expressed proteins rather than a lack of transcription/translation of the cDNA constructs.

The combination of these results with the nearly wild type level of protein and steroid binding activity seen with the 407C rat glucocorticoid receptor (Figs. 1–3) suggests that the steroid binding domain, which is thought to be encoded by sequences between 547 and 795 (22), is required for the steroid binding activity while upstream sequences, such as 407–546, are required for the steroid binding domain to be stable as a protein. One cause of protein instability is improper folding because incorrectly folded proteins are thought to be rapidly degraded (18, 42–44). We therefore asked whether the receptor sequence of 407–536 was unique in stabilizing the carboxyl-terminal region of the receptor.

Protein Stability of Carboxyl-terminal Constructs Can Be Conferred by Non-receptor Sequences—Constructs encoding the three hybrid receptors of Fig. 4*A* were prepared by fusing the intact β-galactosidase gene upstream of various carboxyl-terminal portions of the receptor. Earlier studies on the steroid-induced nuclear localization of Z540C in intact cells (13, 33) showed that this fusion protein was stable and bound steroid. We found that both Z540C and Z537C had the same, or slightly higher, affinity for Dex as did the wild type receptor (Figs. 4*A* *versus* 1). In both cases, the amount of steroid binding was about 4 times that seen for the mock transfection controls. In contrast, Z537–673 did not give any Dex binding (0.79 ± 0.17 (S.D., n = 5)-fold above mock transfections).

Replacement of the β-galactosidase gene in Z537C by the glutathione *S*-transferase (GST537C) gene sequence afforded another stable fusion protein with good steroid binding activity. Transient transfection of GST537C into COS cells gave rise to a 56 ± 22-fold (±S.D., n = 5) increase in the specific binding of [^{3}H]Dex (data not shown).

Another series of hybrid receptors was prepared using dihydrofolate reductase (Fig. 4*B*). The parent receptor construct pMT2D/G (45), here denoted dhfr494C, seemed to have wild type affinity for Dex as witnessed by the reported half-maximal biological response of 10 nM Dex. This was confirmed by the present studies, where Scatchard analysis afforded an affinity comparable to that of wild type receptors (Figs. 4*B* *versus* 1). Similarly, removal of receptor sequences up to amino acid 537, to give dhfr537C, had little effect on the affinity of the resulting hybrid receptor (Fig. 4*B*) or its ability to be covalently labeled by Dex-Mes (data not shown). These data for 537C, Z537C, dhfr537C, and GST537C show that stable molecules with nearly wild type affinity for Dex (and presumably wild type

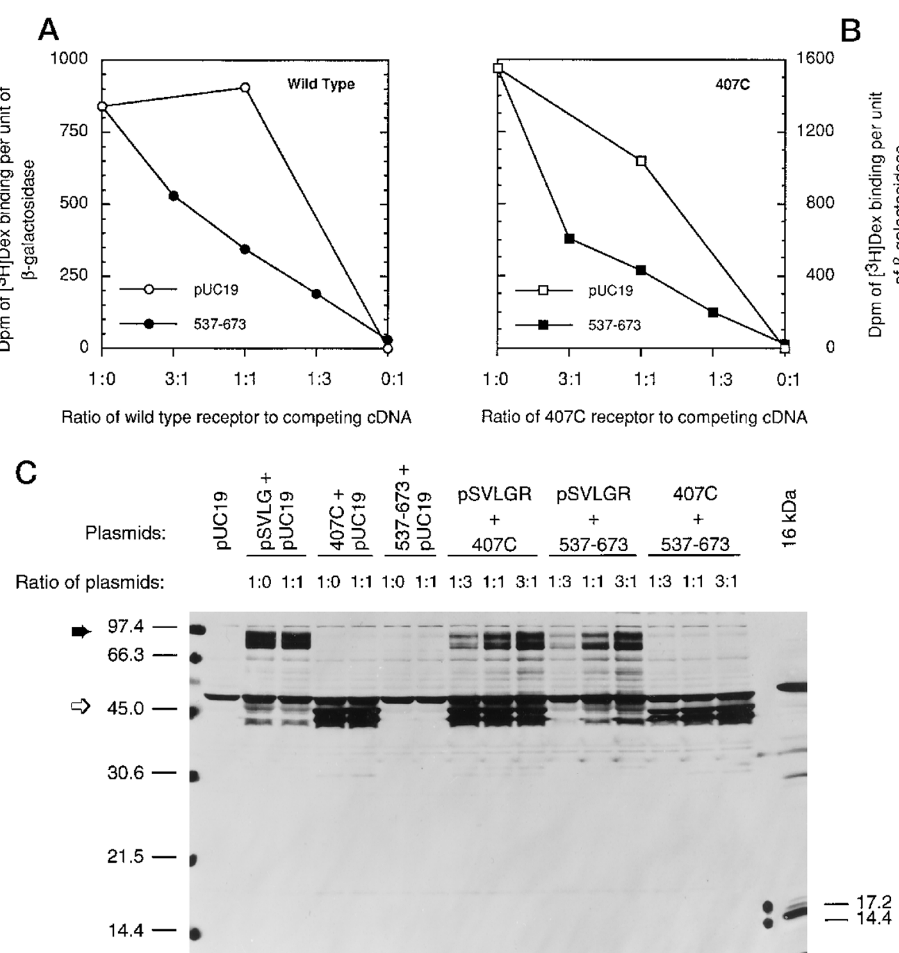

FIG. 3. **Effect of competing receptor cDNA in the whole cell expression of steroid binding activity of transiently transfected receptors.** COS-7 cells were transiently transfected with decreasing amounts of either wild type receptor cDNA (*A*) or truncated (407C) receptor cDNA (*B*), plus increasing amounts of competitor DNA (pUC19 or pSVL537–673) and 0.2 μg of β-galactosidase cDNA (pCMVβ) as an internal control, such that the total amount of added DNA was constant at 10 μg. Aliquots of the transfected cell lysates were then analyzed for steroid binding, and the total specific binding per unit of β-galactosidase activity was plotted as described under "Materials and Methods." *C*, duplicate aliquots of transfected cell lysates from *A* and *B*, with the indicated ratios of plasmids, were separated on an SDS-polyacrylamide (12%) gel. The amount of each receptor protein in various lysates was determined by Western blotting with the anti-receptor antibody aP1 followed by enhanced chemiluminescence using anti-rabbit IgG (location of wild type and 407C receptor is indicated by ➡ and ⇨, respectively). For comparison, a sample of authentic 537–673 (= 16-kDa fragment obtained from trypsin digestion of HTC cell receptors) was included.

tertiary structure) can be obtained with the receptor sequence of 537–795 but only when some protein is fused to the amino-terminal end of the receptor fragment. Thus, the features of rat glucocorticoid receptor from 407–536 are not unique for stabilizing 537–795; many other sequences will work. However, the level of steroid binding activity and the ease of visualization by Western blotting was 4–8-fold greater with the dhfr-containing than the β-galactosidase-containing proteins. Therefore, all subsequent studies were performed with dhfr fusion proteins.

It should be noted that the same dhfr494C was also transiently expressed as a stable, steroid binding fragment from the same pSVLTm vector that failed in Figs. 1 and 2 to yield stable proteins for 537C and 537–673 (data not shown). Therefore, the inability to obtain the latter receptor fragments did not derive from some defect in the vector.

Minimum Sequence Needed for Steroid Binding Activity in Newly Synthesized Receptors—As with Z537–673, dhfr537–673 did not give any Dex binding (Fig. 4*B*; 0.83 ± 0.08 (S.D., $n = 3$) times mock transfection) even though protein of the expected size for dhfr537–673 was evident by Western blotting (data not shown). Two other hybrid receptors with progressively less carboxyl-terminal deletions (dhfr537–710 and dhfr537–766) also afforded no Dex binding (0.9–1.1 times mock transfection ($n = 2$)) although good amounts of each stable protein were produced (Figs. 4*B* and 6, *bottom panel*, and data not shown). The lack of binding with dhfr537–766 was reminiscent of results with the progesterone receptor, where the absence of binding of agonist steroids was associated with the loss of the carboxyl-terminal ≈40 amino acids (46). However, the glucocorticoid receptor carboxyl-terminal sequences are not sufficient for binding as constructs containing the complete carboxyl-terminal sequence but lacking 80 or 15 internal amino acids (dhfr537Δ616–695C or dhfr537Δ690–704C, respectively; Fig. 4*B*) yielded stable proteins that were still unable to bind steroid (0.3–1.1 times mock transfection ($n = 3$)) or be labeled by Dex-Mes (data not shown). This behavior argues that some property of the sequence 674–795 more fundamental than the presence of the carboxyl-terminal ~40 amino acids, such as proper protein folding, is required for steroid binding activity.

A series of amino-terminal deletions of the receptor sequence in dhfr537C was prepared in order to define this boundary of the steroid binding domain. Removal of 10 or 13 amino acids, to give dhfr547C and dhfr550C, did not affect the ability of the hybrid protein to be affinity-labeled by Dex-Mes and caused less than a 3-fold decrease in the affinity for Dex (Fig. 4*B*). However, the deletion of 2 more amino acids to give dhfr552C eliminated Dex binding (1.25 ± 0.64 times mock transfection (S.D., $n = 5$ for two clones)) and dramatically reduced the affinity labeling by Dex-Mes. Further deletions to give dhfr554C and dhfr556C maintained the loss of steroid binding activity (Fig. 4*B*). These results define the amino-terminal end of the steroid binding domain as either 550 or 551 of the rat receptor. Interestingly, a PCR error generated a point mutation in one clone of dhfr550C that changed Arg-732 to a glutamine. This point mutation eliminated the steroid binding activity of dhfr550C (1.0 ± 0.6-fold above mock transfection (S.D., $n = 3$)).

Analysis of Steroid Binding Domain Tertiary Structure by Trypsin Digestion—We previously proposed that the 16-kDa tryptic digest fragment of the rat glucocorticoid receptor, corresponding to amino acids 537–673, binds steroids with high

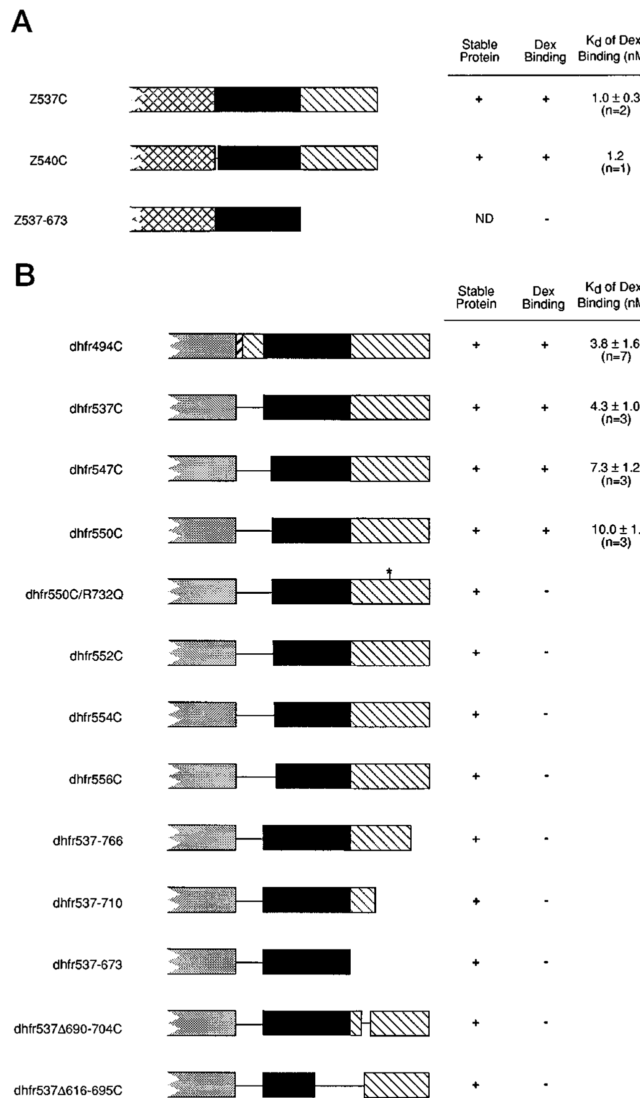

FIG. 4. **Properties of glucocorticoid receptor fusion proteins expressed in COS-7 cells.** cDNAs encoding the indicated regions of the rat glucocorticoid receptor fused to the carboxyl terminus of either β-galactosidase (= Z) (*A*) or dhfr (*B*) were transiently expressed in COS-7 cells and characterized as in Fig. 1. The convention for indentifying the receptor sequences is the same as in Fig. 1. Point mutations are listed after the *diagonal slash*; internal deletions are designated by a Δ followed by the terminal positions of the deleted sequence. The fused proteins, of which only the carboxyl terminus is shown, are as follows: ▩, β-galactosidase; ▤, dhfr. Receptor sequences are coded as in Fig. 1 with gaps in the protein sequences being indicated by a *thin, horizontal line*. Western blots of some of the chimeric receptors are shown below in Fig. 6.

affinity (31) and specificity (32) because the proper tertiary structure had already been achieved and sequences not involved in the actual binding, but which were required for folding, could now be removed. The corollary to this hypothesis is that the 16-kDa fragment should not be formed after trypsin digestion of mutant receptors that do not bind steroid, presumably reflecting an incorrect folding into non-native tertiary structures.

A convenient method for identifying the 16-kDa fragment has been by affinity labeling with Dex-Mes (31). Dex-Mes covalently labels only one amino acid (Cys-656) in the wild type receptor (47). Furthermore, the mutation of Cys-656 to glycine in the full-length receptor not only increases the affinity of [^{3}H]Dex binding (39) but also blocks the covalent labeling of receptors by Dex-Mes (32). As shown in Fig. 5, covalent labeling

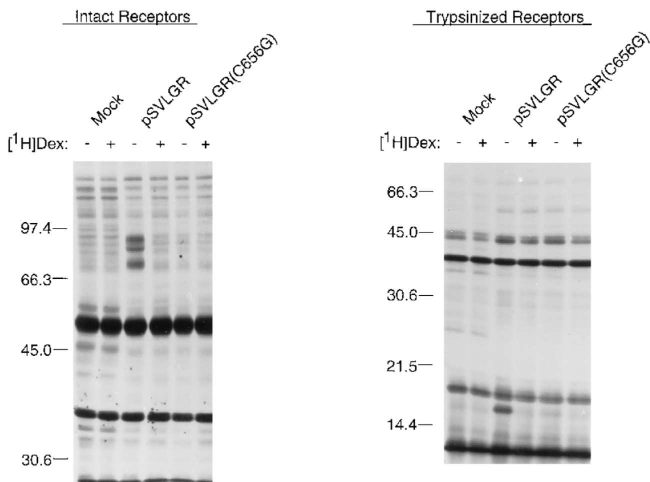

FIG. 5. **Effect of C656G mutation on the affinity labeling of 98-kDa wild type receptors and 16-kDa steroid binding core receptor fragments.** Extracts of COS-7 cells that had been transiently transfected with pSVLGR or pSVLGR(C656G) were incubated for 2.5 h at 0 °C with 5×10^{-8} M [^{3}H]Dex-Mes ± 100-fold excess of nonradioactive Dex before (*left panel*) or after (*right panel*) digestion with trypsin (20 μg for 1 h at 0 °C). Soybean trypsin inhibitor was added to stop further digestion. The labeled proteins were separated on a SDS-polyacrylamide (15%) gel and fluorographed as described under "Materials and Methods." Only the labeling of the full-length and 16-kDa receptor species was prevented by excess nonradioactive Dex. The positions of unmodified protein standards of the indicated molecular mass (kDa) were marked with Ult-Emit on the gel and detected on the fluorographs.

by [^{3}H]Dex-Mes of both the intact receptor and the 16-kDa tryptic fragment was prevented by the Cys-656 to glycine mutation. The fact that the 16-kDa fragment contains two other cysteines that are in close proximity to Cys-656 (32, 48) but were not labeled by [^{3}H]Dex-Mes in the Cys-656 → Gly mutant further argues that the tertiary structure of the 16-kDa fragment is similar to that of the intact receptor.

The tertiary structure of the steroid binding domain in the above mutant receptors was probed by looking at the ability of trypsin digestion to yield the 16-kDa steroid binding fragment. The presence of the 16-kDa fragment was ascertained by a combination of Western blotting with the antibody aP1, [^{3}H]Dex binding, and [^{3}H]Dex-Mes labeling. As expected, mutant receptors that did not bind steroid (pSVLGR1–766, pSVLGR1–673, dhfr537Δ690–704C, dhfr537–766, and dhfr537–673) did not produce a 16-kDa fragment on Western blots (data not shown). Likewise, no [^{3}H]Dex binding was observed after trypsin digestion of pSVLGR1–766 (data not shown). In contrast, trypsin digestion of the steroid binding chimeras dhfr494C and dhfr537C gave, in each case, the correct 16-kDa fragment with good [^{3}H]Dex binding and the correctly sized product on both Western blots (data not shown) and SDS gels of affinity-labeled material (Fig. 6). Thus, the formation of the 16-kDa fragment after trypsin digestion seems to be an accurate probe of receptor tertiary structure.

dhfr537C is missing the lysine at position 536 that would be cleaved by trypsin to generate the 16-kDa fragment (31). However, the dhfr537C contains two lysines in the linker (sequence = KKDAC) between dhfr and the receptor sequences that appear to substitute for Lys-536 in the generation of 16-kDa fragments. Surprisingly, trypsin digestion of two other steroid binding chimeras, dhfr547C and dhfr550C, yielded none of the 16-kDa-like fragment that was expected from trypsin cleavage at the same linker region lysines and eliminated all [^{3}H]Dex binding and [^{3}H]Dex-Mes labeling (Fig. 6 and data not shown). Thus, the ability of trypsin digestion to yield a 16-kDa-like fragment appears to be a more sensitive probe

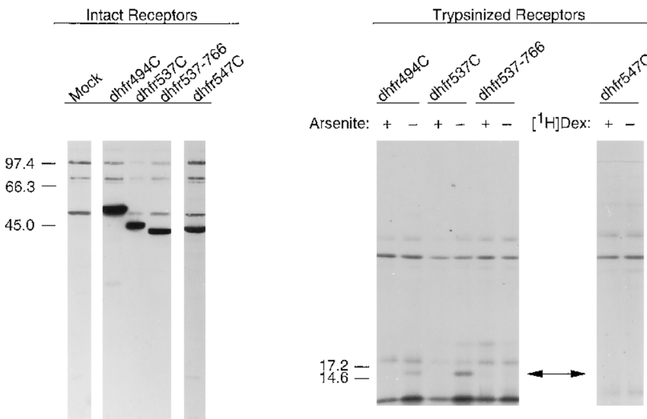

FIG. 6. **Ability of steroid binding, and nonbinding, dhfr-receptor fusion proteins to generate 16-kDa steroid binding core fragments that can be affinity-labeled by Dex-Mes.** Extracts of COS-7 cells that had been transiently transfected with the indicated plasmids were either analyzed by Western blotting or digested with trypsin (20 μg for 1 h at 0 °C) to form the 16-kDa steroid binding core and then labeled with 5×10^{-8} M [^{3}H]Dex-Mes ± 0.1 mM arsenite (40) or 100 fold excess of nonradioactive Dex to block specific labeling of receptors. Intact receptors were detected by enhanced chemiluminescence (= major bands between 40 and 50 kDa) while affinity-labeled proteins were visualized by fluorography (*double-headed arrow* marks the position of the 16-kDa fragment) as in Fig. 5.

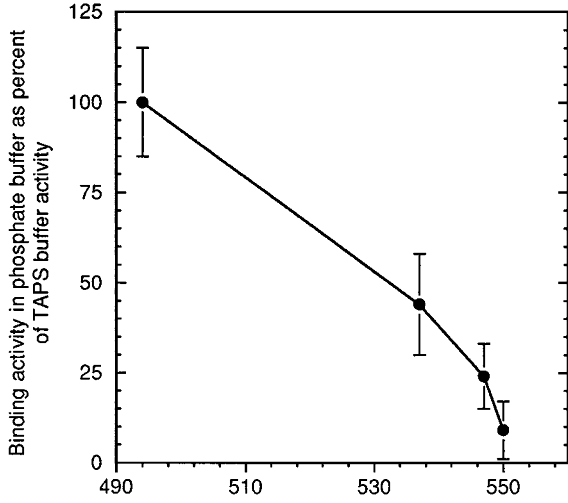

FIG. 7. **Effect of pH 7.4 PBS *versus* pH 8.8 TAPS buffer on steroid binding activity of hybrid receptors.** Extracts of COS-7 cells that had been transiently transfected with the indicated plasmids were diluted in either pH 7.4 PBS, or pH 8.8 TAPS, buffer plus 20 mM sodium molybdate incubated at room temperature for 5 min, chilled to 0° C, and then assayed in duplicate for steroid binding as described under "Materials and Methods." The binding of each hybrid construct in phosphate buffer was then expressed as percent of the binding of the same construct in TAPS buffer in order to normalize for possible differences between constructs in receptor expression. The *error bars* indicate the range of two independent experiments.

than steroid binding activity for changes in protein folding and the tertiary structure of the receptor chimeras.

Tertiary Structure of Receptor Is Stabilized by Sequences Amino-terminal to the Steroid Binding Domain—As a further indication of changes in receptor folding, we examined the stability of the hybrid receptors after brief (5-min) exposure to room temperature in different buffers. In general, the steroid binding activity of the dhfr-receptor constructs was found to be more labile in pH 7.4 phosphate-buffered saline (PBS) than in pH 8.8 TAPS buffer. Furthermore, this lability in PBS increased as more of the amino-terminal receptor sequences were removed (Fig. 7). Thus, receptor sequences outside of the steroid binding domain appear to contribute to the stability of preformed receptors in a manner that cannot be substituted by sequences that prevent the degradation of newly synthesized hybrid proteins.

DISCUSSION

Previous attempts to express the isolated steroid binding domain of the glucocorticoid receptor in a form that retained appreciable amounts of steroid binding activity have been unsuccessful. We now show that the solution to this problem requires the presence of two specific receptor domains. The first domain is the full steroid binding domain corresponding to 550–795; the second is a stabilizing sequence that lies outside of the steroid binding domain.

Our stabilizing sequence does not correspond to the highly conserved region of amino acids 587–643 (rat numbering) (22) within the steroid binding domain that has been reported to decrease proteolysis of mouse glucocorticoid receptors in intact cells (49). This segment of 587–643 was clearly insufficient to stabilize the 537–673 and 537C peptides in our system (Figs. 1–3). Instead, the stabilizing sequence of the rat receptor was found to lie between amino acids 407 and 536 (Fig. 1), which is outside our defined steroid binding domain of 550–795. This positioning was unexpected considering the ability of the functional steroid binding domain to be moved between chimeric proteins in a modular fashion (6, 7). It seems unlikely that there is any unique stabilizing sequence because the effect of the receptor amino acids 407–536 could be mimicked by nonreceptor proteins, such as β-galactosidase (Fig. 4*A*), dihydrofolate reductase (Fig. 4*B*), and glutathione *S*-transferase that contained no homologous sequence (data not shown).

The mechanism of this stabilizing sequence is not known. It could involve protein-protein contacts to block the action of an inactivating region (44), such as a PEST sequence (50) or a "degron" (51), within the sequence of 537–795 (Figs. 1 and 4). According to this scheme, the blockage of a specific inactivating region would have to involve nonspecific interactions of 407–536 to account for the equivalent stabilizing effects seen upon fusion of the unrelated proteins β-galactosidase, dihydrofolate reductase, and glutathione *S*-transferase. Alternatively, a nonspecific stabilization might reflect a weak hydrophobic interaction with the chaperones, such as hsp90, that are required for steroid binding activity (52, 53). Regions upstream of amino acid 537 are not normally thought to be associated with hsp90 (54, 55), but it should be noted that amino acids 506–514 of the receptor, which are recognized by the antibody AP64 (56), are no longer accessible to AP64 after hsp90 binding to receptors (13).

The second domain required for steroid binding is, obviously, the steroid binding domain. However, the precise limits of the steroid binding domain have never been defined (22). The cell-free translated fragment 547–795 displayed very low affinity (1/350 of full-length receptors) (29), presumably due to protein instability since the affinity of the same fragment present as a stable fusion protein with dhfr was reduced by only 2-fold (*dhfr547C* of Fig. 4*B*). The similar high affinity of dhfr550C plus the dramatic loss of steroid binding activity attending the removal of the next 2–6 amino-terminal amino acids in going from dhfr550C to dhfr556C (Fig. 4*B*) reveals the critical nature of residues 550 and/or 551 and marks these two positions as the amino-terminal boundary of the steroid binding domain. As was pointed out earlier (57), this boundary falls in the middle, as opposed to at the end, of an exon of the glucocorticoid receptor gene. The carboxyl terminus has not been as precisely defined. The lack of binding with dhfr537–710 and dhfr537–

766 indicates that it must be somewhere between 767 and 795 (Fig. 4*B*). A 30-fold decrease in affinity was observed after removing the five carboxyl-terminal residues of 407C to give 407–790 (29) while all binding and biological activity disappeared with the deletion of the terminal 14 amino acids (58). Furthermore, internal deletions (dhfr537Δ690–704C and dhfr537Δ616–695C) eliminated Dex binding (Fig. 4) while the removal of the two amino acids 780 and 781 caused a 4-fold decrease in affinity (59). Thus, deletions throughout the carboxyl-terminal half of the receptor can be detrimental to the steroid binding activity without affecting the stability of the final protein. We therefore conclude that the minimum sequence of the steroid binding domain with wild type affinity in a newly synthesized protein can be defined as extending from 550 (or 551) to 795.

The final issue is whether the entire steroid binding domain is needed for steroid binding activity or only for the folding to a conformation that can bind steroid. The fact that the affinity of the 16-kDa glucocorticoid receptor fragment of 537–673 (31, 32) appears to be ~20-fold lower than that of the full-length receptor (31) argues that proteolytically released sequences between 674 and 795 do augment, but are not required for, a significant binding affinity. However, this is to be contrasted with the absence of binding in proteins that are translated without amino acids 674–795, such as Z537–673 or dhfr537–673 (Fig. 4). Thus, the region of 674–795 seems to be required for the proper folding of the steroid binding domain but not for the majority of activity of an already folded domain. We therefore propose that the principal function of the carboxyl-terminal amino acids 674–795 of the glucocorticoid receptor is in facilitating the folding of the mature receptor protein and that the contributions to the steroid binding activity of the final, correctly folded protein are of secondary importance. Several observations support this hypothesis.

First, the folding of the steroid binding domain to give the native tertiary structure appears to be quite sensitive to minor perturbations. Removal of receptor sequences just upstream of the amino terminus of the steroid binding domain minimally affected steroid binding affinity (Fig. 4*B*) but dramatically altered the folding, as indicated by both the instability of steroid binding activity in pH 7.4 phosphate buffer (Fig. 7) and the loss of trypsin cleavage in the linker between dhfr and the receptor to generate a 16 kDa-like fragment (Fig. 6). Larger deletions could be expected to have more notable consequences on folding.

Second, recent x-ray crystallographic data on the steroid binding domain of the steroid-free human retinoid X receptor α (60) and the steroid-bound rat thyroid α_1 (61) and human retinoic acid γ (62) receptors revealed a flexible interlocking network of α helical structures that were proposed to be common for the ligand binding domains of all steroid receptors (63). This network does not require the presence of the carboxyl-terminal 18 amino acids of the glucocorticoid receptor. However, deletion of the terminal 14 amino acids is sufficient to eliminate all steroid binding activity (58). Thus, while amino acids 550–673 may now be regarded as the core of the glucocorticoid receptor steroid binding domain, the entire carboxyl-terminal sequence of 674–795 seems to be required for the folding of the core steroid binding domain into the correct tertiary structure.

Finally, the above arguments assume that steroid binding to the 16-kDa trypsin fragment does not require the noncovalent association of more carboxyl-terminal sequences. In fact, the steroid binding activity of a 17-kDa estrogen receptor fragment was found to be associated with the noncovalent attachment of the adjacent carboxyl-terminal 7 kDa of receptor (64). However, the recent alignment of steroid receptor binding domains (63) indicates that the 17- and 7-kDa estrogen fragments correspond to 538–760 of the rat glucocorticoid receptor, which is completely contained within the nonbinding dhfr537–766 of Fig. 4B. Thus, there is a requirement of extra sequences for steroid binding in the newly synthesized, but not mature, glucocorticoid receptors, just as we have seen above for receptor stability. There, a stabilizing domain amino-terminal to position 537 is essential only for *de novo* synthesized receptors, such as 537–673 (Fig. 1), while the steroid binding 16-kDa tryptic fragment, proposed to be 537–673 (31), is not associated with any upstream sequences (32). Such a dependence on initial conditions is indicative of a folding reaction that is under kinetic control (65). In this respect, the role of the 407–536 and 674–795 regions of the receptor would be analogous to that of proenzyme sequences in folding.

Therefore, two independent domains appear to be required for the production of a stable, active steroid binding domain: the steroid binding domain of 550–795 and an adjacent stabilizing domain that includes at least part of the sequence of 407–536. This requirement of multiple domains for the proper folding of a biologically active protein runs counter to the thesis that individual domains are capable of independent folding (19–21, 66) but has been seen with other proteins, such as apomyoglobin (67) and α-lytic protease (68). Thus, this behavior of glucocorticoid receptors is not unique.

In summary, mutation-induced changes in protein folding and protein stability are constant possible explanations for the loss of activity among genetically engineered proteins. These interpretations are rarely embraced, though, due to the difficulties in substantiating such hypotheses. Such changes could be either direct, due to modifications of the amino acids required for stability of the final folded structure, or indirect by preventing the formation of a necessary folding intermediate (69). We have found that these parameters cannot be ignored and sometimes are the determining factor. Specifically, sequences both within the steroid binding domain of the glucocorticoid receptor, but carboxyl-terminal to the core of the steroid binding structure, and outside of (*i.e.* amino-terminal to) the steroid binding domain are required for proper protein folding and stabilization, respectively. These phenomena nicely explain the reported lack of steroid binding activity in glucocorticoid receptor constructs containing just the steroid binding domain, or portions thereof (29, 59, 70–73), and may account for similar results with other receptors (74, 75). These results of interdomain interactions also complement the growing literature that document functional interactions between separate domains of steroid receptors (76–79).

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Note Added in Proof:—Cadepond *et al.* (73) reported that the internal deletion of amino acids 489-532 of the human glucocorticoid receptor (=508-550 of the rat receptor) eliminated steroid binding. The combination of these data with the present results argue that Leu-550, as opposed to Val-551, of the rat receptor is the amino-terminal end of the steroid binding domain.

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