Structural Characterization of the Trypsinized Estrogen Receptor†

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ABSTRACT: Structural differences between the unoccupied and ligand-occupied rat uterine estrogen receptors (ERs) were investigated using partial proteolysis followed by immunoblotting, affinity labeling, and gel filtration chromatography. Trypsin digestion of the unoccupied ER at 4 °C resulted in retention of 70–80% of high-affinity [^{3}H]estradiol binding. Only two fragments of the rat ER were detected after prolonged trypsin treatment of the unoccupied ER followed by affinity labeling with [^{3}H]tamoxifen aziridine. One fragment represents the intact steroid binding domain (28 kDa), and the other fragment is about 10 kDa. The small 10-kDa fragment of the ER detected by denaturing gel electrophoresis is shown to be held in a large oligomeric complex in solution using gel filtration chromatography. This oligomeric complex probably represents the steroid binding domain, which has its tertiary structure maintained predominantly by noncovalent interactions between the trypsin-generated fragments. The estrogen, anti-estrogen, and unoccupied trypsinized ERs all result in similar patterns of fragments after separation by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and detection by immunoblotting. Although no new trypsin cleavage sites were exposed, the sensitivity of the available trypsin sites was altered by heating the ER and, to a lesser extent, by hormone treatment. Gel filtration chromatography of the trypsinized estradiol- and 4-hydroxytamoxifen-occupied ERs demonstrates similar, diffuse peaks centered at about the correct size for the intact steroid binding domain (28 kDa), whereas the trypsinized unoccupied ER results in a sharp, discrete peak centered at about 80 kDa. We conclude that the unoccupied steroid binding domain has a different conformation than either the estradiol- or the 4-hydroxytamoxifen-occupied steroid binding domains.

Early studies using trypsinization of the occupied estrogen receptor (ER)[1] revealed that it was composed of distinct functional domains (Sherman, 1984), which was confirmed by studies of the ER cDNA sequence (Kumar et al., 1987). Mutational analysis of the steroid binding domain revealed that the entire domain was necessary for hormone binding (Kumar et al., 1986; Lees et al., 1989; Tora et al., 1989). Even small deletions or point mutations could decrease the ability of the steroid binding domain to bind hormone. The biochemical properties responsible for maintaining a structurally and functionally intact steroid binding domain are unknown.

Both the unoccupied and anti-estrogen-occupied ER complexes must differ from the estrogen-occupied ER because the latter activates transcription, whereas the others do not. We showed in a previous study (Fritsch et al., 1992) that upon binding 17β-estradiol (E_2) or the anti-estrogen 4-hydroxytamoxifen (4-OHT), the steroid binding domain undergoes a conformational change characterized by a decrease in surface hydrophobicity. The steroid binding domain also possesses a transcriptional activation function that becomes active upon binding estrogen but not anti-estrogen (Lees et al., 1989; Webster et al., 1988). Although several studies have shown similarities and differences in the physicochemical properties of the estrogen- and anti-estrogen-occupied receptor complexes (Nelson et al., 1988; Jordan & Murphy, 1990), almost no studies have compared the unoccupied receptor directly to the anti-estrogen-occupied and estrogen-occupied receptors. Few studies have examined the trypsin-generated, unoccupied steroid binding domain (Pavlik & Katzenellenbogen, 1980). Structurally, the unoccupied and anti-estrogen-occupied steroid binding domains are not identical, as previously demonstrated using affinity partitioning (Fritsch et al., 1992). Functionally, the transcriptional inactivity of the unoccupied and anti-estrogen-occupied ERs may occur as a result of different mechanisms.

Not only is the nature of the conformational differences between the estrogen-occupied, anti-estrogen-occupied, and unoccupied steroid binding domains unknown, but the functional significances of these conformational differences are unclear. Receptor interaction with other proteins is probably important for building the final functional transcription complex that leads to increased transcription from estrogen-responsive genes. The ER must make contact with the basal transcription machinery either directly or indirectly through other transcription factors (Carson-Jurica et al., 1990; Murdoch & Gorski, 1991). The unoccupied and anti-estrogen-bound steroid binding domains probably have conformations that do not allow for the proper receptor–protein interactions necessary to initiate transcription.

In this study, we demonstrate that when the steroid binding domains of the unoccupied, estrogen-, and anti-estrogen-occupied ERs are cleaved by trypsin, they remain structurally and functionally intact. We believe that the tertiary structure is maintained by noncovalent interactions between the various

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[1] Abbreviations: ER, estrogen receptor; E_2, 17β-estradiol; 4-OHT, 4-hydroxytamoxifen; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethanesulfonyl fluoride; TAMZ, tamoxifen aziridine; HAP, hydroxylapatite; BCIP, 5-bromo-4-chloro-3-indolyl 1-phosphate; NBT, nitro blue tetrazolium; DES, diethylstilbestrol; DTT, dithiothreitol; hsp, heat shock protein; a.a., amino acid(s).

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secondary structures. There are minimal differences in the trypsin digestion patterns between the unoccupied, anti-estrogen-occupied, or estrogen-occupied steroid binding domains on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). However, there is a difference in the oligomeric state between the unoccupied and estrogen- or anti-estrogen-occupied, trypsin-generated steroid binding domains in solution. We propose that the mechanisms by which the unoccupied and anti-estrogen-occupied steroid binding domains result in transcriptional inactivity are different at the molecular level.

MATERIALS AND METHODS

Materials. Trypsin, aprotinin, phenylmethanesulfonyl fluoride (PMSF), Blue Dextran (M_r 2 000 000), and Sephacryl S200 were purchased from Sigma (St. Louis, MO). 17β-[2,4,6,7-^{3}H]Estradiol (90–110 Ci/mmol), (*Z*)-4-[*N-methyl*-^{3}H]hydroxytamoxifen (80–100 Ci/mmol), and [*ring*-^{3}H]-tamoxifen aziridine (TAMZ) (10–30 Ci/mmol) were obtained from New England Nuclear (Boston, MA) and Amersham (Arlington Heights, IL). Immature Sprague–Dawley female rats (19 days of age) were from Harlan Sprague Dawley (Madison, WI). Bio-Gel HT HAP (hydroxylapatite) was from Bio-Rad Laboratories (Richmond, CA). Ready Safe scintillation cocktail was obtained from Beckman (Fullerton, CA). BCIP (5-bromo-4-chloro-3-indolyl 1-phosphate) and NBT (nitro blue tetrazolium) were from Promega (Madison, WI). ENHANCE was from New England Nuclear. All other chemicals were reagent grade. All procedures were performed at 4 °C unless otherwise indicated.

Preparation of Rat Uterine Cytosolic ER. Preparation of rat uterine cytosolic ER has been previously described (Murdoch et al., 1990; Hansen & Gorski, 1985). In brief, uteri from 19-day-old rats were homogenized in 10 mM Tris-HCl (pH 7.5 at 25 °C), 1.5 mM EDTA (ethylenediaminetetraacetate), and 10 mM mercaptoethanol (TEM) buffer at 3 uteri/mL and centrifuged at 436000*g* for 10 min to obtain cytosol containing unoccupied ER. The unoccupied ER was incubated with 5–10 nM ^{3}H-E_2 or ^{3}H-4-OHT for 1.5 h at 4 °C. Heated forms were obtained by placing the unoccupied or ligand-occupied (45 min at 4 °C) ERs at 30 °C for 45 min. The resulting ER forms were unoccupied heated, E_2-occupied heated, and 4-OHT-occupied heated. Parallel incubation of samples containing 200-fold molar excess of unlabeled DES was used to determine nonspecific binding for all ER forms.

Trypsin Generation of the Steroid Binding Domain of the Cytosolic ER. Stock trypsin solution (0.5 mg/mL) was made 3–5 h before use by dissolving solid trypsin in TEM at 4 °C. The "freshness" of the trypsin solution affected the percent recovery of the ER. If the trypsin stock solution (0.5 mg/mL) was made within 15 min before adding it to the cytosol, significantly more of the ^{3}H-E_2 binding activity was lost after 60-min incubation at 4 °C (data not shown). A mixed stock solution of aprotinin (0.5 mg/mL) and PMSF (1.5 mM) was prepared in TEM at 4 °C. For each ER form, 1 μL of trypsin stock was mixed with every 100 μL of sample for 1 h at 4 °C, with the final trypsin concentration at 5 μg/mL unless otherwise noted. One microliter of the aprotinin/PMSF mixture was added to every 100 μL of sample to inhibit trypsin activity, with the final concentrations being 2.5 μg/mL aprotinin and 15 μM PMSF. This ratio of trypsin to protease inhibitor mix was demonstrated to completely inhibit trypsin activity (data not shown).

Hydroxylapatite Assay. The HAP assay was performed as previously described (Murdoch et al., 1990; Hansen & Gorski, 1985), except that after washing with buffer, the hydroxylapatite (HAP) pellets were extracted with 0.75 mL of ethanol at room temperature. Aliquots of 0.5 mL were counted in 3.5 mL of Ready Safe scintillation cocktail at 36–40% efficiency.

Modified HAP Assay. A modified HAP assay was used to determine the elution profile shown in Figure 7A using ^{3}H-TAMZ. Ethanol will not elute the covalently bound ^{3}H-TAMZ from the ER in the final step of the conventional HAP assay. To every third fraction from the S200 column was added 250 μL of 70% HAP plus 2.5 mL of TEM for 30 min with mixing at 4 °C. The HAP was pelleted and washed once with 2.4 mL of T buffer (50 mM Tris-HCl, pH 7.5). The final HAP pellet was resuspended in 250 μL of TEM, 300 μL of the HAP/TEM slurry was placed directly into a scintillation vial, and 4 mL of scintillation cocktail was added. The amount of hydroxylapatite present in the scintillation vial had no significant effect on the efficiency of tritium counting (data not shown).

Immunoblotting. All samples were diluted 1:1 with 2× sample buffer (15% glycerol, 62.5 mM Tris-HCl, pH 6.8, 0.7 M mercaptoethanol, 3% SDS, and 0.05% bromophenol blue), boiled for 5 min, and stored at −20 °C prior to separation by SDS–PAGE. The separated proteins were transferred to nitrocellulose, and the ER bands were identified using affinity-purified polyclonal rabbit antibody ER715 (1:1000) raised against a synthetic peptide with a sequence derived from the D region of the rat ER (Furlow et al., 1990). The second antibody was a goat anti-rabbit IgG (1:5000) linked to alkaline phosphatase, and the substrate 5-bromo-4-chloro-3-indolyl 1-phosphate/nitro blue tetrazolium (BCIP/NBT) was added for color development. The molecular mass standards were from Bio-Rad and included rabbit muscle phosphorylase *b* (97 kDa), bovine serum albumin (66 kDa), hen egg white ovalbumin (43 kDa), bovine carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21 kDa), and hen egg white lysozyme (14 kDa).

Affinity Labeling the ER with ^{3}H-TAMZ. ER samples were covalently labeled with the anti-estrogen ^{3}H-TAMZ (Katzenellenbogen et al., 1983) as described in the text, and the proteins were then separated by SDS–PAGE. The gel was fixed using 10% (v/v) glacial acetic acid, 50% methanol, and 40% ddH_2O for 1 h at 25 °C, incubated in the fluor ENHANCE for 1 h at 25 °C, and incubated with cold ddH_2O for 25–30 min. The gel was placed on 3 MM Whatman filter paper, covered with plastic wrap, and dried for 2.5 h under low heat (60 °C). The dried gel was exposed to XAR-5 film (Kodak, Rochester, NY) for 7–14 days at −70 °C.

Saturation Binding Analysis. Aliquots of rat uterine cytosolic ER were diluted 57.5-fold (40 μL of cytosol in a final volume of 2.3 mL) into TEM buffer containing 0.03–24 nM ^{3}H-E_2 ±200-fold molar excess of unlabeled diethylstilbestrol (DES) at 4 °C for 1.5 h. Each sample had 500 μL of 70% HAP added for 30 min at 4 °C with several mixings. The HAP was pelleted, and an aliquot (150 μL) of the supernatant was removed and placed directly into a scintillation vial with 4 mL of scintillation fluid to directly determine the concentration of free ^{3}H-E_2. The HAP pellets were washed as usual, the amount of specific ^{3}H-E_2 bound was determined, and the equilibrium dissociation constant (K_d) was estimated using the LIGAND computer program (Biosoft, Milltown, NJ) (Munson & Rodbard, 1980).

Sephacryl S200 Gel Filtration Chromatography. Gel filtration was performed on a 1.2 × 47.5 cm column of Sephacryl S200 equilibrated in 10% glycerol, 50 mM KCl, 10

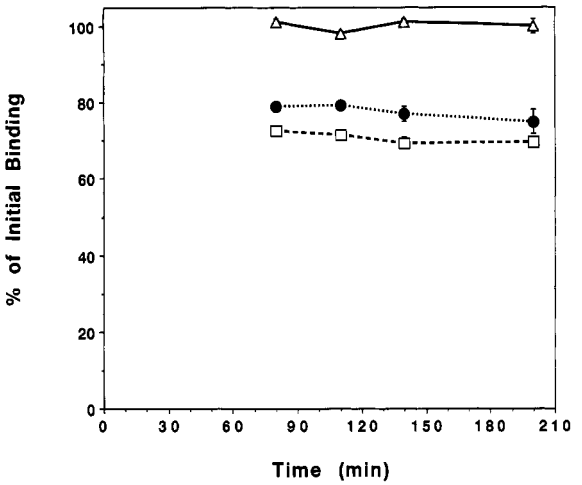

FIGURE 1: Time course of the percent of initial binding of ^{3}H-E_2 following typsin treatment of the unoccupied ER. Unoccupied ER was incubated for the indicated times at 4 °C with 0 (△), 5 (●), or 15 (□) μg/mL trypsin followed by the addition of aprotinin/PMSF to inhibit trypsin activity. The controls were treated with trypsin (5 μg/mL) that had been premixed with aprotinin and PMSF for 3 h at 4 °C, which resulted in no loss of the full-length ER (66 kDa) by immunoblotting (data not shown). Samples were incubated with 10 nM ^{3}H-E_2 for 1.5 h at 4 °C followed by HAP assay. Parallel samples were incubated with 10 nM ^{3}H-E_2 and 200-fold molar excess DES to determine nonspecific binding. The initial binding values were determined by the specific ^{3}H-E_2 binding activity in the absence of trypsin, or in the presence of trypsin (5 or 15 μg/mL) premixed with aprotinin and PMSF. The initial binding value was 1960 ± 10 fmol of ER/mL for the controls. The values shown were the means of duplicate samples ± the standard error of the means.

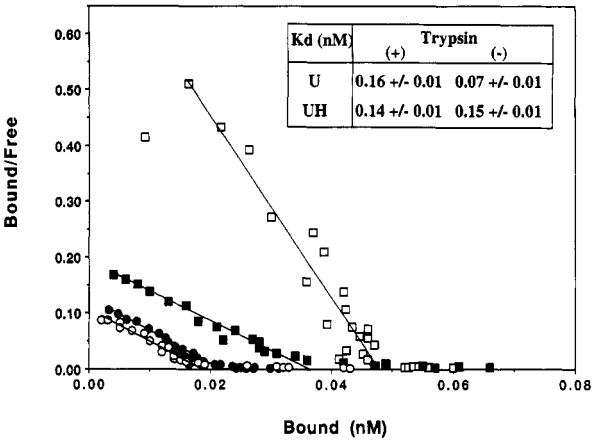

FIGURE 2: Saturation binding analysis of the nontrypsinized–unoccupied (□), the nontrypsinized–unoccupied–heated (○), the trypsinized–unoccupied (■), and the trypsinized–unoccupied–heated (●) ERs. The various ER forms were prepared as described under Materials and Methods. The trypsin treatment (5 μg/mL) was for 1 h at 4 °C followed by the addition of aprotinin/PMSF. The samples were then diluted 50–60-fold into buffer with a final concentration of ^{3}H-E_2 ranging from 0 to 25 nM ±200-fold molar excess of DES for 1.5 h at 4 °C, and the ^{3}H-E_2 binding activity was determined by HAP assay. The free ^{3}H-E_2 concentrations were measured directly and the data plotted using the method of Scatchard (1949). The table insert summarizes the equilibrium dissociation binding constants for each ER form.

mM Tris-HCl (pH 7.5 at 25 °C), 1.5 mM EDTA, and 1 mM dithiothreitol (DTT) at 4 °C. Samples were applied in a volume of 260 μL, containing Blue Dextran (M_r 2 000 000), to mark the void volume (V_0). The column was eluted at a flow rate of 0.15 mL/min, and 0.35-mL fractions were collected. The column was calibrated using bovine IgG (M_r 152 000; Stokes radius 5.2 nm) in the absence of DTT, dansylated albumin (M_r 66 000; Stokes radius 3.6 nm), ovalbumin (M_r 43 000; Stokes radius 3.05 nm), carbonic anhydrase (M_r 31 000; Stokes radius 2.4 nm), and cytochrome *c* (M_r 12 000; Stokes radius 1.65 nm). Bovine IgG, ovalbumin, carbonic anhydrase, and cytochrome *c* were detected by measuring the absorbance at 280 nm, whereas dansylated albumin was detected by fluorescence under a UV lamp. Ten milliliters of the void volume was collected in a graduated tube and discarded, and the fraction collector was started. For the ER preparation prelabeled with 5 nM ^{3}H-E_2 or 1 nM ^{3}H-4-OHT ±200-fold molar excess of DES, the amount of ^{3}H-E_2 or ^{3}H-4-OHT binding was determined by hydroxylapatite (HAP) assay for each fraction. For the unoccupied ER sample, 0.3 mL of 10 nM ^{3}H-E_2 was added to each odd-numbered fraction, and 0.3 mL of 10 nM ^{3}H-E_2 plus a 200-fold molar excess of unlabeled DES was added to each even-numbered fraction to determine the nonspecific binding, and the samples were incubated at 4 °C for 1.5 h. The amount of ^{3}H-E_2 binding was determined using the HAP assay. The amount of specific ^{3}H-E_2 binding was calculated by subtracting the binding activity in the even fractions from the binding activity in the odd fractions.

RESULTS

Estradiol Binding Activity of the Unoccupied ER Is Stable after Trypsin Treatment. Figure 1 shows a time course of ^{3}H-E_2 binding after treatment of the unoccupied, nonheated rat uterine ER at 4 °C with 0, 5, or 15 μg/mL final trypsin concentration. Even with trypsin concentrations of 15 μg/mL for over 3 h, more than 70% of the ^{3}H-E_2 binding activity was recovered. In order to determine whether the 70–80% of ^{3}H-E_2 binding activity recovered following trypsin treatment still represented a single, high-affinity binding site, we performed saturation binding analysis using ^{3}H-E_2 with the unoccupied, nonheated ER ±trypsin and with the unoccupied, heated ER ±trypsin. Heating cytosol results in dissociation of heat shock protein 90 (hsp90) from the unoccupied or occupied ERs as determined by sucrose density gradient analysis (data not shown). Figure 2 shows the saturation binding data plotted by the method of Scatchard (1949). The equilibrium dissociation binding constant (K_d) for ^{3}H-E_2 for each ER form was determined, and the results are summarized in the insert of Figure 2. The nontrypsinized, unoccupied–nonheated ER had a slightly higher affinity for ^{3}H-E_2 than the other three ER forms. The maximum binding for the nontrypsinized, unoccupied–heated ER was about 50% of the value for the nontrypsinized, unoccupied–nonheated ER. The trypsinized ER forms retained 70–80% of the E_2 binding activity of the nontrypsinized ER forms, which is consistent with Figure 1. Thus, neither heating nor trypsinizing the unoccupied ER greatly altered the ability of the steroid binding domain to bind E_2 with high affinity, as demonstrated by only a relatively small decrease in the binding affinity for ^{3}H-E_2 of about 2–3-fold.

Size of the Fragments Generated by Trypsinization of the Unoccupied ER. Affinity labeling of the steroid binding domain was used to examine the products of trypsin treatment of the unoccupied ER (Figure 3). The unoccupied ER was subjected to trypsin treatment for the indicated times, the anti-estrogenic affinity label ^{3}H-TAMZ ±200-fold molar excess DES was added, and the labeled fragments were separated by SDS–PAGE. When the cytosol was treated with a premix of trypsin and protease inhibitors to inactivate the trypsin, the full-length ER at approximately 66 kDa was the predominant band, with small amounts of 50- and 37-kDa fragments observed, as previously reported (Katzenellenbogen

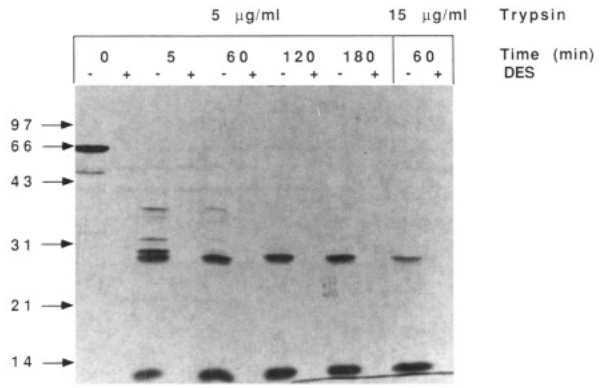

FIGURE 3: Autoradiograph of a time course of trypsin treatment of the unoccupied ER. The unoccupied rat uterine cytosolic ER was treated with trypsin (5 or 15 μg/mL) for the indicated times at 4 °C. Trypsin digestion was stopped as in Figure 1. The samples were then incubated with 25 nM ^{3}H-TAMZ without (–) DES or with (+) 200-fold molar excess DES. An equal volume of 2× sample buffer was added, and the samples were boiled and stored at –20 °C. The proteins in the samples were separated by SDS–PAGE (12% acrylamide), and the gel was fixed, treated with ENHANCE, dried, and exposed to film for 35 days to produce the autoradiograph shown. The migrated positions of the molecular mass markers (in kDa) are shown to the left.

et al., 1987). There was no endogenous protease activity detected even when the unoccupied and occupied cytosols were heated to 30 °C for 1 h (data not shown). Over the course of 180 min at 5 μg/mL or 60 min at 15 μg/mL trypsin at 4 °C, there were only two major fragments that bound ^{3}H-TAMZ. No nonspecific binding of ^{3}H-TAMZ was observed for any of the various treatments. One fragment was 28 kDa in size, which is approximately the expected size for the intact steroid binding domain, and the other was about 10 kDa in size. The 10-kDa fragment is not on the linear part of the gel with respect to molecular mass and probably represents the 6–8-kDa fragment previously observed after trypsin treatment of the ^{3}H-TAMZ-occupied ER using gradient gel electrophoresis (Katzenellenbogen et al., 1987; Elliston & Katzenellenbogen, 1988); however, we shall refer to it as the 10-kDa fragment in this paper. There are 25 potential trypsin sites (lysine and arginine residues) in the steroid binding domain of the rat ER between amino acids (a.a.) 307 and 557 (Koike et al., 1987). The absence of additional proteolysis of the 28- and 10-kDa fragments, despite these additional potential cleavage sites, demonstrates that the steroid binding domain is relatively resistant to trypsin treatment. ^{3}H-TAMZ binding occurs to some proteins that do not enter the gel. This binding is partially competed by DES, but not completely and not on all gels (data not shown). There is no difference in the intensity of these bands at the top of the gels induced by trypsin, ligand, or heating, and immunoblots do not detect these bands. Therefore, these bands probably do not represent ER protein.

Trypsin Generates Stepwise Cleavage of the ER. Figure 4A shows the pattern of receptor fragments obtained at different times after limited trypsin digestion of the ^{3}H-TAMZ-occupied ER at 4 °C. Receptor fragments of approximately 37, 35, 31, 29, 28, and 10 kDa were generated by trypsin treatment from the 66-kDa ER and from the small amount of 50-kDa ERs, which were present in the control, untreated samples. At the longest time point and highest concentration of trypsin, all the ER is in the 28- and 10-kDa fragments. To determine which part of the ER these various fragments represented, the remainder of the samples used for Figure 4A were separated on a different gel and immunoblotted with an affinity-purified antibody directed against a synthetic

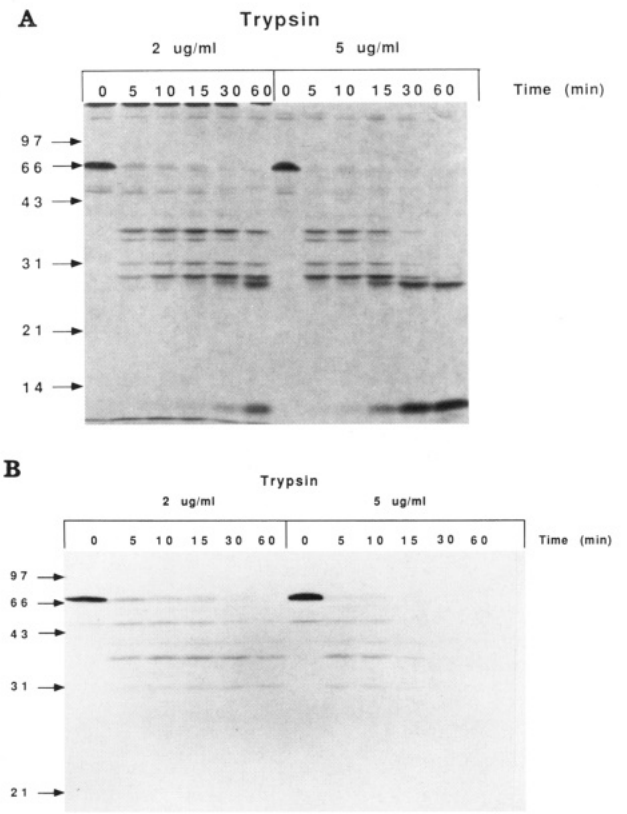

FIGURE 4: Autoradiograph and immunoblot of a time course of trypsin treatment of the ^{3}H-TAMZ-occupied ER. (A) The ER was occupied with 25 nM ^{3}H-TAMZ for 1.5 h at 4 °C, followed by treatment with trypsin at 2 or 5 μg/mL for the indicated times, and trypsin activity was stopped as described previously. The proteins in half of the sample were separated by SDS–PAGE (13% acrylamide), and the gel was processed as in Figure 3. The autoradiograph is a 10-day exposure. (B) The proteins in the remaining half of the sample were separated on a different SDS–PAGE (12% acrylamide), transferred to nitrocellulose, and immunoblotted using affinity-purified antibody ER715 (Furlow et al., 1990). The migrated positions of the molecular mass markers (in kDa) are shown to the left.

peptide derived from the D region (a.a. 270–284) of the rat ER (Furlow et al., 1990). As seen in Figure 4B, a series of fragments was produced: 66, 50, 37, and 31 kDa. The fragments at 35, 29, 28, and 10 kDa were not observed on the immunoblot and thus must have lost the antigenic site for the antibody in the D region of the ER. Presumably, the 35-kDa fragment has retained the F region of the ER, whereas the smaller fragment at 31 kDa has lost the F region (Figure 5).

These data, using ligand and antibody binding, allow partial mapping of each trypsin-generated fragment to the full-length ER (Figure 5). Since ^{3}H-TAMZ has been shown to covalently bind to a cysteine residue, Cys-530, of the human ER (Harlow et al., 1989) (and presumably a.a. Cys-535 for the rat ER), and the polyclonal antiserum was raised against a 15 a.a. sequence in the D region of the rat ER (Leu-270 to Cys-284), both of these amino acid sequences must be present to detect a fragment on both blots. The fragments at 37, 35, 31, 29, and 28 kDa probably represent at least an intact steroid binding domain because they are clearly observed using the ^{3}H-TAMZ affinity label. The absence of the 35-, 29-, 28-, and 10-kDa fragments on the immunoblot suggests that the antigenic site for ER715 (the D domain) is missing but a.a. 535 must be present. The entire steroid binding domain and the F region together comprise 294 a.a. (a.a. 307–600) for the rat ER, or about 33.2 kDa. Although some or all of the F region may be cleaved in some of these fragments, the pattern of fragments

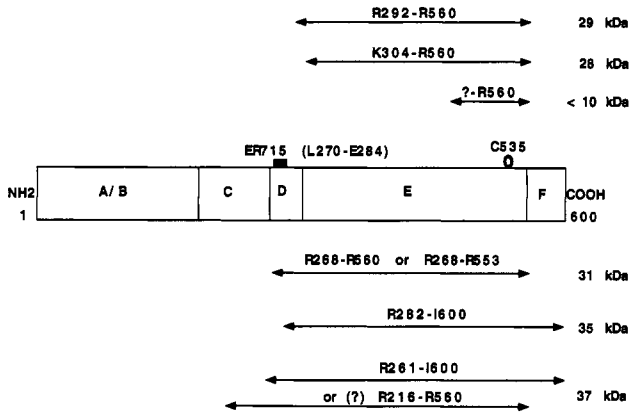

FIGURE 5: ER structure with mapping of potential trypsin sites. The various functional domains are designated A through F. The potential site of covalent attachment of ^{3}H-TAMZ is indicated by an open circle, and the antigenic site for antibody ER715 is indicated by the solid bar. The predicted trypsin-generated fragments are shown above and below the ER with the sizes determined by SDS–PAGE.

suggests the predominant cleavage of trypsin with time occurs from the amino terminus toward the carboxyl terminus, leaving the steroid binding domain relatively intact and resistant to proteolysis. The predicted sites for trypsin action on the ER are shown in Figure 5.

The effects of ligand and heat on the pattern of fragments produced by immunoblotting were investigated. Time courses and trypsin dose–responses identical to those in the immunoblot shown in Figure 4B were performed on the unoccupied, unoccupied–heated, E_2-occupied, and E_2-occupied–heated ER forms from rat uterine cytosol (data not shown). Only fragments of the sizes reported above were observed for all four ER forms, although the intensities of the observed bands were affected by ligand and heat. Thus, the ER can only be digested at certain trypsin sites, but these sites become more or less available to trypsin, depending on preheating the sample and whether ligand is present. The relative sensitivity to trypsin treatment was as follows: unoccupied–heated ER > E_2-occupied–heated ER > E_2-occupied ER ≥ unoccupied ER = ^{3}H-TAMZ occupied. Heating made the trypsin sites localized within the A/B/C/D regions of the ER much more available, which is consistent with our previous observation that the amino terminus and/or the DNA binding domain of the ER may be affected by heating in addition to the loss of hsp90 from the steroid binding domain (Fritsch et al., 1992). Although ligands (agonists and antagonists) have been shown to stabilize the binding ability of the ER following trypsinization at 30 °C (Pavlik & Katzenellenbogen, 1980), our data suggest that ligand had only minor effects on the trypsin sensitivity at 4 °C of the sites in the A/B/C/D domains, which is also consistent with the observation that ligand binding has little effect on the DNA binding domain's ability to interact with DNA (Murdoch et al., 1990).

Gel Filtration Chromatography of Various Forms of the ER. To determine the size of the trypsinized ER in solution under varying conditions of ligand and heat, the elution profiles from a Sephacryl S200 gel filtration column were investigated. Figure 6A–E shows the various forms of the ER either without trypsin treatment or following trypsin treatment. The most interesting observation from Figure 6 is that following trypsin treatment, both the unoccupied (Figure 6A) and the unoccupied–heated (Figure 6B) ERs eluted as single sharp peaks at about 80 kDa relative to the molecular mass standards whereas after trypsin treatment, both the E_2-occupied (Figure 6C) and E_2-occupied–heated (Figure 6D) ERs eluted as broad peaks between 12 and 70 kDa. The 4-OHT-occupied (Figure 6E) ER after trypsin treatment eluted almost identically to the trypsin-treated, E_2-occupied ER. One possible explanation for this difference between the unoccupied ER and the E_2/4-OHT-occupied ERs could be their relative sensitivities to trypsin; however, our previous data show that both the unoccupied and the anti-estrogen-occupied steroid binding domains exist as the same two fragments of 28 and 10 kDa after 1 h of trypsin treatment as determined by denaturing electrophoresis. These same two fragments also have been reported for an affinity labeling estrogen bound to the trypsinized ER (Elliston & Katzenellenbogen, 1988). Therefore, we conclude that there is a ligand-induced difference in the ability of the steroid binding domain to interact with other proteins or protein fragments in the cytosol. This suggests a difference in the surface conformation between the unoccupied steroid binding domain and the E_2- or 4-OHT-occupied steroid binding domain. Alternatively, the difference in the elution patterns from the S200 column could be due to a difference in the shape of the trypsin-generated, unoccupied steroid binding domain and the E_2/4-OHT-occupied steroid binding domains (Sherman, 1984).

The 10-kDa Fragment of the ER Is Not Free in Solution. The ability of the 10-kDa fragment of the ER to bind ^{3}H-TAMZ was surprising (Figure 3). One possible explanation is that the 10-kDa fragment of the steroid binding domain represents the core sequence of amino acids necessary to bind hormone. Our initial studies failed to reveal any free 10-kDa fragment that could bind ^{3}H-E_2 for the unoccupied or unoccupied–heated ERs, as demonstrated by the lack of a peak of ^{3}H-E_2 binding activity at 10 kDa on the gel filtration elution profiles in Figure 6A,B. This does not support the above model. The alternative model suggests that the 10-kDa fragment remains in its native state, held in place by the other fragments of the steroid binding domain via noncovalent interactions. Despite the primary amino acid sequence being disrupted by trypsin treatment, the secondary and tertiary structures of the steroid binding domain might remain intact, allowing for the retention of high-affinity ^{3}H-E_2 binding (Figure 2) and for covalent ^{3}H-TAMZ binding at the appropriate amino acid residue.

To distinguish between these two models, we separated the trypsin-generated ^{3}H-TAMZ-labeled ER fragments by gel filtration followed by analysis of the fragment sizes by SDS–PAGE to identify any free 10-kDa fragment in solution. An aliquot of rat uterine cytosolic ER was incubated with ^{3}H-TAMZ and heated to dissociate hsp90s. The heated ^{3}H-TAMZ-occupied ER was then treated with trypsin to generate a mixture of the two fragments of 28 and 10 kDa. This sample was then fractionated by gel filtration chromatography using Sephacryl S200. The elution profile of ^{3}H-TAMZ is shown in Figure 7A. The initial peak of ^{3}H-TAMZ in the void volume represented nonreceptor-bound ^{3}H-TAMZ, whereas the ^{3}H-TAMZ bound to ER eluted with a peak at about 40 kDa trailing to 12 kDa.

Groups of four fractions from Figure 7A were then pooled and concentrated, and separated by SDS–PAGE. We demonstrated that none of the 10-kDa fragment was lost while concentrating the sample (data not shown). Figure 7B shows an autoradiograph of the gel with controls in the first four lanes. The first two lanes show the nontrypsinized cytosol incubated with ^{3}H-TAMZ ±200-fold molar excess DES as competitor. Heating rat uterine cytosol in the presence of the affinity label ^{3}H-TAMZ greatly increased the number of nonspecific proteins that bound ^{3}H-TAMZ. Of the labeled

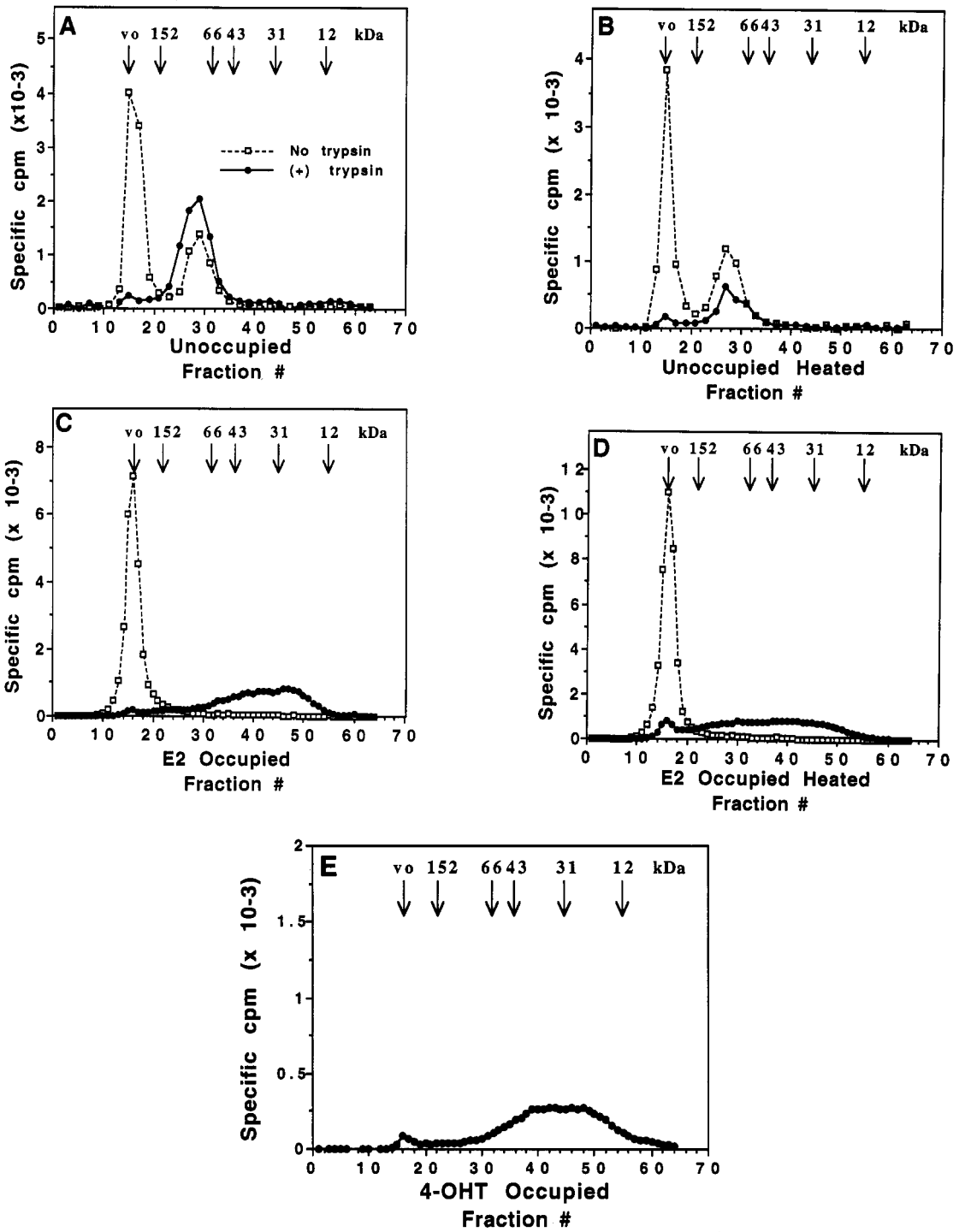

FIGURE 6: Gel filtration chromatography of the five ER forms ± trypsin treatment. The five ER forms were prepared as described under Materials and Methods: unoccupied (A), unoccupied–heated (B), E_2-occupied (C), E_2-occupied–heated (D), 4-OHT-occupied (E), and then treated with trypsin (5 μg/mL) for 1 h at 4 °C (solid circles and lines) or with no trypsin (open squares and dashed lines) before the proteins were separated on a Sephacryl S200 gel filtration column. After the fractions were collected, the specific counts per minute were determined as described under Materials and Methods. The peak fractions in which the protein standards were used to calibrate the column eluted are indicated by arrows at the top with the molecular mass (in kDa) for each protein shown above the arrows. The percent recoveries from the S200 column for each ER form ± trypsin were the following: unoccupied ER, 56% [1]/57% [1]; unoccupied–heated ER, 36% [2]/108% [5]; E_2-occupied ER, 59% [2]/82% [1]; E_2-occupied–heated ER, 72% [2]/108% [1]; 4-OHT-occupied ER, 63% [1]/–, where the number of determinations for each ER form is shown in brackets.

bands in lane 1, only the ER bands at 66, 50, and 37 kDa are competed by DES in lane 2 and are therefore identified as ER fragments. Samples of ^{3}H-TAMZ-occupied–heated cytosols were trypsinized (lanes 3 and 4), and only two major bands were competed by DES, one at 28 kDa and one at about 10 kDa. The 10-kDa band was diffuse in this preparation.

In Figure 7B, lanes a through h represent the pooled and concentrated fractions from the S200 column. The majority of the 28- and 10-kDa ER fragments elute from the column in pooled fractions d and e, which represent a molecular mass range from 66 to 31 kDa as determined from the globular protein standards separated using the S200 gel filtration column. Small amounts of the 10- and 28-kDa ER fragments were observed on SDS–PAGE in pooled fraction f, which is centered at about 25 kDa on the column. The relative ratio of the amount of 28- to 10-kDa fragments remains constant

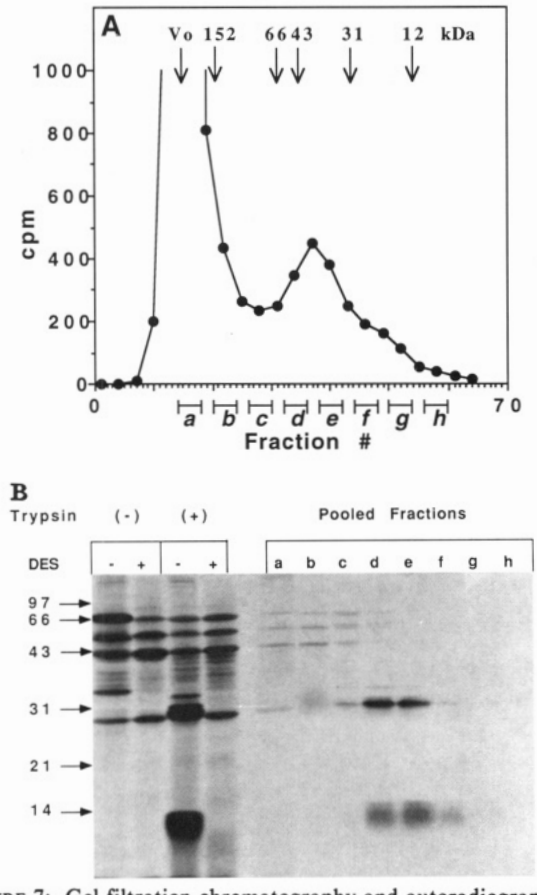

FIGURE 7: Gel filtration chromatography and autoradiography of the trypsin-treated ^{3}H-TAMZ-occupied–heated ER. (A) ER was occupied with 25 nM ^{3}H-TAMZ for 45 min at 4 °C, followed by 45 min at 30 °C, and then placed back on ice. The sample was treated with trypsin (5 μg/mL) for 1 h at 4 °C, followed by the addition of aprotinin/PMSF. The proteins were separated on a Sephacryl S200 gel filtration column, fractions were collected, and the counts per minute in every third fraction were determined by a modified HAP assay (see Materials and Methods). The peak fractions in which the external molecular mass standards eluted are shown at the top in kilodaltons. The remaining fractions were pooled as groups of four (labeled a through h). These grouped samples were concentrated in a size-exclusion (10-kDa exclusion limited) filtration device to 100–200 μL, sample buffer was added, the samples were boiled and stored at −20 °C. (B) Autoradiography of the pooled concentrated samples from (A). Half of the volume of b–h and one-sixth of the volume of a were placed on an SDS–PAGE (13% acrylamide), and the gel was processed as described previously. The nontrypsinized ^{3}H-TAMZ-occupied–heated cytosol without DES (–, lane 1) or with 200-fold molar excess DES (+, lane 2) and the trypsinized ^{3}H-TAMZ-occupied–heated cytosol without DES (–, lane 3) or with DES (+, lane 4) were also placed on the gel. The migrated positions of the molecular mass markers (in kDa) are shown to the left. The data shown are representative of three independent studies.

in each set of pooled fractions. The 10-kDa fragment is not enriched relative to the 28-kDa fragment in the smaller molecular mass range of the S200 column, suggesting a similar size for the 28- and 10-kDa fragments in solution.

Although we do not know whether the 10-kDa fragment behaves strictly as a globular protein, we believe shape plays a minimal role in the elution of the 10-kDa fragment from the gel filtration column. We previously reported that the trypsinized ER (trypsinization conditions were identical to those described above) partitions in an aqueous two-phase partitioning system as a single homogeneous population independent of heat or ligand effects (Fritsch et al., 1992), suggesting no heterogeneity of the steroid binding domain's surface features for the 28- and 10-kDa fragments. Similarly, from Figure 6, if the 10-kDa fragment were a different shape from the 28-kDa fragment, two discrete peaks would be expected, unless they coincidentally eluted at the same volume. However, since the elution pattern is very different for the unoccupied and occupied trypsinized ERs, it is unlikely that the 10- and 28-kDa fragments would coincidentally elute together under different ligand conditions if the fragments were different in shape. The more likely explanation is that the 10- and 28-kDa fragments are approximately the same size in solution.

None of the small fragment (<10 kDa) was observed in pooled fractions g and h, whereas if 10-kDa fragments were free in solution and globular in shape, they should have been observed in g and h. These data suggest that the 10-kDa fragment detected on denaturing gels was not free in solution, but was complexed with other protein fragments and eluted in the molecular mass range of 31–66 kDa. Thus, our data support the model in which the 10-kDa fragment is complexed to other protein fragments and these probably represent maintenance of the secondary and tertiary structures of the steroid binding domain in solution despite the primary amino acid sequence being disrupted.

DISCUSSION

These results demonstrate that the steroid binding domain of the unoccupied ER is relatively resistant to trypsin cleavage and that even after cleavage the fragments retain the secondary and tertiary structures necessary for normal steroid binding in solution. The results also demonstrate a difference in the conformation between the unoccupied and the hormone or antihormone-bound, trypsin-generated steroid binding domains from the rat uterine cytosolic ER.

The structural stability of the steroid binding domain is an obvious necessity for the retention of function. Despite the cleavage of the steroid binding domain into fragments of 28 and 10 kDa as demonstrated by denaturing gel electrophoresis, the steroid binding domain retained its ability to bind hormone with high affinity. Although the primary amino acid sequence was disrupted with trypsin, the secondary and tertiary structures necessary for hormone binding were preserved in solution. The trypsin-generated steroid binding domain was previously shown to retain the ability to undergo a conformational change upon binding hormone as reflected by a loss in surface hydrophobicity, measured using affinity partitioning (Fritsch et al., 1992). Our data are consistent with the reported studies using mutational analysis of the cDNA for the ER illustrating that the entire steroid binding domain is essential in order to maintain steroid binding ability (Lees et al., 1989; Tora et al., 1989; Webster et al., 1988). However, a recent paper demonstrates that a small portion of the carboxyl terminus of the steroid binding domain of the progesterone receptor may be important for distinguishing between hormone and antihormone activities (Vegeto et al., 1992). Similarly, it was recently shown that a conserved region in the carboxyl terminus of the steroid binding domain of the ER is required for hormone-dependent transcriptional activation but not for the binding of hormone (Danielian et al., 1992).

Our data suggest that the 10-kDa fragment from the ER steroid binding domain does not represent the core sequence of amino acids necessary to bind hormone. The 10-kDa fragment was not free in solution and therefore probably continued to interact with other protein fragments, presumably from the steroid binding domain of the ER, to retain a structurally and functionally intact steroid binding domain. However, for the glucocorticoid receptor, a 16-kDa trypsin-generated core sequence of amino acids from the steroid

binding domain was shown to be the smallest protein fragment still capable of binding hormone with relatively high affinity (about 23-fold lower than the wild type) (Simons et al., 1989). It has also been clearly demonstrated that this 16-kDa fragment is in a larger oligomeric state in solution with hsp90 (Chakraborti & Simons, 1991). Whether any other fragments of the glucocorticoid binding domain remain bound to this 16-kDa fragment is unclear. Our studies clearly indicate that hsp90s or hsp90 fragments cannot be present in the oligomeric complex. The heated ER forms ± trypsin are capable of binding hormone with high affinity and no longer have hsp90 bound to them, as demonstrated by sucrose density gradient analysis (data not shown). This demonstrates that hsp90 is not necessary to maintain high-affinity hormone binding for the ER. We cannot exclude the presence of fragments from other non-hsp90 proteins in the oligomeric complexes observed by gel filtration, especially for the unoccupied trypsinized ER. However, a functional significance for these non-ER protein fragments in hormone binding is unlikely since no other protein has yet been identified as being necessary for hormone binding to the ER. Only an intact steroid binding domain has been shown to be essential for high-affinity hormone binding (Lees et al., 1989; Tora et al., 1989; Webster et al., 1988).

The secondary and tertiary structures of the steroid binding domain could be maintained through one of the following mechanisms. The 10-kDa fragment of the steroid binding domain could remain noncovalently bound to the other trypsin-generated fragments of the domain, most likely by hydrophobic interactions between secondary structures (Jaenicke, 1991). However, we cannot completely exclude a role for disulfide bridges holding the fragments together. All our studies were performed in the presence of 10 mM mercaptoethanol or 1 mM DTT, but a buried disulfide bridge may not have been accessible to the solvent for reduction by these agents. Others have shown that reduced sulfhydryl groups in the steroid binding domain of the glucocorticoid receptor are essential for the maintenance of high-affinity hormone binding (Bresnick et al., 1988; Meshinchi et al., 1990). However, in our studies with the ER, a disulfide bridge (the oxidized form of the sulfhydryl group) would be necessary to bind the 10-kDa fragment to the other fragments and thereby maintain the steroid binding domain's structure and function in solution. Thus, we favor the model in which noncovalent interactions (probably hydrophobic interactions) are the most important forces keeping the 10-kDa fragment bound to other fragments of the steroid binding domain, and disulfide bridges are unlikely maintaining the structural integrity of the domain following trypsin treatment.

The steroid binding domain does not show any difference in the generated trypsin fragments (28 and 10 kDa) using denaturing gel electrophoresis when the ER is unoccupied, occupied by the antiestrogen affinity label (Figures 2 and 3), or occupied by an affinity labeling estrogen (Elliston & Katzenellenbogen, 1988), suggesting that new trypsin sites are not exposed or buried upon binding hormone. A study reporting the elution profiles from sucrose gradients and gel filtration columns of the in vivo occupied ER followed by trypsin treatment demonstrated very different patterns of fragments generated between the estrogen- and anti-estrogen-occupied ER complexes (Attardi & Happe, 1986). The differences observed were proposed to represent conformational differences between the E_2- and 4-OHT-occupied receptors. The study did not assay the unoccupied ER.

We showed previously (Fritsch et al., 1992) that the steroid binding domain undergoes a major decrease in surface hydrophobicity upon binding ligand. Decreases in hydrophobicity have been correlated with decreases in the surface area of proteins (Chothia, 1976; Chothia & Finkelstein, 1990). Therefore, upon binding hormone, the receptor may actually lose surface residues rather than expose new ones. The decrease in surface hydrophobicity previously reported (Fritsch et al., 1992) occurred for both estrogen- and anti-estrogen-occupied receptors; therefore, the transcriptional activation function must be regulated by more than a loss in surface hydrophobicity. We propose that the transcriptional activation function is probably created during the burying of hydrophobic residues by an estrogen-induced rearrangement of the surface amino acid residues and occurs with minimal exposure of new residues. When antihormone binds, the hydrophobic residues are also buried, but the rearrangement of amino acid residues on the surface is spatially different than for the estrogen-occupied steroid binding domain. Thus, the conformational difference between hormone and antihormone bound to the steroid binding domain lies in the three-dimensional arrangement of the same amino acid side chains on the surface of the domain.

The ER has been proposed to interact with other proteins (Carson-Jurica et al., 1990). A ligand-dependent difference in the heat-induced oligomeric state of the intact cytosolic ER was previously reported (Nelson et al., 1989). After the E_2-occupied ER was heated in vitro, the receptor eluted as a very large molecular weight species on high-performance liquid chromatography. However, if the receptor was heated in an unoccupied state and then occupied with E_2, the very large complex was not observed. The authors suggested a ligand-specific interaction between the ER and other cytosolic proteins. We observed a ligand-dependent difference in the elution profiles between the unoccupied–trypsin-generated steroid binding domain and the E_2- or 4-OHT-occupied steroid binding domains (Figure 6). These differences in the oligomeric states of the steroid binding domains are clearly heat-independent and therefore represent alterations in the ER other than the heat-induced formation of a large oligomeric complex previously reported (Nelson et al., 1989).

The elution profiles of the trypsin-generated rat uterine cytosolic ER from the gel filtration column shown in Figure 6 are different than the previously reported elution profiles of the human ER's recombinant steroid binding domain overproduced by *Escherichia coli* (Fritsch et al., 1992). The unoccupied and occupied–recombinant steroid binding domains eluted similarly on the S200 gel filtration column. The protein environment in which the ER is assayed may determine the receptor–protein interactions that occur. The results using the recombinant steroid binding domain suggest that the shapes of the unoccupied and occupied steroid binding domains were similar and that the ligand-dependent differences in the elution patterns of the trypsinized rat ER reported here are probably due to changes in the oligomeric state of the steroid binding domain. The other proteins involved in the oligomeric complex with the rat uterine unoccupied steroid binding domains could not be hsp90 or hsp90 fragments because the trypsin-generated, unoccupied–heated steroid binding domain, which is hsp90-free, had the same elution profile as the trypsin-generated, unoccupied steroid binding domain on the gel filtration column. The exact oligomeric nature of the complex at 80 kDa for the unoccupied and unoccupied–heated, trypsin-generated steroid binding domains is not known.

These data demonstrate the ability of the trypsinized, rat uterine cytosolic steroid binding domain to retain structural and functional integrity despite the primary amino acid sequence being disrupted. A difference in the oligomeric state between the trypsin-generated–unoccupied and E_2/4-OHT-occupied steroid binding domains in solution reflects a potential difference in conformation. Our data support a model in which the process of transcriptional inactivity by the unoccupied steroid binding domain is different from that of the anti-estrogen-occupied steroid binding domain. Part of the difference between the unoccupied, the estrogen-, and the anti-estrogen-occupied steroid binding domains may be in their respective ability to form the proper three-dimensional surfaces that allow for receptor–protein interactions.

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