The Steroid Binding Domain of Porcine Estrogen Receptor†

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ABSTRACT: For the purpose of characterizing the estrogen binding domain of porcine estrogen receptor (ER), we have made use of affinity labeling of partially purified ER with [^{3}H]tamoxifen aziridine. The labeling is very efficient and selective particularly after partial purification of ER. A 65 000-dalton (65-kDa) band was detected on the fluorogram of a sodium dodecyl sulfate–polyacrylamide gel, together with a 50-kDa band and a few more smaller bands. The 50-kDa protein appears to be a degradation product of the 65-kDa protein in view of the similar peptide map. ER was affinity labeled before or after controlled limited proteolysis with either trypsin, papain, or α-chymotrypsin. The labeling patterns of limited digests indicate that a fragment of about 30 kDa is relatively resistant to proteases and has a full and specific binding activity to estrogen, whereas smaller fragments have lost much of the binding activity. This fragment is very hydrophobic and probably corresponds to the carboxy half of ER.

Steroid hormone action is mediated by specific receptors present in the target cells. When a cell is stimulated by a steroid hormone, the hormone–receptor complex activates the transcription of hormone-sensitive genes (Yamamoto, 1985). The mechanism of steroid hormone action is not well understood because of some experimental difficulties. Jensen et al. (1968) have first proposed a generally acceptable model. According to this model, unactivated receptor present in the cytoplasm binds the steroid hormone which has passed through the plasma membrane by diffusion. After binding with hormone, the activated hormone–receptor complex is translocated into the nucleus and binds to the specific DNA sequence on the inducible genes. Since exclusive localization of the estrogen receptor (ER) in the nucleus was demonstrated recently (King & Greene, 1984; Welshons et al., 1984), the cytoplasmic localization may just represent the looser binding of unoccupied ER to the chromatin. The possibility that the association and dissociation of receptor with the regulatory subunit component regulate the activation phenomenon is also pointed out (Vedickis, 1983; Mendel et al., 1986). Phosphorylation of receptors also appears to play important roles in the regulation of receptor functions (Groul et al., 1986; Migliaccio et al., 1984). A more refined model must be established to explain the molecular events involved in the ER regulation of gene expression.

A steroid receptor molecule consists of at least two functional domains. One is the steroid binding domain, which receives the hormonal signal from outside of the cell. This signal is transmitted to the other functional domain, the DNA binding domain. The receptor molecule is converted to the active form, which is now able to bind to the specific DNA sequence and activate the transcription of hormone-sensitive genes. The separation of these two domains by limited proteolysis was reported (Wrange & Gustafsson, 1978; Carlstedt-Duke et al., 1982; Allegretto & Pike, 1985). Several DNA sequences to which different steroid receptors bind have been reported (Jost et al., 1984; Compton et al., 1983; Dean et al., 1983; Karin et al., 1984; Moore et al., 1985; Schreidereit et al., 1983; Payvar et al., 1983; Renkawitz et al., 1984). Recently, cDNA clones of estrogen and glucocorticoid receptors (GR) have been isolated and primary structures of these receptors deduced (Green et al., 1986; Krust et al., 1986; Hollenberg et al., 1985; Weinberger et al., 1985). Sequence homologies among ER, GR, and v-erbA oncogene product were also demonstrated.

To clarify the mechanism of steroid hormone action, further studies of receptor proteins including molecular properties, behavior, structure of the functional domains, and their interactions with ligands and DNA sequences are indispensable. Covalent labeling using a radioactive ligand is quite useful for the study of an unstable and low-abundance protein which is otherwise difficult to purify. In this paper, we have investigated the structure of the estrogen binding domain of ER using the techniques of affinity labeling with [^{3}H]tamoxifen aziridine[1] and controlled limited proteolysis.

Materials and Methods

Preparation of Partially Purified ER. Porcine uteri were purchased from a local slaughterhouse. Tissues were frozen in liquid nitrogen as immediately as possible and stored at −80 °C. Tissues were homogenized in 3 volumes of TED [10 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) (pH 7.5), 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol (DTT)] with a Waring blender. Cytosol was obtained by centrifugation at 100000*g* for 1 h at 2 °C with a Hitachi RP-42 rotor. Ammonium sulfate precipitation was performed by slowly adding 0.144 g of finely powdered $(NH_4)_2SO_4$/mL (25% saturation) with continuous stirring. The suspension was centrifuged at 10000*g* for 20 min at 2 °C in a Sorvall SS-34 rotor. The precipitate was dissolved in one-third the original volume of TED and centrifuged briefly to remove insoluble materials. Supernatant was mixed with one-fifth volume of heparin–Sepharose which was prepared as described (Morinali et al., 1977) at 4 °C for 1 h with continuous shaking. After the resin was washed with TED, resin was packed in a column and receptor fraction was eluted by 0.5 M KCl in TED.

Protein Assay. Protein was determined by the method of Bradford (1976).

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[1] The common name tamoxifen aziridine is used in this paper for the compound (*Z*)-1-[2-[4-(1,2-diphenyl-1-butenyl)phenoxy]ethyl]aziridine.

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Estrogen Binding Assay. [^{3}H]Estradiol (47.8 Ci/mmol) was purchased from New England Nuclear. Estrogen binding activity was measured by either the dextran-coated charcoal method, the hydroxyapatite method, or the protamine sulfate precipitation method as described (Scharder & O'Malley, 1982).

Scatchard Analysis of ER. Dissociation constants of undigested or digested ER were determined according to the method of Scatchard (1949).

Affinity Labeling. [^{3}H]Tamoxifen aziridine (21 Ci/mmol) was purchased from Amersham. Affinity labeling was carried out by adding ethanol solution of [^{3}H]tamoxifen aziridine into the heparin–Sepharose eluates to a concentration of 10 nM and allowing to stand at 4 °C for 1 h.

Analysis of Estrogen Binding Domain by Controlled Limited Proteolysis. Heparin–Sepharose eluates were subjected to controlled limited proteolysis with one of the following enzymes: trypsin [Sigma, N^{α}-tosylphenylalanine chloromethyl ketone (TPCK) treated], α-chymotrypsin (Worthington, 3× crystallized), or papain (Sigma), at 25 °C for 2 h under the conditions described in the figure legends. The products of limited digestion were subjected to affinity labeling with tamoxifen aziridine followed by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (Laemmli, 1970) or were bound with [^{3}H]estradiol. After gels were stained with Coomassie Brilliant Blue R-250, they were soaked in EN^{3}HANCE (New England Nuclear) for 1 h and then in distilled water for 1 h. Gels were dried and fluorographed by using Kodak X-Omat AR film and intensifying screen (Du Pont Cronex Lightning-plus) at −80 °C for 3 days.

Results

Affinity Labeling of ER. Initial attempts to affinity label the ER with [^{3}H]tamoxifen aziridine using the cytosol fraction resulted in a relatively high background of labeled bands presumably due to the proteins with low affinity but high abundance. After a number of trials, we found that the following partial purification could effectively eliminate the nonspecific labeling, providing an excellent starting material for further purification and study of the estrogen binding domain of ER. We have purified the ER 60-fold with ammonium sulfate precipitation at 25% saturation followed by heparin–Sepharose column chromatography. Partially purified ER was labeled with [^{3}H]tamoxifen aziridine and electrophoresed on a 10% SDS–polyacrylamide gel. The 65- and 50-kDa bands and, in some preparations, two or three bands of about 35 kDa were detected by fluorography. Labeling was inhibited by preincubation with an excess amount of estrogen but not by other steroids, such as androgen, progesterone, and glucocorticoid (Figure 1). When ER was denatured by heat, $HgCl_2$, or urea to various degrees, the labeling efficiency of partially denatured ER with tamoxifen aziridine was decreased in a parallel manner with that of estradiol binding activity (data not shown). From these results, we conclude that this labeling is ER specific and that tamoxifen aziridine binds to the estrogen binding site. The efficiency of labeling the receptor is calculated to be about 80%, assuming that the number of estradiol-bound molecules under saturating conditions corresponds to 100%. Nonspecific labeling was less than 10% of the total labeling in the optimum conditions.

Peptide Mapping of Labeled ER. We and others have identified ERs of different molecular weight (Van Oosbree et al., 1984; Lubahn et al., 1985; Katzenellenbogen et al., 1983). The relationship or the physiological role of these forms of ERs is not yet clarified well. To elucidate the relationship between the 65- and 50-kDa proteins, we have compared the

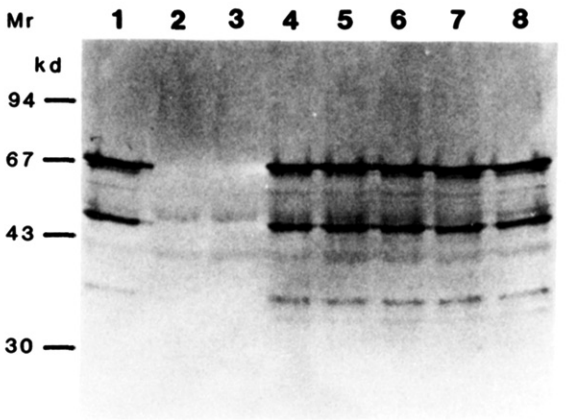

FIGURE 1: Fluorogram of tamoxifen aziridine labeled ER. Partially purified ER was labeled with 10 nM [^{3}H]tamoxifen aziridine. Before affinity labeling, samples were incubated without any further addition (lane 1) or with 1 μM each of estradiol (lane 2), diethylstilbestrol (lane 3), testosterone (lane 4), 5α-dihydrotestosterone (lane 5), progesterone (lane 6), dexamethasone (lane 7), or triamcinolone acetonide (lane 8), respectively, at 0 °C for 30 min.

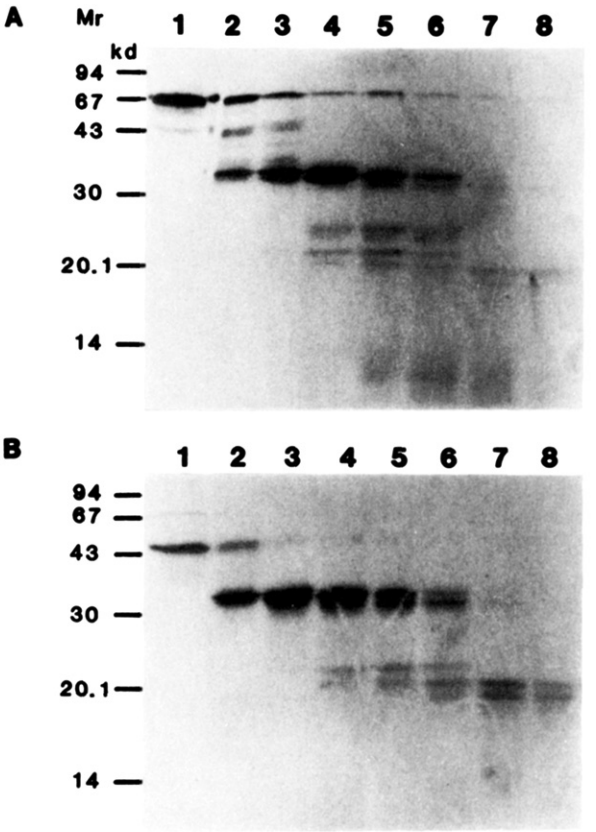

FIGURE 2: Peptide mapping of trypsin digests of 65- (A) and 50-kDa (B) proteins. The 65- and 50-kDa proteins were separated by gel filtration with a TSK G3000 SW column. Aliquots (40 μg) were digested with 0 (lane 1), 4 ng (lane 2), 12 ng (lane 3), 40 ng (lane 4), 120 ng (lane 5), 400 ng (lane 6), 1.2 μg (lane 7), or 4 μg (lane 8) of trypsin, respectively, at 25 °C for 2 h. Samples were precipitated with 10% trichloroacetic acid (TCA), washed with acetone, and resolved by 15% SDS–polyacrylamide gel.

partial proteolysis patterns of these proteins. This peptide mapping technique is quite powerful in identifying or distinguishing proteins (Cleveland et al., 1977). Panels A and B of Figure 2 show the peptide maps of trypsin digests of the 65- and 50-kDa proteins, respectively. The patterns of digested ER are almost identical. These results indicate that the structures of the 65-kDa protein and the 50-kDa protein are common or very similar. As the digestion proceeds, 34-kDa

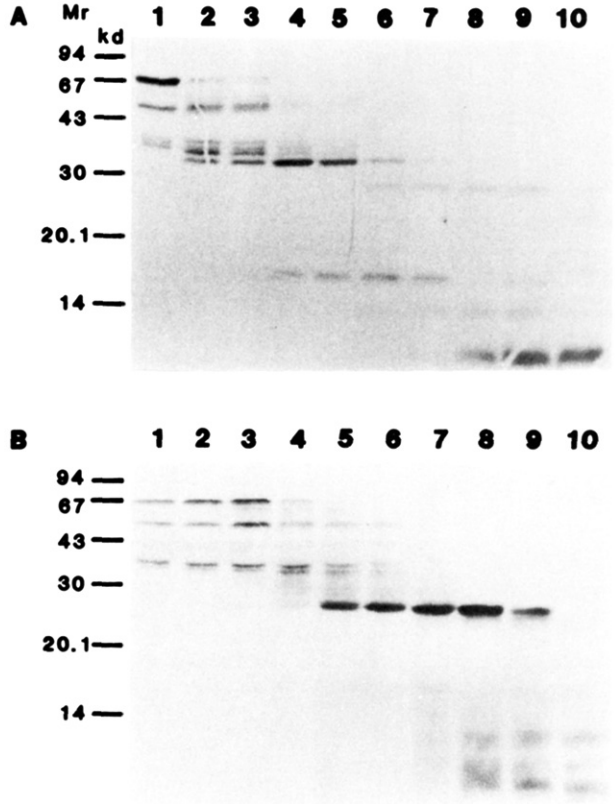

FIGURE 3: Digestion of ER with α-chymotrypsin (A) and papain (B). Affinity-labeled ER (100 μg) was digested with 0 (lane 1), 10 ng (lane 2), 30 ng (lane 3), 100 ng (lane 4), 300 ng (lane 5), 1 μg (lane 6), 3 μg (lane 7), 10 μg (lane 8), 30 μg (lane 9), or 100 μg (lane 10) of α-chymotrypsin or papain, respectively, at 25 °C for 2 h.

bands accumulated and then they were degraded to smaller molecules in both cases. The accumulation of 34-kDa band indicates the existence of a structure that is relatively resistant to the trypsin, and the estrogen binding site is located within this region.

Presence of Protease-Resistant Region. We have also investigated the α-chymotrypsin and papain digests of tamoxifen aziridine labeled ER (panels A and B, respectively, of Figure 3). When ER was digested with α-chymotrypsin and papain, accumulation of 32- and 27-kDa bands was observed respectively. To investigate whether the fragments produced by the digestion of these proteases contain a common region, we have digested the ER with a mixture of the three enzymes (Figure 4). Accumulation of 27–34-kDa bands, almost the same size as the single digestions, were observed as shown in lane 2. This result suggests that these fragments contain the same region which is relatively resistant to proteases.

Estrogen Binding Domain of ER. To determine the region that is required for estrogen binding, we have done a limited digestion of ER and investigated the relationship between the estrogen binding activity and the size of ER molecules. Partially purified ER was subjected to controlled limited proteolysis, and estrogen binding activity was determined. To avoid experimental error, estrogen binding activities were measured by three different methods, i.e., the dextran-coated charcoal method, the hydroxyapatite method, and the protamine sulfate precipitation method. The sizes of digested receptor fragments were determined by SDS–polyacrylamide gel electrophoresis.

Figure 5A′ shows the estrogen binding activities of ER fragments produced by various amounts of trypsin. After each treatment, the digest was labeled with tamoxifen aziridine and analyzed by SDS–polyacrylamide gel electrophoresis (Figure

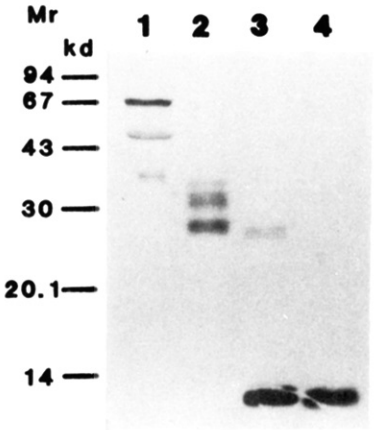

FIGURE 4: Digestion of ER with a mixture of trypsin, α-chymotrypsin, and papain. Affinity-labeled ER (100 μg) was digested with a mixture of three proteases containing 0 (lane 1), 10 ng (lane 2), 100 ng (lane 3), or 1 μg (lane 4) of each enzyme, respectively, at 25 °C for 2 h.

5A, lanes 1–5). As a control, samples that had been labeled before digestion were loaded on lanes 6–10. The conditions of digestion in lanes 6–10 exactly correspond to those of lanes 1–5, respectively. A 34-kDa product which was accumulated on digestion with trypsin [1/1000 trypsin/heparin–Sepharose eluates (w/w)] retained a full estrogen binding activity (Figure 5A, lanes 3 and 8). When ER was digested to a smaller fragment, binding activity decreased dramatically as shown by a very weak 17-kDa band in lane 4. These results indicate that the 34-kDa fragment is large enough to maintain a full estrogen binding activity but the 17-kDa fragment is not. A part of the estrogen binding domain must have been destroyed in the latter. When ER is digested with papain, a similar result can be observed (Figure 5B,B′). A 27-kDa fragment appears to have a full binding capacity. When ER is digested with α-chymotrypsin, 32- and 17-kDa bands are detected (Figure 5C, lane 3). As seen in lane 4, about 50% activity remained (Figure 5C′), and smaller bands are detectable with reduced intensities (Figure 5C). The smallest fragment that has a significant estrogen binding activity is the α-chymotrypsin-digested 17-kDa fragment.

Characterization of Estrogen Binding Domain. Next, we determined the specificity and the affinity of fragmented ERs. Figure 6 shows that the labeling of digests of all three proteases can be competed out only by estrogen, indicating that the fragments produced by these enzymes contain a binding site specific for estrogen. Dissociation constants of these fragments turned out to be in the same range [$(\sim 0.5\text{–}1.0) \times 10^{-9}$ M] as that of undigested ER (Figure 7). Thus, these fragments of about 30 kDa containing the estrogen binding domain have not only full estrogen binding activity but also ligand specificity.

We have tried further characterization of papain-digested 27-kDa fragment. The 27-kDa fragment was purified on a TSK G3000 SW column, and eluates were analyzed by reverse-phase high-performance liquid chromatography. The peak fractions from gel filtration were applied on a TSK gel ODS 120T column that had been equilibrated with 10 mM potassium phosphate buffer (pH 2.2) and eluted by a 10–80% linear gradient of acetonitrile. Radioactivity was eluted at 60% acetonitrile while most of the proteins were eluted at its lower concentrations (Figure 8), indicating a very high hydrophobicity of this molecule.

Discussion

Tamoxifen is known as a potent anti-estrogen that functions by occupying the estrogen binding site of ER. The affinity

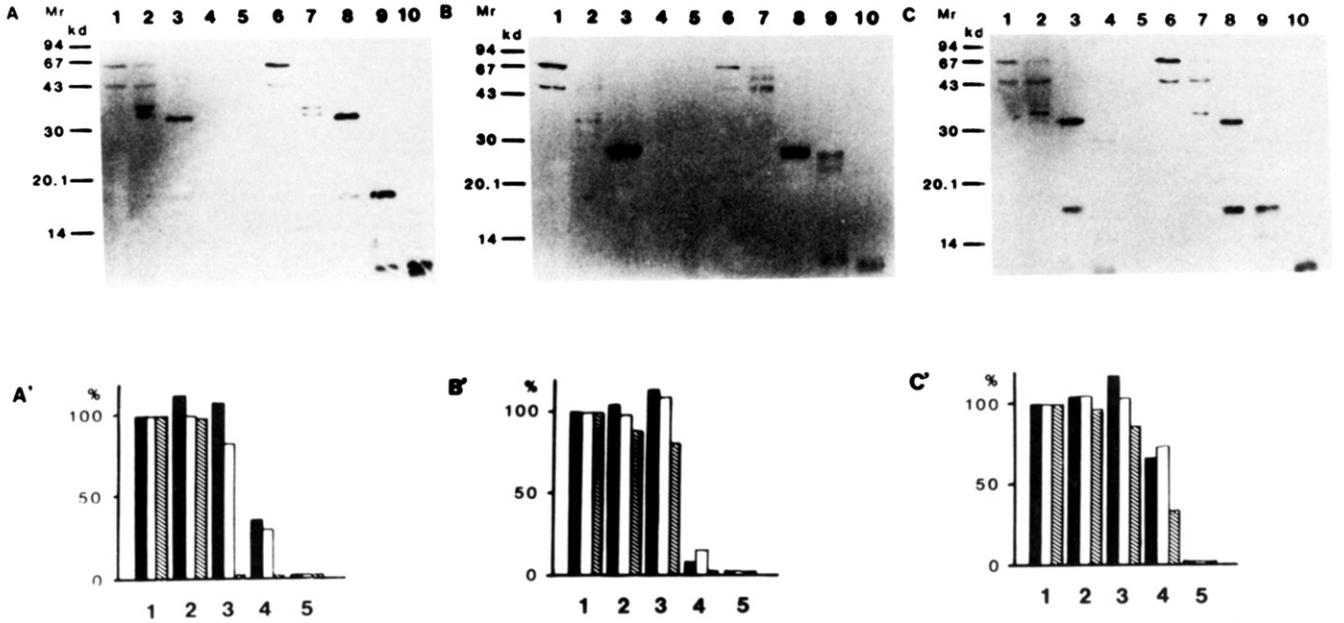

FIGURE 5: Analysis of estrogen binding domain. Partially purified ER (50 μg) was digested with 0 (lane 1), 5 ng (lane 2), 50 ng (lane 3), 500 ng (lane 4), or 5 μg (lane 5), respectively, of trypsin (A), papain (B), or α-chymotrypsin (C) at 25 °C for 2 h and then labeled with [^{3}H]tamoxifen aziridine, or estradiol binding activity was measured (panels A′, B′, and C′ respectively). Solid bar, open bar, and hatched bar indicate the activities measured by dextran-coated charcoal assay, hydroxyapatite assay, and protamine sulfate precipitation assay, respectively. In lanes 6–10, affinity-labeled ERs digested thereafter under the same conditions as those of lanes 1–5 are shown as control.

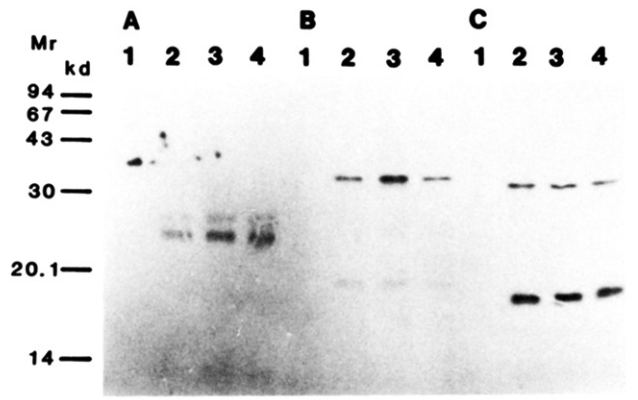

FIGURE 6: Ligand specificity of digested fragments. Partially purified ER was digested with papain (A), trypsin (B), or α-chymotrypsin (C) [1/1000 protease/heparin–Sepharose eluates (w/w)] and then labeled with [^{3}H]tamoxifen aziridine in the presence of a 100-fold excess of estradiol (lane 1), testosterone (lane 2), progesterone (lane 3), or dexamethasone (lane 4).

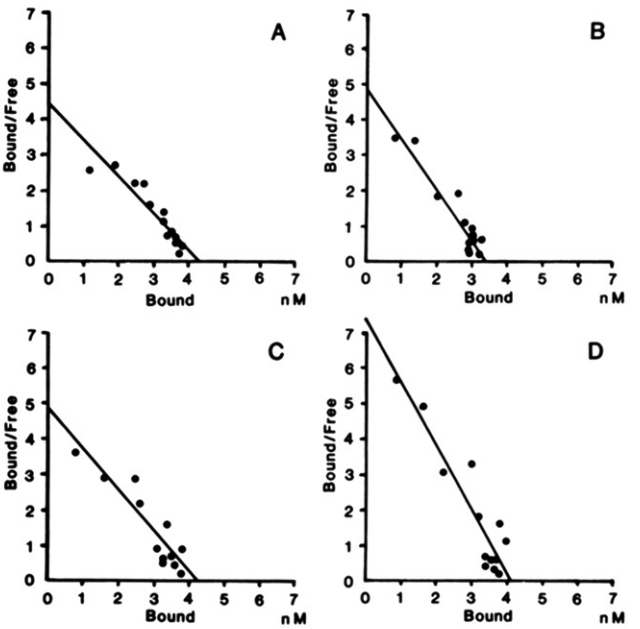

FIGURE 7: Scatchard analysis of digested fragments. ER was digested with either papain (B), trypsin (C), or α-chymotrypsin (D) as described in legend for Figure 6. Estrogen binding activities of undigested (A) and digested ER were measured by the estradiol binding assay (dextran-coated charcoal method) at concentrations of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 16, and 20 nM and ploted by the method of Scatchard (1949).

labeling of ER with tamoxifen aziridine, a derivative of tamoxifen, was first reported by Katzenellenbogen et al. (1983). We have applied this method on porcine uterine cytosol ER. The affinity labeling of ER with tamoxifen aziridine after a certain partial purification is highly efficient and selective, providing a powerful tool for tracing this unstable protein present in very low amounts in the cell.

By affinity labeling, we could obtain considerable information on the structure of ER which was not purified to homogeneity. We have detected the two bands, of 65 and 50 kDa, on the SDS gel of affinity-labeled ER. Limited digests of both the 65- and 50-kDa proteins show almost identical maps of degradation. The similarity of the two peptide maps suggests that the two forms of ER have common or at least very similar structure. Probably, 50- and 35-kDa proteins are derived from the 65-kDa protein by degradation. Consistent with our data, two forms of ER were detected by Katzenellenbogen et al. (1983), Van Oosbree et al. (1984), and Lubahn et al. (1985) in MCF-7 cell or uterine cytosol of various animals. The size of larger one is 65–70 kDa, and that of the smaller one is 50 kDa. Furthermore, Lubahn et al. demonstrated that these two forms of ER cross-reacted with a monoclonal antibody raised against human ER. The existence of the same antigenicity suggests that smaller form is a part of the larger molecule.

We have demonstrated the presence of a protease-resistant region in porcine utrerine ER. Sherman et al. (1978) previously found the presence of similar-sized fragments that could bind estrogen after Ca^{2+}-dependent proteolysis. Our results are in good agreement with theirs.

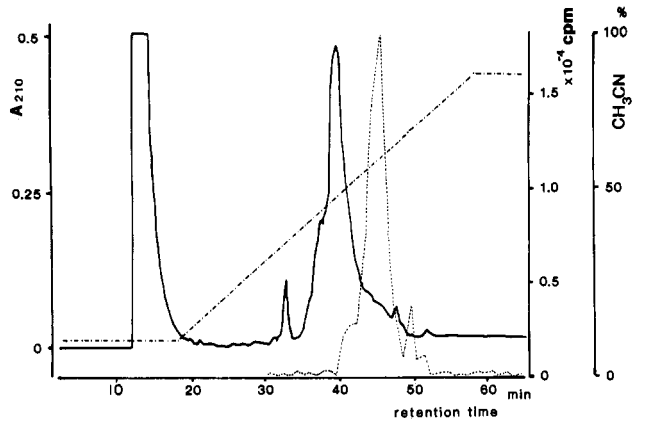

FIGURE 8: Characterization of papain-digested 27-kDa fragment by reverse-phase HPLC. The labeled ER were digested with papain [1/1000 papain/heparin–Sepharose eluates (w/w)] at 37 °C for 2 h. The 27-kDa fragment was separated on a TSK G3000 SW column (Toyo Soda) in 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 0.2 M NaCl, and 0.1% SDS. The 27-kDa fraction was collected, dialyzed against distilled water, and lyophilized. The sample was dissolved in 6 M urea and applied on a reverse-phase high-performance liquid chromatography column, TSK gel ODS 120T (Toyo Soda), equipped with a Hitachi 638-30 liquid chromatography and a Hitachi 635M LC detector. The column was equilibrated with 10 mM potassium phosphate buffer (pH 2.2), and the elution was done by a 10–80% linear gradient of acetonitrile at a flow rate of 1.5 mL/min. Absorbance at 210 nm, radioactivity, and the concentration of acetonitrile are indicated by solid line, dotted line, and dot-and-broken line, respectively.

We have investigated the size of the region required to have an estrogen binding activity using a controlled limited proteolysis combined with affinity labeling. When receptor was digested to smaller than about 30 kDa by three different proteases, estrogen binding activity was severely inhibited. These results suggest that the estrogen binding domain becomes more unstable or suseptible to proteases once the 30-kDa domain is destroyed. Thus, it is possible that this 30-kDa region which is relatively resistant to proteases forms an integral functional domain for estrogen binding and is required to elicit a full binding activity. The data of Lubahn et al. (1985) suggesting that the minimum size of ER that was purified by affinity chromatography was about 30 kDa are also compatible with our conclusion. The dissociation constants of the fragments containing this region are not significantly different from that of undigested receptor. Furthermore, the ligand specificity is also retained.

This 30-kDa domain corresponds to nearly the half-size of ER. Since estrogen is a very small molecule compared with ER, the site that contacts estrogen must be folded in a very small region. Presumably, only a part of this 30-kDa region may form the binding site specific for the estrogen molecule, and the remaining portion may contribute to keep the binding site in a correct conformation. When ER is digested with α-chymotrypsin, a 17-kDa band can be detected with estrogen binding activity. We suppose that, in this case, the domain has been digested in a manner so as not to influence seriously the required specific conformation.

Recently, primary structures of ER and GR have been deduced from the nucleotide sequence of cDNA clones. A correspondence between the domain structure and the amino acid sequence is proposed. A region rich in basic amino acid residues was found that shared a high homology among ER, GR, and erbA protein. In this region, cysteine-containing motifs were found that were homologous to a sequence in TFIIIA and other regulatory proteins (Miller et al., 1985; Rosenberg et al., 1986; Laughan & Gesteland, 1985; Kammerer et al., 1984; Hartshorne et al., 1986; Vincent et al., 1985). This sequence binds Zn^{2+} ion and shows DNA binding activity. Thus, this region is suggested to form a finger to bind with a specific portion of DNA double strand.

The estrogen binding domain is believed to be located in the hydrophobic region in the C-terminal half. Greene et al. (1980, 1984) have produced monoclonal antibodies that recognize the specific domains of ER. Using D75 monoclonal antibody which reacts with a vicinity of the estrogen binding site of ER, cDNA clones encoding the C-terminal region of ER have been screened by using expression vector λgt 11 (Walter et al., 1985). By comparison of ER sequences among human (Green et al., 1986), chicken (Krust et al., 1986), and rat (Koike et al., 1987), more than 90% homology was detected in the hydrophobic C-terminal-half region. Since this region is a candidate for the estrogen binding domain, we assume that this conservation is due to the selective pressure to the hormone binding function. If this assumption is correct, the size of the domain obtained from our data well matches the size of this conserved region. In our purification procedure, the papain-digested 27-kDa fragment was eluted at a very hydrophobic fraction by reverse-phase HPLC. This result is consistent with the hypothesis that the estrogen binding domain is in the C-terminal-half region of ER, which is also hydrophobic.

Wrange et al. (1984) and Reichman et al. (1984) have presented the results of limited proteolysis of photoaffinity- or affinity-labeled GR. The patterns of digested fragments of GR are similar to our results. A structure that is relatively resistant to proteases containing glucocorticoid binding site also exists in GR. The similarity of the sizes of protease-resistant regions suggests that structures of a similar category exist in ER and GR. Indeed, 30% sequence homology is also found in the C-terminal-half region of human ER and GR (Krust et al., 1986). The hydrophobic structure of this region may be the reason for the protease resistance. Hollenberg et al. (1985) isolated two types of GR cDNA clones. They were identical except for the C-terminal region. One (α-form) has 70 amino acid residues to the C-terminal side of Glu-727. The other (β-form) has a different set of 15 amino acid residues in that place. The former binds glucocorticoid while the latter does not. They assume from this result that the glucocorticoid binding site is located in the extreme C-terminal region. More recently, Giguère et al. (1986) have reported that the steroid binding activity of α-form GR protein was lost when the C-terminal region extending for at least 200 amino acids was disrupted by the insertion of 3 or 4 amino acids. This suggests that the binding site can not be localized in the extreme C-terminal region alone but the conformation of the relatively large C-terminal region of GR is important. Perhaps, the importance of the relatively large region for steroid binding activity is common nature of all steroid hormone receptors. The exact steroid binding site may be determined by purifying and sequencing the covalently labeled small peptide.

More detailed analysis of the structure of the estrogen binding domain should help in understanding the molecular basis of the specific binding of this protein to the ligand and the nature of activation of this molecule for interaction with specific gene sequences.

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Registry No. Tamoxifen aziridine, 79642-44-7; estradiol, 50-28-2.

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