van der Werf, S., Bradley, J., Wimmer, E., Studier, F. W., & Dunn, J. J. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 2330-2334.
Wireman, J. W., & Sypherd, P. S. (1974) *Biochemistry 13*, 1215-1221.
Wittmann, H. G. (1982) *Annu. Rev. Biochem. 51*, 155-183.
Zamir, A., Miskin, R., Vogel, Z., & Elson, D. (1982) *Methods Enzymol. 30*, 406-426.
Zwieb, C., & Dahlberg, A. E. (1984) *Nucleic Acids Res. 12*, 4361-4375.
Zwieb, C., Jemiolo, D. K., Jacob, W. F., Wagner, R., & Dahlberg, A. E. (1986) *Mol. Gen. Genet. 203*, 256-264.

Structural Analysis of Covalently Labeled Estrogen Receptors by Limited Proteolysis and Monoclonal Antibody Reactivity†

Benita S. Katzenellenbogen,* Jonathan F. Elliston, Frederick J. Monsma, Jr., Penelope A. Springer, and Yvonne S. Ziegler

Department of Physiology and Biophysics, University of Illinois and University of Illinois College of Medicine, Urbana, Illinois 61801

Geoffrey L. Greene

Ben May Laboratory, University of Chicago, Chicago, Illinois 60637

Received August 19, 1986; Revised Manuscript Received November 13, 1986

ABSTRACT: We have used limited proteolysis of affinity-labeled estrogen receptors (ER), coupled with antireceptor antibody immunoreactivity, to assess structural features of ER and the relatedness of ER from MCF-7 human breast cancer and rat uterine cells. MCF-7 ER preparations covalently labeled with [^{3}H]tamoxifen aziridine ([^{3}H]TAZ) were treated with trypsin (T), α-chymotrypsin (C), or *Staphylococcus aureus* V8 protease prior to electrophoresis on sodium dodecyl sulfate gels. Fluorography revealed a distinctive ladder of ER fragments containing TAZ for each protease generated from the M_r 66 000 ER: for T, fragments of 50K, 38K, 36K, 31K, 29K, and 28K that with longer exposure generated a 6K fragment; for C, fragments of 50K, 38K, 35K, 33K, 31K, 19K, and 18K that with longer exposure generated 14K and 6K fragments; and for V8, ca. 10 fragments between 62K and 28K. Two-dimensional gels revealed charge heterogeneity (two to three spots between p*I* 5.5 and 6.2) of the 66K ER and the T-generated 28K meroreceptor form. Immunoblot detection with the primate-specific antibody D75P3γ revealed that all immunoreactive fragments corresponded to TAZ-labeled fragments but that some small TAZ-labeled fragments (V8-generated forms <47K and T-generated forms <31K) were no longer immunoreactive. In contrast, use of the antibody H222Spγ revealed a correspondence between TAZ-labeled and immunoreactive fragments down to the smallest fragments generated, ca. 6K for T and C and 28K for V8. MCF-7 nuclear and cytosol ER showed very similar digest patterns, and there was a remarkable similarity in the TAZ-labeled and H222-immunoreactive fragments generated by proteolysis of both MCF-7 and rat uterine ER. These findings reveal great structural similarities between the human (breast cancer) and rat (uterine) ER and between nuclear and cytosol ER, indicate charge heterogeneity of ER, and allow a comparison of the immunoreactive and hormone attachment site domains of the ER. The observation that T and C generate a ca. 6K TAZ-labeled fragment that is also detectable with the H222 antibody should be of interest in studies determining the hormone binding domain of the ER and in amino acid sequencing of this region.

The estrogen receptor is an intracellular protein that appears to be responsible for mediating the biochemical and physiological effects of estrogens in estrogen target tissues and cells. While a great deal is known about the tissue distribution and intracellular dynamics of this protein (Gorski & Gannon, 1976; Katzenellenbogen, 1980; King & Greene, 1984; Welshons et al., 1984, Katzenellenbogen et al., 1985), only recently is information becoming available about the detailed biochemistry of this protein (Green, G. L., et al., 1986; Greene, S., et al., 1986).

Since the estrogen receptor is present in very low concentrations in target cells, and most estrogen-based ligands bind reversibly to the receptor, the receptor has proven difficult to purify to the extent required for detailed protein analyses. The recent availability of two reagents—a covalent labeling ligand for the receptor, tamoxifen aziridine (Katzenellenbogen et al., 1983; Katzenellenbogen & Katzenellenbogen, 1984), and monoclonal antibodies to the estrogen receptor (Greene, 1984; Greene et al., 1984)—has now made possible a detailed analysis of the structural and functional domains of this important regulatory protein.

In this paper, we report a detailed structural analysis of the estrogen receptor using proteolytic digestion and monoclonal antibodies to define the hormone binding and immunoreactive domains of this protein. For these studies, we use estrogen receptors from an estrogen-responsive human breast cancer cell line (MCF-7) and from rat uterus. Our findings provide

† This work was supported by National Institutes of Health Grants CA 18119 (B.S.K.), 5T32 HD7028 (J.F.E.), and CA 02897 (G.L.G.) and by American Cancer Society Grant BC-86 and Abbott Laboratories (G.L.G.). A preliminary report on these studies was presented at the 68th Annual Endocrine Society Meeting, June 1986 (Katzenellenbogen et al., 1986).

* Correspondence should be addressed to this author.

0006-2960/87/0426-2364$01.50/0

evidence for great similarities between these two mammalian receptors and between nuclear and cytosol receptor forms and indicate charge heterogeneity of this protein, and our data allow one to compare the immunoreactive and hormone attachment site domains of the receptor protein.

Materials and Methods

[^{3}H]Tamoxifen aziridine [*Z* or trans isomer ([^{3}H]TAZ)][1] was synthesized as detailed previously (Katzenellenbogen et al., 1983). It had a radiochemical purity of 93–96% and a specific activity of 20 Ci/mmol determined by quantitative high-performance liquid chromatography–UV analysis. Other biochemicals were obtained from the following sources. Acrylamide, *N,N'*-methylenebis(acrylamide) (Bis), sodium dodecyl sulfate (SDS), glycine, *N,N,N',N'*-tetramethylethylenediamine (TEMED), ammonium persulfate, and molecular weight standards (phosphorylase *b*, 92 500; bovine serum albumin, 66 200; ovalbumin, 45 000; carbonic anhydrase, 31 000; soybean trypsin inhibitor, 21 500; lysozyme, 14 400) were from Bio-Rad. Tris was from Boehringer Mannheim. Ethanolamine, 2-mercaptoethanol, and Photo-flo were from Kodak. Ampholines pH 3.5–10 and pH 5–8 were from LKB. *Staphylococcus aureus* V8 protease was from Miles. Trypsin (TPCK treated) and α-chymotrypsin (TLCK treated) were from Worthington. Soybean trypsin inhibitor, phenylmethanesulfonyl fluoride (PMSF), 17β-estradiol, and molecular weight markers (myosin, 205 000; β-galactosidase, 116 000; phosphorylase *b*, 97 400; bovine serum albumin, 66 000; ovalbumin, 45 000; carbonic anhydrase, 29 000; aprotinin, 6500) were from Sigma. Ultrapure urea was from Schwarz/Mann. Protosol and Enlighten were from New England Nuclear.

Buffers and Solutions. TETG buffer contained 10 mM Tris-HCl, 1.5 mM EDTA, 10 mM thioglycerol, and 10% (v/v) glycerol, pH 7.4 at 4 °C. Two-fold-concentrated sample buffer for electrophoresis contained 0.125 M Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, and bromophenol blue. Stock solutions of [^{3}H]TAZ (in ethanol) were stored at −80 °C, as were aliquots of protease stock solutions (10 mg/mL). Fresh aliquots of proteases were used in each experiment.

Cell Culture. MCF-7 cells, obtained from Dr. Charles McGrath of the Michigan Cancer Foundation (Detroit, MI), were maintained as previously described (Eckert & Katzenellenbogen, 1982; Katzenellenbogen et al., 1984) in closed, 150 cm^2 Corning T-flasks at 37 °C. Cells were grown in Eagle's minimal essential medium (MEM) with 5% calf serum and were transferred to Eagle's MEM with 5% charcoal–dextran-treated calf serum (CDCS) for 7–10 days before harvesting.

Preparation of Rat Uterine Estrogen Receptors. Uteri were excised from 19–23-day-old Sprague-Dawley Holtzman rats (Holtzman Co., Madison, WI) and were homogenized at 8 uteri/mL of TETG buffer. Homogenates were centrifuged at 180000*g* for 30 min at 0–4 °C, and the supernatant was carefully collected and then filtered through a Millipore 0.22-μm filter to remove lipids. This uterine cytosol was then incubated for 2 h at 0 °C with 20 nM [^{3}H]TAZ in the absence and presence of a 100-fold excess of radioinert estradiol. Receptor was precipitated with 40% ammonium sulfate, and the precipitate was redissolved in half of the original volume and then treated with 10% charcoal–dextran to remove free ligand. Protein concentration of the preparations was determined from the 280 nm/260 nm absorbance ratio, and protease treatments were then conducted at 22 °C with protease concentrations and times as indicated in the text.

Preparation of MCF-7 Estrogen Receptors. For the preparation of nuclear receptor extracts, near-confluent flasks of MCF-7 cells (ca. 20 × 10^6 cells/T-150 flask) were harvested (using Hank's balanced salt solution containing 1 mM EDTA) and up to four flasks of cells resuspended in 10 mL of Eagle's MEM containing 5% CDCS. The suspended cells were labeled with 20 nM [^{3}H]TAZ for 1 h at 37 °C in the absence and presence of a 100-fold excess of radioinert estradiol. The labeled cells were washed twice with 10 mL of TETG and homogenized in 1 mL of TETG (50 strokes in a Dounce homogenizer with the B pestle). The homogenate was centrifuged (800*g*, 10 min) and the pellet washed twice with an equal volume of TETG buffer. The crude nuclear pellet was then resuspended in TETG buffer containing 0.6 M NaCl, pH 8.5, and incubated for 1 h at 0–4 °C with resuspension every 15 min. The suspension was then centrifuged at 180000*g* for 30 min to give the nuclear extract supernatant.

MCF-7 cell cytosol was prepared by harvesting near-confluent T-150 flasks and washing the cells twice with TETG buffer containing 20 mM sodium molybdate. The cells were then homogenized in the same buffer, and the homogenate was centrifuged for 30 min at 180000*g*. The supernatant (cytosol) was then labeled with 20 nM [^{3}H]TAZ for 2–3 h at 0–4 °C. Determination of nonspecific binding was accomplished by including a 200-fold excess of unlabeled estradiol in parallel incubations. After the incubations, excess ligand was removed by incubation with charcoal–dextran slurry (1 part slurry to 9 parts cytosol) for 10 min at 0–4 °C, and the charcoal was then removed by centrifugation at 15600*g* (5 min). The labeled cytosol and nuclear receptor preparations were used for most studies without subsequent purification. Partial purification of nuclear or cytosol receptors for some experiments was achieved by precipitation with $(NH_4)_2SO_4$ (30% saturation, 20 min, 0–4 °C). The precipitate was collected by centrifugation at 48000*g* for 20 min and the pellet dissolved in an appropriate volume of TETG buffer.

Limited Proteolytic Digestion. Freshly prepared [^{3}H]-TAZ-labeled cytosol or nuclear receptors were incubated with several concentrations of proteases at 22 °C for various amounts of time. At specified time points, aliquots of samples digested with trypsin or chymotrypsin were removed and added to tubes containing soybean trypsin inhibitor (final concentration 50 μg/mL) on ice. Samples digested with V8 protease were added to tubes containing PMSF (final concentration 1 mM) on ice. Some aliquots of samples were mixed with an equal volume of 2-fold-concentrated SDS sample buffer and incubated at 100 °C for 2 min. All tubes were quick-frozen on dry ice and stored at −80 °C for subsequent analysis by SDS–PAGE.

SDS–Polyacrylamide Gel Electrophoresis, Electroblotting, and Immune Overlay Procedures. Samples to be analyzed, after mixing with an equal volume of 2-fold-concentrated SDS sample buffer were incubated at 100 °C for 2 min. Gels were prepared according to Laemmli (1970) and contained 10% total acrylamides (T) with 2.7% bis(acrylamide) (C) in the

[1] Abbreviations: TAZ, tamoxifen aziridine, (*Z*)-1-[4-[2-(*N*-aziridinyl)ethoxy]phenyl]-1,2-diphenyl-1-butene; E_2, estradiol, 1,3,5(10)-estratriene-3,17β-diol; SDS, sodium dodecyl sulfate; ER, estrogen receptor(s); T, trypsin; C, α-chymotrypsin; Bis, *N,N'*-methylenebis(acrylamide); TEMED, *N,N,N',N'*-tetramethylethylenediamine; Tris, tris(hydroxymethyl)aminomethane; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; TLCK, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone; PMSF, phenylmethanesulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid; CDCS, charcoal–dextran-treated calf serum; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; IEF, isoelectric focusing.

resolving gel. Electrophoresis was conducted in a 16 cm × 18 cm Protean gel apparatus (Bio-Rad Laboratories) at 30 mA per slab gel.

Gels to be used for autoradiography were fixed and stained in Destain 1 [50% (v/v) MeOH–10% (v/v) acetic acid] plus 0.125% w/v Coomassie Blue R250 overnight and then destained in Destain 1, followed by Destain 2 [5% (v/v) MeOH–7% (v/v) acetic acid]. After the stained gel was photographed, it was treated for 30 min with Enlighten rapid autoradiography enhancer and then dried at 60 °C under vacuum with a Bio-Rad gel slab drier. The dried gels were exposed to Dupont Cronex X-ray film at −80 °C for 10–30 days.

Receptor digests to be used for electroblotting were separated on slab gels and then were blotted from the gel to a nitrocellulose filter using a Bio-Rad Trans blot electrophoretic transfer cell following the method of Towbin et al. (1979). This procedure involved equilibrating the gel in a transfer buffer [25 mM Tris, 192 mM glycine, and 20% (v/v) methanol, pH 8.3] for 30 min at room temperature. Proteins were then electrophoretically transferred from the gel to a 0.25-μm nitrocellulose filter at 4 °C by applying 70 V overnight.

After electroblotting, the nitrocellulose filter was soaked in 3% bovine serum albumin in Tris-buffered saline (TBS: 0.9% NaCl–10 mM Tris, pH 7.4) for 2 h at room temperature and then treated with antibody to estrogen receptor. Radiolabeled ^{35}S-H222Spγ, as well as unlabeled H222Spγ and D75P3γ monoclonal antibodies, was used in immunoblot assays.

For immunoblots using ^{35}S-H222Spγ, 10 mL of BSA buffer containing 2 × 10^6 cpm of the ^{35}S-labeled H222Spγ and 10% carrier serum was incubated with the blot in a glass Pyrex dish at room temperature for 4 h with gentle shaking. The blot was then washed with 100 mL of Tris-buffered saline with 0.05% Triton X-100 for 2 h. The wash buffer was changed after 15-min intervals. The blot was incubated with Enlightening solution for 30 min and then dried overnight and exposed to Kodak X-Omat AR film at −80 °C for 10 days.

For immunoblots using unlabeled H222Spγ or D75P3γ, a second detector antibody was used. Each filter was first incubated with 2 or 4 μL of monoclonal antibody (D75P3γ or H222Spγ, 1 mg/mL stock solution; Greene, 1984), 1.8 mL of BSA–TBS buffer, and 200 μL of rabbit serum overnight at room temperature. The nitrocellulose filter was washed in TBS and then reacted with a 4-μL peroxidase-linked rabbit anti-rat IgG (Miles Laboratories) diluted 1:500 in 1.8 mL of BSA buffer with 200 μL of rabbit serum for 2 h at room temperature. Following five washes in TBS buffer, the position of the receptor was identified by a peroxidase enzyme assay using 20 mL of 3 mg/mL 4-chloro-1-naphthol dissolved in methanol and 60 μL of 30% H_2O_2 in 100 mL of 10 mM Tris-HCl, pH 7.4 (Reiner & Katzenellenbogen, 1986). After the development of the violet-colored band, the reaction was quenched by washing the filter with tap water.

Discontinuous SDS–8 M Urea–Polyacrylamide Slab Gel Electrophoresis and SDS–Polyacrylamide Gradient Gel Electrophoresis To Characterize Low Molecular Weight Receptor Peptides. The SDS-8 M urea–polyacrylamide gel electrophoresis system of Anderson et al. (1983) was used to obtain better resolution of proteolytic fragments of receptor below M_r 30 000. The resolving gel contains 8% total acrylamides, 5% bis(acrylamide) cross-linker, 8 M urea, 0.025% SDS, and 0.2 M Tris–sulfate, pH 7.8, and the stacking gel contains 3.125% acrylamide, 20% cross-linker, 0.025% SDS, and 0.2 M Tris–sulfate, pH 7.8. Each 8% slab gel was 16 cm × 18 cm × 1.5 mm thick. Gels and samples were prepared exactly as described (Anderson et al., 1983). Gels were run at a constant power of 20 W/gel with rigorous temperature maintenance at 10 °C. It took 4–5 h for the tracking dye (Pyronin Y) to reach within 1 cm from the bottom of the slab gel. The upper buffer was removed after 2 h and replaced with fresh upper buffer for the remainder of the electrophoretic run. Gels were then silver stained, or lanes were cut out and then sliced into 2-mm gel slices with a razor blade slicer for counting of radioactivity. The mobility of the estrogen receptor peptides was compared to that of the molecular weight standards from Bio-Rad covering the range from 92 500 to 14 400 and included, in addition, aprotinin and the β and α chains of bovine insulin with molecular weights of 6500, 3400, and 2300, respectively.

Although the Anderson et al. (1983) gel system gave nice resolution of low molecular weight receptor fragments, we found it difficult to electroblot proteins from these gels onto nitrocellulose paper. Therefore, we used, in addition, SDS containing 10–20% polyacrylamide gradient slab gels since these resolved over a wide molecular weight range and also gave efficient transfer of proteins for subsequent immunoblotting. These acrylamide gradient (10–20%) separating gels contained 0.37 M Tris (pH 8.8) and *N*,*N*′-dimethylenebisacrylamide (0.675–1.8%), and the stacking gel contained 0.4% acrylamide, 0.036% *N*,*N*′-dimethylenebis(acrylamide), 1% agarose, 0.1% SDS, and 0.125 M Tris, pH 6.8. Samples were electrophoresed (30 mA/gel) until the tracking dye front reached the end of the gel.

Isoelectric Focusing and Two-Dimensional Polyacrylamide Gel Electrophoresis. Isoelectric focusing was done as previously described (Monsma et al., 1984), with minor modifications. Gels (6 × 85 mm) containing 5% total acrylamides, 15% cross-linker, 2% ampholytes (pH 3.5–10), and 8 M urea were used. The upper buffer contained 0.4% ethanolamine, and the lower reservoir contained 0.5 M acetic acid. Samples were brought to 8 M in urea and either loaded on the top of the polymerized gel or mixed with the gel components prior to polymerization. Focusing was done at 1 mA/gel with a voltage limit of 400 V, for 18 h at 4 °C. After the gels were focused, they either were frozen and sliced for determination of pH and radioactivity profiles or were placed on top of the stacking gel of an SDS–polyacrylamide gel of the same composition as used elsewhere in these studies. The IEF gel was sealed in place with 1% agarose in sample buffer and electrophoresed until the dye front reached 1 cm from the bottom of the gel.

RESULTS

Two-Dimensional Gel Analysis of the Estrogen Receptor. Exposure of MCF-7 cells to [^{3}H]TAZ results in labeling of essentially all of the cell estrogen receptors, and the intact covalently labeled ER extracted from the nucleus migrates on two-dimensional gels with an apparent molecular weight (M_r) of 66 000 (Figure 1 and Figure 2, lane 1). This two-dimensional IEF–SDS gel analysis reveals some charge heterogeneity (two to three spots between p*I* 5.5 and 6.2) of the M_r 66 000 [^{3}H]TAZ-labeled estrogen receptor. It is also clear from Figure 1 that, despite the fact that these nuclear estrogen receptor preparations contain many proteins, as shown by the protein stain pattern, [^{3}H]TAZ selectively labels only the 66K estrogen receptor. In receptor preparations from cells labeled with [^{3}H]TAZ in the presence of a 100-fold excess of radioinert estradiol, the labeling of the M_r 66 000 protein (all charge species) is completely prevented (data not shown).

Partial Proteolytic Digestion with Trypsin: Tamoxifen Aziridine Labeled and Immunoreactive Receptor Fragments.

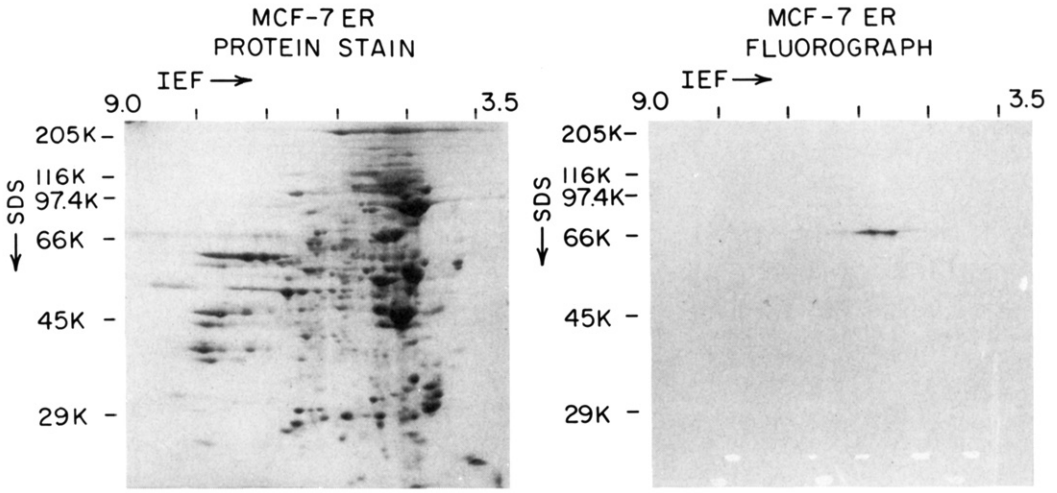

FIGURE 1: Two-dimensional gel analysis of MCF-7 nuclear receptor. MCF-7 estrogen receptor covalently labeled with [^{3}H]TAZ was run on an isoelectric focusing gel (pH 3.5–10) in the presence of 8 M urea for the first dimension. This was then applied to the top of an SDS–10% polyacrylamide slab gel and electrophoresed in the vertical direction as described under Materials and Methods. Several IEF gels were run in parallel. One was sliced for determination of pH and radioactivity, and another was electrophoresed on an SDS gel. (Left) Coomassie Blue stain of two-dimensional gel used for fluorography. (Right) Fluorogram of gel in left panel.

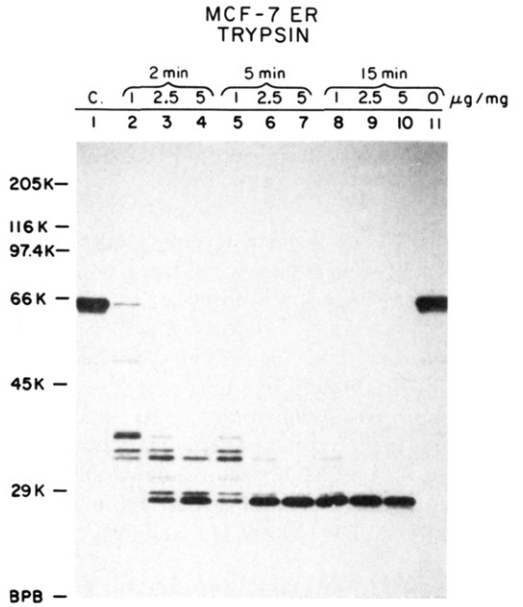

FIGURE 2: Trypsin proteolysis. MCF-7 cells were incubated with 20 nM [^{3}H]TAZ, and the MCF-7 nuclear receptor covalently labeled with [^{3}H]TAZ was extracted with 0.6 M NaCl. Receptors were precipitated with $(NH_4)_2SO_4$ at 30% saturation, redissolved, and incubated as indicated with various amounts of trypsin and analyzed by SDS–PAGE (10% polyacrylamide slab gel) and fluorography as detailed under Materials and Methods. Lane 1, no enzyme treatment; lanes 2–4, incubation for 2 min, 22 °C, with 1, 2.5, and 5 μg of enzyme/mg of protein, respectively; lanes 5–7, incubation for 5 min, 22 °C, with 1, 2.5, and 5 μg of enzyme/mg of protein, respectively; lanes 8–11, incubation for 15 min, 22 °C, with 1, 2.5, 5, and 0 μg of enzyme/mg of protein, respectively.

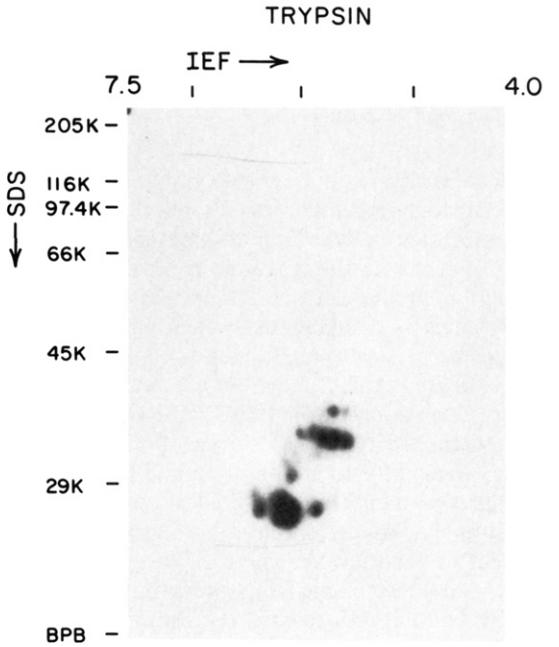

FIGURE 3: Two-dimensional gel analysis of the MCF-7 nuclear receptor after proteolytic digestion with trypsin. MCF-7 cells were incubated with [^{3}H]TAZ, and the nuclear salt extracted receptor was incubated with trypsin (2.5 μg/mg of protein) for 5 min at 22 °C and then electrophoresed on an isoelectric focusing gel (pH 4.0–7.5) in the presence of 8 M urea for the first dimension. This gel was then applied to the top of an SDS–10% polyacrylamide slab gel and electrophoresed in the vertical direction. A fluorogram of the gel is shown.

The [^{3}H]TAZ-labeled estrogen receptor found in the nuclear salt extract, while not subject to endogenous proteolytic degradation (Figure 2, lanes 1 and 11), is none-the-less quickly degraded by treatment with exogenous proteases. When nuclear receptor extracts are incubated with varying concentrations of trypsin and then analyzed by SDS–PAGE followed by fluorography, a distinct ladder of proteolytic fragments is observed (Figure 2). Significant proteolysis of receptor occurs within 2 min at 22 °C when treated with 1 μg of trypsin/mg of protein (Figure 2), and after 15 min at 22 °C with 1 μg of trypsin/mg of protein, virtually all receptor has been reduced to M_r 28 000, a form which is digested to smaller forms only with very much more forcing conditions (see below, Figure 8). Increasing the concentration of trypsin results only in the apparent acceleration of the rate of degradation. That is, the same bands and relative intensities are seen, but at earlier times with increasing enzyme concentration. The smallest stable form of receptor seen is 28K (meroreceptor). On two-dimensional gels, charge heterogeneity of the trypsin-generated 35 000 and 28 000 (mero) receptor forms is evident (Figure 3).

Trypsin-digested receptor preparations were also analyzed by SDS–PAGE followed by electroblotting and immunodetection with the ER monoclonal antibody D75P3γ (Figure 6, lanes 2–4). It is seen that only bands of M_r 31 000 or larger

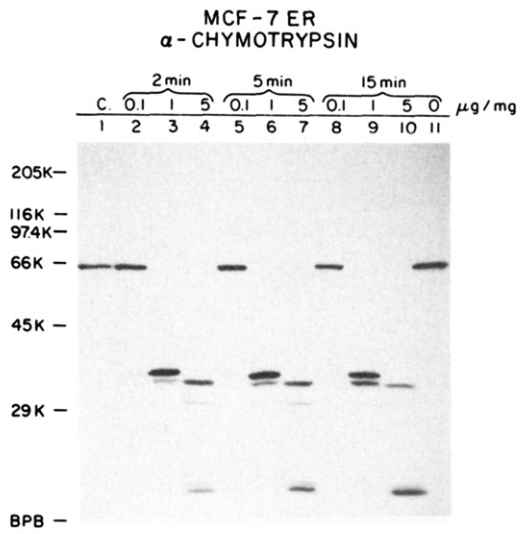

FIGURE 4: α-Chymotrypsin proteolysis. MCF-7 nuclear receptors covalently labeled with [^{3}H]TAZ were incubated with the indicated amounts of α-chymotrypsin at 22 °C for various lengths of time and analyzed by SDS–PAGE and fluorography as detailed under Materials and Methods. Lane 1, untreated nuclear extract; lanes 2–4, incubation for 2 min, 22 °C, with 0.1, 1, and 5 μg of enzyme/mg of protein, respectively; lanes 5–7, incubation for 5 min, 22 °C, with 0.1, 1, and 5 μg of enzyme/mg of protein, respectively; lanes 8–11, incubation for 15 min, 22 °C, with 0.1, 1, and 5 μg of enzyme/mg of protein or with no enzyme, respectively.

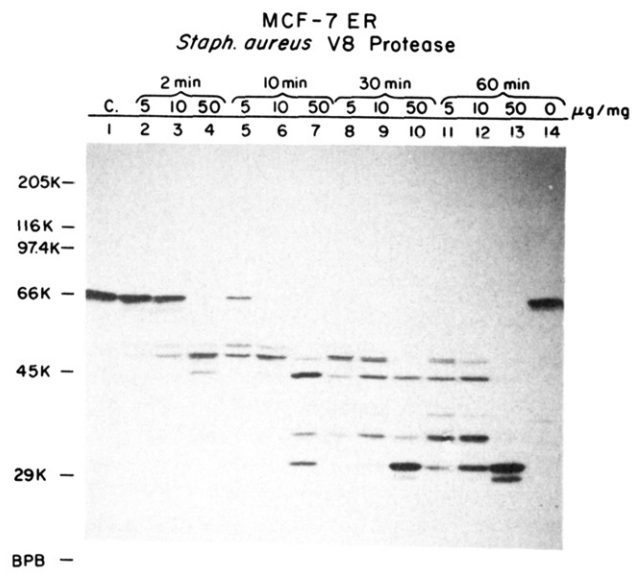

FIGURE 5: *Staph. aureus* V8 protease digestion. MCF-7 nuclear receptors covalently labeled with [^{3}H]TAZ were incubated as indicated with various amounts of *S. aureus* V8 protease and analyzed by SDS–PAGE and fluorography, as detailed under Materials and Methods. Lane 1, nuclear extract, no treatment; lanes 2–4, incubation for 2 min, 22 °C, with 5, 10, and 50 μg of enzyme/mg of protein, respectively; lanes 5–7, incubation for 10 min, 22 °C, with 5, 10, and 50 μg of enzyme/mg of protein, respectively; lanes 8–10, incubation for 30 min with 5, 10, and 50 μg of enzyme/mg of protein, respectively; lanes 11–14, incubation for 60 min with 5, 10, and 50 μg of enzyme/mg of protein or with no enzyme, respectively.

react with the antibody. In fragments smaller than M_r 31 000, the antibody domain is lost. It is of note that all fragments which exhibit antibody binding correspond to bands of [^{3}H]TAZ receptor radioactivity seen on the fluorograms. Consequently, we did not detect antibody binding on any fragments which did not contain covalently bound [^{3}H]TAZ.

Partial Proteolytic Digestion with α-Chymotrypsin: Tamoxifen Aziridine Labeled and Immunoreactive Receptor Fragments. Treatment of the [^{3}H]TAZ-labeled MCF-7 nuclear salt extracted ER with 1–5 μg of TLCK-treated α-chymotrypsin/mg of protein results in rapid proteolysis of the 66K estrogen receptor (Figure 4). The cleavage of the receptor is strongly enzyme concentration dependent and nearly independent of time. When the receptor preparations are incubated with 0.1 μg of α-chymotrypsin/mg of protein, only a very faint band at 35K is observed with the remainder of the receptor still at 66K. Treatment with 1 μg of α-chymotrypsin for 2–15 min, 22 °C, results in the formation of a major band of M_r 35K and a minor band of M_r 33K. When receptor preparations are treated with 5 μg of α-chymotrypsin/mg of protein for 2–15 min, fragments of 33K, 30K, ca. 19K, and 18K molecular weight are formed.

When receptor preparations were examined by electroblotting the SDS gel and detecting with the monoclonal antibody D75P3γ (Figure 6, lanes 5–7), only certain fragments could be detected. The original 66K receptor form and the 33K, 19K, and 18K forms all reacted with this antibody while the 35K and 30K fragments did not. As with the trypsin-treated preparations, the fragments which reacted with the antibody also contained covalently attached [^{3}H]TAZ, and there were no fragments observed which bound antibody and were not associated with [^{3}H]TAZ.

Partial Proteolytic Digestion with Staphylococcus aureus V8 Protease: Tamoxifen Aziridine Labeled and Immunoreactive Receptor Fragments. Proteolysis of the [^{3}H]TAZ-labeled MCF-7 nuclear salt extracted receptors required much higher concentrations of *Staph. aureus* V8 protease and more prolonged incubation times to achieve the same extent of digestion as with trypsin or chymotrypsin (Figure 5). A minor amount of degradation occurs when these preparations are treated with 5 or 10 μg of V8 protease/mg of protein for 2 min, 22 °C. The initial products formed by V8 protease digestion are several fairly large, but short-lived fragments from 58K to 48K (three to five bands have been observed). Further digestion (10–60 min) with 5 or 10 μg of protease/mg of protein results in the appearance of numerous bands between 48K and 29K. At a higher concentration of V8 protease (50 μg/mg of protein), degradation is more rapid and complete such that by 60 min, 22 °C, only 31K and 29K fragments are present.

Analysis by electroblotting and immunodetection with monoclonal antibody D75P3γ of receptor digested with V8 protease (Figure 6) indicated that only fragments of M_r 47 000 or greater were immunoreactive. As with the trypsin and α-chymotrypsin receptor products, all fragments detected with the D75P3γ antibody were also seen on the fluorograms and thus contained covalently bound [^{3}H]TAZ.

Comparison of Cytosol Receptor and Nuclear Receptor Proteolytic Digestion Patterns. A comparison of the patterns produced by protease digestion of MCF-7 cytosol and nuclear receptors indicates virtual identity in the fragments produced from these receptors (Figure 7). With trypsin, the same sequence of digestion and the same size fragments are produced from nuclear and cytosol receptors, although the rate of degradation in the cytosol preparations is somewhat faster. However, by 30 min at 22 °C with 1 μg of trypsin/mg of protein, both the cytosolic and nuclear receptors have reached the M_r 28 000 form.

With α-chymotrypsin or V8 protease digestion, the fragments produced from the cytosol receptor are all the same size as corresponding fragments observed from the nuclear receptor. Analysis by electroblotting and antibody binding (not shown) also indicates that the same fragments from cytosol and nuclear receptors react with monoclonal antibody D75P3γ.

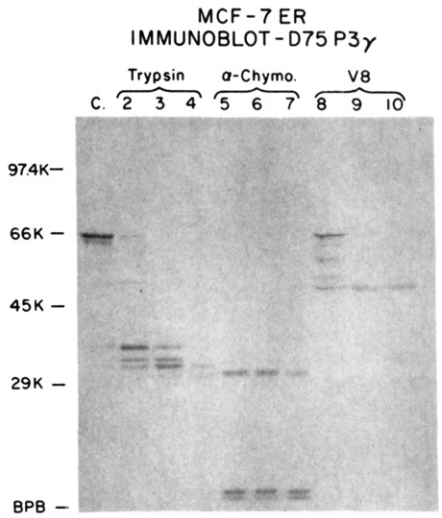

FIGURE 6: Western blot of partially proteolyzed estrogen receptor detected with the monoclonal antibody D75P3γ. MCF-7 nuclear receptor was treated with protease and electrophoresed on an SDS–10% polyacrylamide slab gel. The proteins were then electroblotted onto nitrocellulose and detected with the monoclonal antibody D75P3γ as detailed under Materials and Methods. Lane 1, untreated receptor; lanes 2–4, receptor treated with 1 μg of trypsin/mg of protein for 2, 5, and 15 min, respectively; lanes 5–7, receptor treated with 5 μg of α-chymotrypsin/mg of protein for 2, 5, and 15 min, respectively; lanes 8–10, receptor treated with *S. aureus* V8 protease, 10 μg of enzyme/mg of protein for 2 min, 50 μg/mg of protein for 2 min, and 10 μg/mg of protein for 30 min, respectively. All enzyme treatments were at 22 °C.

Limit Digests of the Estrogen Receptor with Trypsin, α-Chymotrypsin, and V8 Protease and Immunoreactivity of Proteolytic Receptor Fragments. With more forcing enzyme conditions, i.e., treatment either with higher concentrations of enzymes or for longer time periods, trypsin and chymotrypsin were found to generate a small receptor fragment that still contained the [^{3}H]TAZ binding region (Figure 8). With a long exposure to trypsin, the 28K receptor was reduced to a predominant 6K fragment; with chymotrypsin, longer exposures generated prominent 14K and 6K fragments. However, exposure to high concentrations of V8 protease for long periods of time failed to generate forms smaller than 28K that were [^{3}H]TAZ labeled.

Immunoreactivity of these receptor fragments (Figure 9), determined by using the monoclonal antibody H222Spγ, indicated that the smallest fragments generated, ca. 6K for trypsin and chymotrypsin and ca. 28K for V8, retained H222 immunoreactivity.

Comparison of Covalently (TAZ) Labeled and H222-Immunoreactive Fragments Generated by Proteolysis of Rat Uterine Estrogen Receptor. Figure 10A reveals that the pattern of TAZ-labeled fragments generated by proteolysis of rat uterine estrogen receptor is similar to that observed for the MCF-7 human breast cancer estrogen receptor. The untreated rat uterine estrogen receptor migrated as a major 66K protein with some 52K receptor form. The [^{3}H]TAZ labeling of this 52K form is estradiol competable, as is the labeling of the intact 66K receptor. The 52K species represents some endogenous proteolytic clipping of receptor that occurs during sample preparation, and its formation can be prevented by the use of protease inhibitors such as leupeptin (Monsma et al., 1984; Nardulli & Katzenellenbogen, 1986). Trypsin proteolysis generated prominent 28K and 29K receptor fragments which with more forcing conditions yielded an 8–9K and a 6K form. Chymotrypsin proteolysis generated major 35K, 32K, and 18K forms, which upon more prolonged treatment resulted in 14K and 6K [^{3}H]TAZ-labeled fragments. V8 protease at high concentrations generated prominent 35–36K and 30K forms which were converted to a 26K fragment. This latter fragment was not seen in MCF-7 estrogen receptor digests, and it was not immunoreactive with the H222 monoclonal antibody. The major [^{3}H]TAZ-labeled species (Figure 10A) corresponded to H222 immunoreactive species (Figure 10B). Interestingly, the smallest receptor fragments generated by trypsin and chymotrypsin, of M_r 6000, showed weak immunoreactivity compared to that observed for the next larger species of 8K (for trypsin) and 14K (for chymotrypsin).

Discussion

These studies reveal great structural similarities between human (breast cancer) and rat (uterine) estrogen receptors and between nuclear and cytosol estrogen receptors, and they provide several pieces of interesting information about the estrogen receptor protein.

Digestion of estrogen receptors from either receptor source (human or rat), with three different proteases, generated for each protease very similar [^{3}H]TAZ-labeled fragments. In addition, each protease yielded a relatively "resistant" receptor fragment of ca. 30 000 daltons (the 28K meroreceptor form in the case of trypsin, and ca. 30K forms in the case of chymotrypsin and V8 protease). The meroreceptor form has been identified previously and is considered to be the smallest receptor form containing an intact hormone binding site (Sherman & Stevens, 1984). Interestingly, however, use of higher trypsin or chymotrypsin concentrations or longer incubation times with the native estrogen receptor generates much smaller limit digest products that still contain the TAZ-labeled receptor binding site: 6K in the case of trypsin and 14K and 6K species in the case of chymotrypsin. In addition, the observation of identical molecular weights of nuclear receptors covalently labeled with TAZ in the intact cell at 37 °C and of cytosol receptors covalently labeled with [^{3}H]TAZ in vitro at 0–4 °C, and virtually identical protease digest patterns, indicates that the receptors are not markedly altered in their three-dimensional structure upon ligand binding in vivo. Our observations would be consistent with findings on glucocorticoid receptors from liver cells that have shown identical protease digestion patterns of unactivated and activated glucocorticoid receptors (Reichman et al., 1984).

Our studies comparing the immunoreactivity of estrogen receptor proteolytic fragments using the monoclonal antibodies D75P3γ and H222Spγ indicate a close correspondence between TAZ-labeled and H222-immunoreactive fragments down to the smallest fragments generated (M_r 6000 for trypsin and chymotrypsin and M_r 28 000 for V8 protease). Hence, the epitope for H222 antibody recognition must lie close to the hormone binding site on the receptor. In contrast, immunoprobing with the primate-specific antibody D75P3γ revealed that all immunoreactive fragments were TAZ labeled but that [^{3}H]TAZ can be found on receptor fragments (V8-generated peptides <47K and trypsin-generated peptides <31K) that no longer contain the D75 antibody recognition epitope. Hence, the hormone binding site and epitope for recognition of this antibody must be more distantly separated on the MCF-7 receptor.

A model showing our findings regarding the placement of the antibody recognition sites and TAZ attachment site on the MCF-7 estrogen receptor is presented in Figure 11. This figure presents for each protease the smallest receptor fragment obtained by proteolysis containing the TAZ attachment site (6K for chymotrypsin, 6K for trypsin, and 28K for V8 pro-

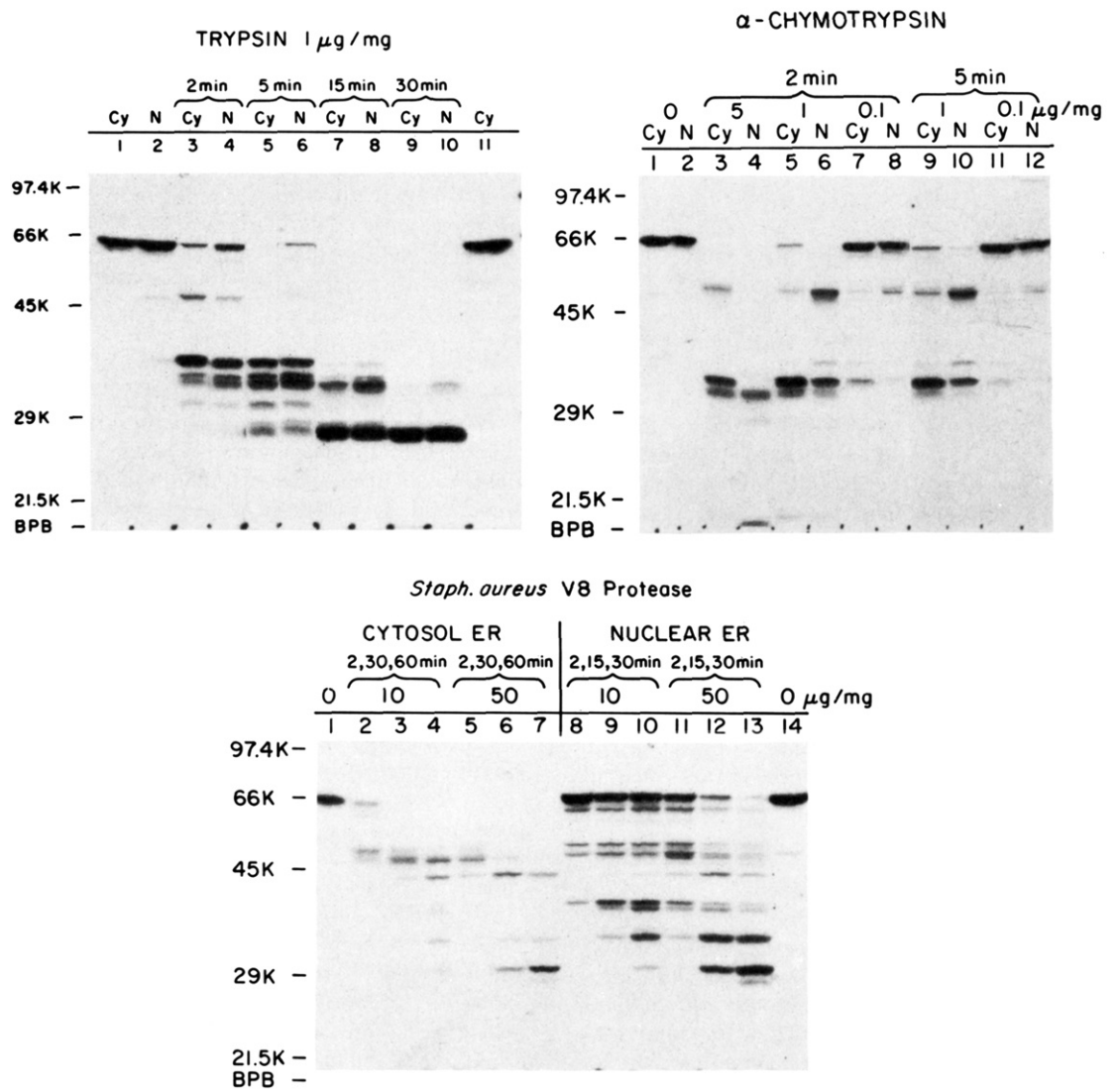

FIGURE 7: Comparison of the proteolytic digestion with trypsin, α-chymotrypsin, or *Staph. aureus* V8 protease of MCF-7 cytosol and nuclear receptors labeled with [^{3}H]TAZ. MCF-7 cytosol receptors and nuclear salt extracted receptors covalently labeled with [^{3}H]TAZ were precipitated with ammonium sulfate, and the resuspended precipitate was treated with 1 μg of trypsin/mg of protein for 2–30 min at 22 °C, or with various amounts of α-chymotrypsin for 2 and 5 min, 22 °C, or with 10 or 50 μg of V8 protease/mg of protein for 2–60 min at 22 °C and analyzed side by side on SDS–PAGE followed by fluorography. Treatments are of cytosol (Cy) and nuclear receptors (N), respectively. For trypsin: lanes 1 and 2, untreated; lanes 3 and 4, 2 min, 22 °C; lanes 5 and 6, 5 min, 22 °C; lanes 7 and 8, 15 min, 22 °C; lanes 9 and 10, 30 min, 22 °C. For chymotrypsin: lanes 1 and 2, untreated; lanes 3 and 4, 5 μg of α-chymotrypsin/mg of protein, 2 min; lanes 5 and 6, 1 μg of α-chymotrypsin/mg of protein, 2 min; lanes 7 and 8, 0.1 μg of α-chymotrypsin/mg of protein, 2 min; lanes 9–12, same concentrations as lanes 5–8, respectively, for 5 min, 22 °C. For V8 protease: lanes 1 and 14, untreated cytosol and nuclear receptors; lanes 2–4, cytosol treated with 10 μg of V8 protease/mg of protein for 2, 30, and 60 min, respectively; lanes 5–7, cytosol treated with 50 μg of V8 protease/mg of protein for 2, 30, and 60 min, respectively; lanes 8–10, nuclear receptor treated with 10 μg of V8 protease/mg of protein for 2, 15, and 30 min, respectively; lanes 11–13, nuclear receptor treated with 50 μg of V8 protease/mg of protein for 2, 15, and 30 min, respectively.

tease) and the smallest receptor fragment containing the TAZ attachment site and the epitopes for the antibodies H222 and D75 (6K for chymotrypsin, 32K for trypsin, and 47K for V8). Hence, the 6K chymotrypsin-generated receptor fragment allows us to state that these three domains (TAZ binding and H222 and D75 antibody recognition) are within 6K of each other on the receptor protein. Likewise, the 6K trypsin-generated receptor fragment and the 28K V8-generated receptor fragment, both of which lack the D75 antibody binding domain while retaining TAZ and H222 binding sites, indicate that the D75 antibody recognition region is not between the TAZ and H222 binding regions. Also, the 28K V8 fragment enables us to state that the region between TAZ/H222 and D75 binding sites cannot be closer than 28K from the amino-terminal end of the receptor. We know from other studies that the hormone binding domain is in the carboxy-terminal half of the receptor (Krust et al., 1986), and hence, we have indicated the TAZ binding site toward the carboxy terminus. Also, previous cloning studies have suggested the D75 antibody recognition region to be near the carboxy terminus (Walter et al., 1985), consistent with how we have drawn our model here. However, several aspects are still uncertain. We do not know the relative orientation of the TAZ binding and H222-immunoreactive regions, and this uncertainty is indicated in our model (Figure 11). We do not know the relative position/orientation of trypsin and V8 cuts that divide the TAZ/H222 and D75 domains. Also, while we do know that the D75 recognition region is close to the carboxy terminus relative to hormone binding (Walter et al., 1985), we do not know from our protease digest data whether the receptor fragments containing the D75 recognition region (6K chymotrypsin-generated fragment, 32K trypsin-generated fragment, and 47K V8-generated fragment) actually include the carboxy terminus or not; hence, a region including possible

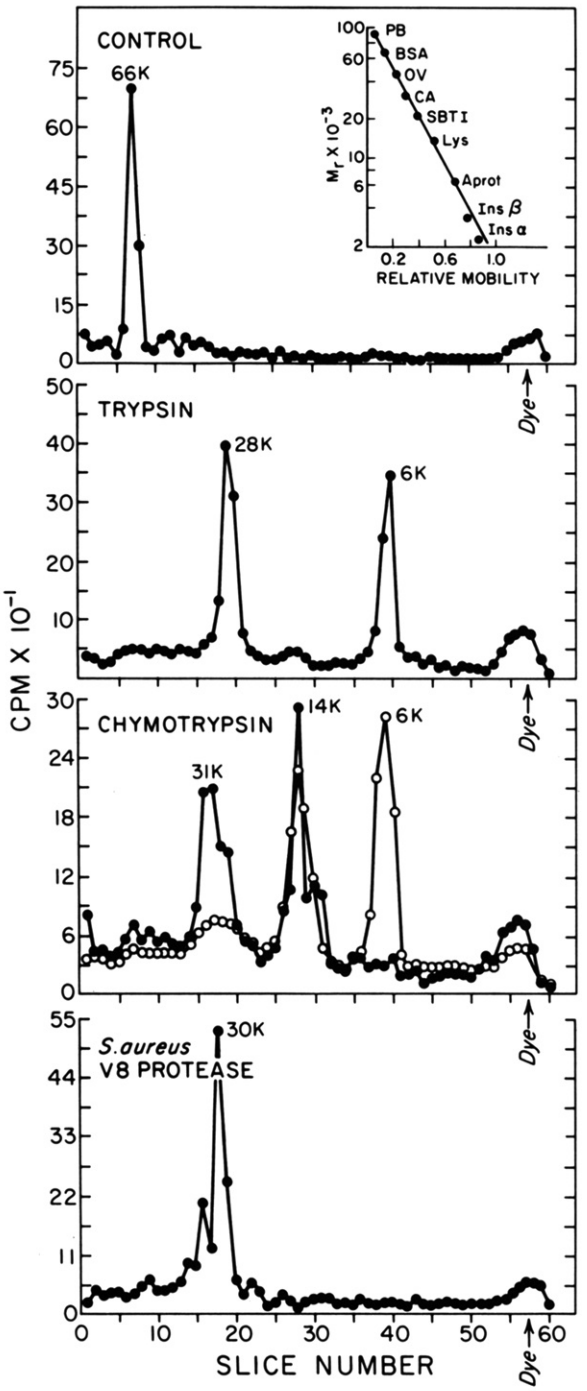

FIGURE 8: Limit digestion of the MCF-7 nuclear estrogen receptor by trypsin, α-chymotrypsin, and *S. aureus* V8 protease. The experimental design was identical with that described in the legend to Figure 2, except that more forcing enzyme conditions were used. Incubation of receptor preparations was with 10 μg of trypsin/mg of protein for 20 min at 22 °C, or with 10 μg of α-chymotrypsin/mg of protein for 20 min (closed circles) or 60 min (open circles) at 22 °C, or with 100 μg of V8 protease/mg of protein for 20 min at 22 °C prior to electrophoresis in the SDS–8 M urea gel system of Anderson et al. (1983). Control receptor preparations were incubated for 20 min at 22 °C in the absence of enzyme. Gel lanes were sliced into 2-mm segments, and radioactivity was determined by scintillation counting. Protein standards were electrophoresed in parallel gel lanes, and their relative mobilities (inset of top panel) are shown.

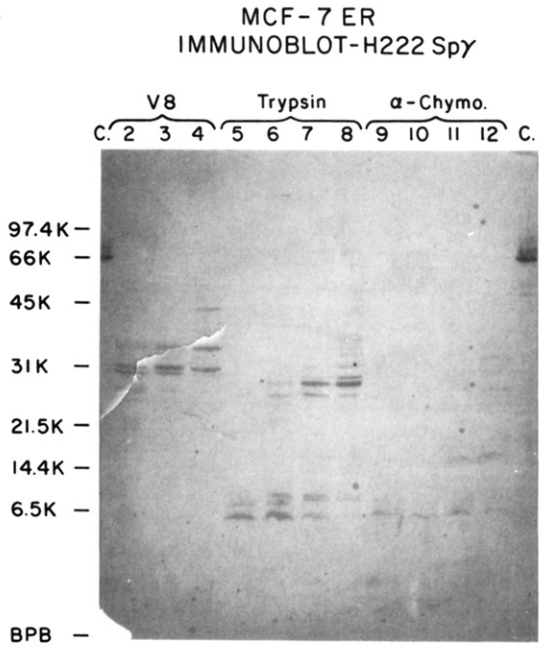

FIGURE 9: Western blot of partially proteolyzed estrogen receptor detected with the monoclonal antibody H222Spγ. MCF-7 nuclear receptor was treated with protease and electrophoresed on an SDS–10–20% polyacrylamide gradient slab gel. The proteins were then electroblotted onto nitrocellulose and detected with the monoclonal antibody H222Spγ as detailed under Materials and Methods. Lanes 1 and 13, control (C), untreated receptor incubated for 15 min at 22 °C; lanes 2–4, receptor treated with 20 μg of V8 protease/mg of protein for 60, 30, and 10 min, respectively; lanes 5–8, receptor treated with 10 μg of trypsin/mg of protein for 60, 30, 10, and 2 min, respectively; lanes 9–12, α-chymotrypsin treatment at 10 μg/mg of protein for 60, 30, 10, and 2 min, respectively.

chymotrypsin, trypsin, and V8 cleavage sites is shown in brackets between the D75 recognition region and the carboxy-terminal end of the receptor to indicate this possibility.

Since the D75 monoclonal antibody does not cross-react with estrogen receptors from the rat, it could not be used to compare human and rat estrogen receptors. However, the H222 monoclonal antibody has high affinity for both rat and human estrogen receptors (Greene, 1984; Greene et al., 1984), and the comparative studies reported here reveal similar immunoreactive fragments generated by controlled proteolysis of these two receptors. The relatively weak immunodetection reaction with the small receptor peptide fragments most likely is a reflection of the fact that an antibody raised against the native, larger protein usually binds peptide fragments of the protein with lower affinity (Benjamin, 1984). In addition, it should be stressed that the M_r ~6000 trypsin- and chymotrypsin-generated receptor fragments must be different (Figure 11) because although both retain H222 antibody recognition, the MCF-7 chymotryptic-generated fragment is also reactive with the D75 monoclonal antibody, whereas the tryptic fragment is not immunochemically reactive with the D75 antibody. [D75 antibody reactivity is lost in trypsin-generated receptor fragments smaller than M_r 31 000 (Figure 6).]

Although the primary amino acid sequence of the MCF-7 estrogen receptor deduced from receptor cDNAs has recently been reported by two groups (Green, G. L., et al., 1986; Greene S., et al., 1986), rather little is yet known about the tertiary structure of the receptor and the hormone binding domain of the receptor, although deletion experiments indicate that the carboxy-terminal half of the receptor is required for estrogen binding (Krust et al., 1986). Our observation that trypsin and chymotrypsin generate a M_r ~6000 TAZ-labeled fragment that is also detectable with the H222 monoclonal antibody should be helpful in defining the hormone binding domain of the estrogen receptor and in amino acid sequencing of this region. In addition, it is clear that the covalent labeling by TAZ is limited to a relatively small region in the receptor.

Comparison of the proteolytic cleavage fragments of the estrogen receptor with those reported previously for the glu-

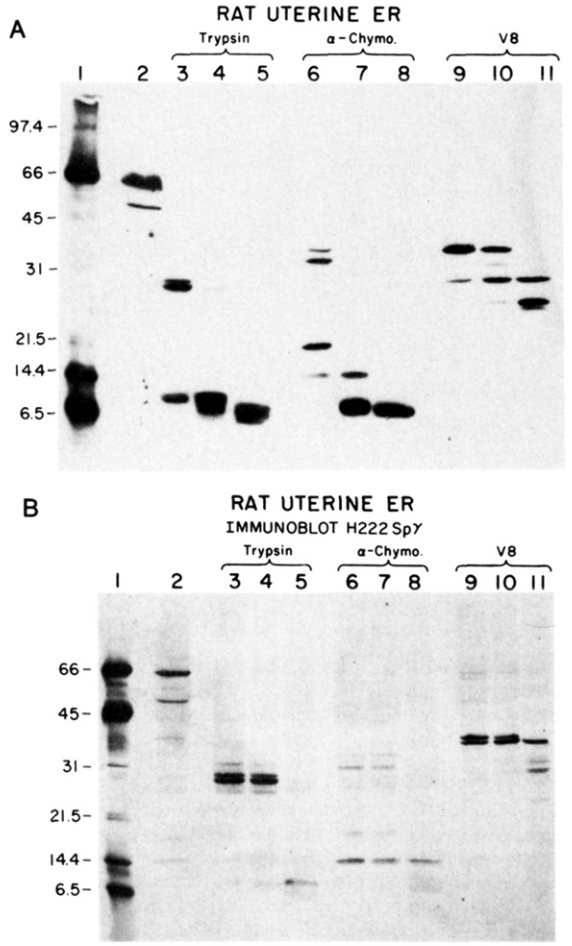

FIGURE 10: Analysis of rat uterine estrogen receptor subjected to proteolysis with trypsin, α-chymotrypsin, or V8 protease, by [^{3}H]TAZ fluorography (panel A) and monoclonal antibody H222Spγ immunoreactivity (panel B). Rat uterine cytosol receptor was covalently labeled with [^{3}H]TAZ, precipitated with 40% ammonium sulfate, redissolved, treated with protease, and electrophoresed on SDS–10–20% polyacrylamide gradient slab gels. Panel A shows a fluorograph revealing [^{3}H]TAZ-labeled receptor fragments. Panel B shows an immunoblot of receptor fragments after electroblotting onto nitrocellulose and detection with ^{35}S-labeled monoclonal antibody H222Spγ. The data in panels A and B derive from separate experiments. Lane 1, ^{14}C-labeled protein standards as molecular weight × 10^{-3} are phosphorylase *b* (97.4), bovine serum albumin (66), ovalbumin (45), carbonic anhydrase (31), soybean trypsin inhibitor (21.5), lysozyme (14.4), and aprotinin (6.5); lane 2, untreated receptor incubated 30 min at 22 °C; lanes 3–5, receptor treated with 1, 10, or 50 μg of trypsin/mg of protein for 15 min, respectively; lanes 6–8, receptor treated with 1, 10, or 50 μg of α-chymotrypsin/mg of protein for 15 min, respectively; lanes 9–11, receptor treated with 1, 10, or 100 μg of V8 protease/mg of protein for 30 min, respectively.

cocorticoid receptor affinity labeled with dexamethasone 21-mesylate (Reichman et al., 1984) and for the progesterone receptor (Birnbaumer et al., 1983; Puri & Toft, 1986) reveals some similarity in the size fragments generated. For example, trypsin treatment of each of these three receptors generates prominent meroreceptor forms of M_r 30 000 and 28 000, implying some structural similarity not only in their primary structure (Hollenberg et al., 1985; Green, G. L., et al., 1986; Greene, S., et al., 1986) but also in their higher order structure.

It is of note that the estrogen receptor shows some charge heterogeneity (Figure 1) and was resolved into two to three species of M_r 66 000 with p*I* values between 5.5 and 6.2. Steroid receptors including the progesterone receptor (Dougherty et al., 1982; Logeat et al., 1985), glucocorticoid receptor (Housely & Pratt, 1983), and estrogen receptor (Migliaccio et al., 1982, 1984) have been shown to undergo

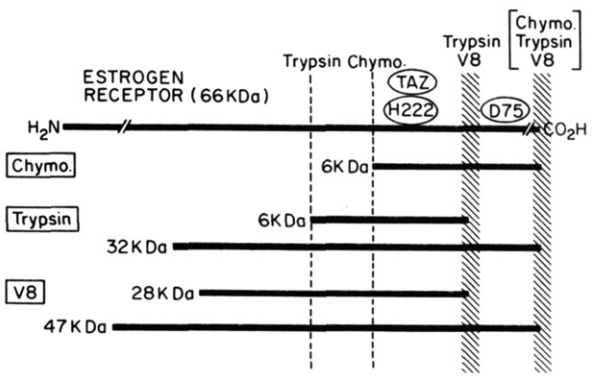

FIGURE 11: Model, derived from our protease digest data, indicating the relative positions of the TAZ attachment site and H222 and D75 antibody recognition sites on the MCF-7 estrogen receptor. The figure also shows, for each protease, the smallest fragment containing the TAZ attachment site and the smallest fragment generated by each protease that contains the TAZ attachment site and both antibody recognition sites.

phosphorylation reactions, and it is possible that charge heterogeneity may reflect different degrees of attachment of charge-modifying groups such as phosphate.

In both rat uterine and MCF-7 cells, the largest estrogen receptor species detected by antibody binding or covalent labeling with TAZ appears at an apparent molecular weight of 66 000 on SDS gels (Monsma et al., 1984; Katzenellenbogen et al., 1983). This 66 000 molecular weight receptor size is the same molecular weight as calculated for the MCF-7 estrogen receptor by analysis of cDNA for the estrogen receptor (Green, G. L., et al., 1986; Greene, S., et al., 1986). While our MCF-7 receptor extracts showed little endogenous proteolytic activity over the times studied, uterine estrogen receptor preparations frequently showed a M_r 52 000 species along with the predominant 66K form. We believe that this lower molecular weight fragment represents receptor that is proteolyzed during homogenization as its formation is completely prevented by homogenization in the presence of 20 mM molybdate and 1 mg/mL leupeptin (Monsma et al., 1984; Nardulli & Katzenellenbogen, 1986). Interestingly, treatment of affinity-labeled rat uterine and MCF-7 estrogen receptors with exogenous proteases generates prominent 52K and 30K receptor forms, species of the same size as are detected during purification of estrogen receptor preparations from uteri from a variety of mammalian species (Lubahn et al., 1985).

These studies, the first on controlled proteolysis of covalently labeled estrogen receptors, thus reveal great similarity between estrogen receptors from two different target tissues (breast and uterus) and two mammalian species (human and rat), and, in combination with antibody immunoreactivity profiles, they provide information allowing one to place these sites within the primary linear sequence of the receptor protein. We have shown that the tamoxifen aziridine binding site can be found on a small fragment of the receptor, of M_r ~6000, representing less than 10% of the intact receptor. This information will be useful in further studies on estrogen receptor structure and particularly in analysis of the amino acids in the hormone binding site of the receptor.

Registry No. TAZ, 79642-44-7.

REFERENCES

Anderson, B. L., Berry, R. W., & Telser, A. (1983) *Anal. Biochem. 132*, 365–375.

Benjamin, D. C. (1984) *Annu. Rev. Immunol. 2*, 31–50.

Birnbaumer, M. E., Schrader, W. T., & O'Malley, B. W. (1983) *J. Biol. Chem. 258*, 7331–7337.

Dougherty, J. J., Puri, R. K., & Toft, D. O. (1982) *J. Biol. Chem. 257*, 14226–14230.

Eckert, R. L., & Katzenellenbogen, B. S. (1982) *J. Biol. Chem. 257*, 8840–8846.

Gorski, J., & Gannon, F. (1976) *Annu. Rev. Physiol. 38*, 425–440.

Green, S., Walter, P., Kumar, V., Krust, A., Bornert, J.-M., Argos, P., & Chambon, P. (1986) *Nature (London) 320*, 134–139.

Greene, G. L. (1984) *Biochem. Actions Horm. 11*, 207–239.

Greene, G. L., Sobel, N. B., King, W. J., & Jensen, E. V. (1984) *J. Steroid Biochem. 20*, 51–56.

Greene, G. L., Gilna, P., Waterfield, M., Baker, A., Hort, Y., & Shine, J. (1986) *Science (Washington, D.C.) 231*, 1150–1154.

Hollenberg, S. M., Weinberger, C., Ong, E. S., Cerelli, G., Oro, G., Lebo, R., Thompson, E. B., Rosenfeld, M. G., & Evans, R. M. (1985) *Nature (London) 318*, 635–641.

Housley, P. R., & Pratt, W. B. (1983) *J. Biol. Chem. 258*, 4630–4636.

Katzenellenbogen, B. S. (1980) *Annu. Rev. Physiol. 42*, 17–35.

Katzenellenbogen, B. S., Norman, M. J., Eckert, R. E., Peltz, S. W., & Mangel, W. F. (1984) *Cancer Res. 44*, 112–119.

Katzenellenbogen, B. S., Miller, M. A., Mullick, A., & Sheen, Y. Y. (1985) *Breast Cancer Res. Treat. 5*, 231–243.

Katzenellenbogen, B. S., Elliston, J., Monsma, F. J., Jr., & Greene, G. L. (1986) *Endocrinology (Baltimore) 118 (Suppl.)*, 95.

Katzenellenbogen, J. A., & Katzenellenbogen, B. S. (1984) *Vitam. Horm. (N.Y.) 41*, 213–274.

Katzenellenbogen, J. A., Carlson, K. E., Heiman, D. F., Robertson, D. W., Wei, L. L., & Katzenellenbogen, B. S. (1983) *J. Biol. Chem. 258*, 3487–3495.

King, W. J., & Greene, G. L. (1984) *Nature (London) 307*, 745–747.

Krust, A., Green, S., Argos, P., Kumar, V., Walter, P., Bornert, J.-M., & Chambon, P. (1986) *EMBO J. 5*, 891–897.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–684.

Logeat, F., LeCunff, M., Pamphile, R., & Milgrom, E. (1985) *Biochem. Biophys. Res. Commun. 131*, 421–427.

Lubahn, D. B., McCarty, K. S., Jr., & McCarty, K. S., Sr. (1985) *J. Biol. Chem. 260*, 2515–2526.

Migliaccio, A., Lastoria, S., Moncharmont, B., Rotondi, A., & Auricchio, F. (1982) *Biochem. Biophys. Res. Commun. 109*, 1002–1007.

Migliaccio, A., Rotondi, A., & Auricchio, F. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 5921–5925.

Monsma, F. J., Katzenellenbogen, B. S., Miller, M. A., Ziegler, Y. S., & Katzenellenbogen, J. A. (1984) *Endocrinology (Baltimore) 115*, 143–153.

Nardulli, A. M., & Katzenellenbogen, B. S. (1986) *Endocrinology (Baltimore) 119*, 2038–2046.

Puri, R. K., & Toft, D. O. (1986) *J. Biol. Chem. 261*, 5651–5657.

Reichman, M. E., Foster, C. M., Eisen, L. P., Eisen, H. J. Torain, B. F, & Simons, S. S., Jr. (1984) *Biochemistry 23*, 5376–5384.

Reiner, G. C. A., & Katzenellenbogen, B. S. (1986) *Cancer Res. 46*, 1124–1131.

Sherman, M. R., & Stevens, J. (1984) *Annu. Rev. Physiol. 46*, 83–105.

Towbin, H., Staehelin, T., & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350–4355.

Walter, P., Green, S., Greene, G. L., Krust, A., Bornert, J. M., Jeltsch, J. M., Staub, A., Jensen, E., Scrace, G., Waterfield, M., & Chambon, P. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 7889–7893.

Welshons, W. V., Lieberman, M. E., & Gorski, J. (1984) *Nature (London) 307*, 747–749.