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Nuclear estrogen receptor molecular heterogeneity in the mouse uterus

(protein modification/molecular forms/estradiol/steroid hormone/nuclear receptor)

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ABSTRACT **Holomeric estrogen receptor (ER) prepared from ovariectomized mouse uteri displays heterogeneous electrophoretic mobility when analyzed by NaDodSO$_4$/PAGE. ER derived from nuclei (ER$_n$) appears as a closely spaced doublet having apparent molecular masses of 66.4 and 65 kDa, while ER from the cytosolic compartment (ER$_c$) has a single band of 65 kDa. Both partially purified ER$_c$ and the 8S form of unactivated ER$_c$ show only the 65-kDa band. The appearance of the ER$_n$ doublet is hormonally inducible, and the relative proportions of the two doublet bands are influenced by the type of hormone treatment, with weakly estrogenic compounds yielding the lower band as predominant while potent estrogens increase the proportion of the upper band. Steroid binding of the ER$_n$ doublet was determined by [^{3}H]tamoxifen aziridine affinity labeling of both the 66.4- and the 65-kDa peptides; binding to the 65-kDa peptide was predominant. The ER$_n$ doublet displays a time dependency after estrogen administration with maximal amounts occurring in a bimodal fashion at 1 and 8 hr.**

Steroid hormones exert some of their actions in target tissues through a specific receptor mechanism (1). Although the precise molecular mechanisms of action are not well understood, the generally accepted hypothesis is that the hormone binds to a specific receptor protein. This binding causes a conformational change in the receptor that produces a nuclear-residing form with increased affinity for DNA. This hormone–receptor complex then interacts with specific DNA and chromatin sites, causing modulation of gene expression for certain proteins. A number of reports have described the molecular characterization of the estrogen protein including gross physical determination of molecular weight and pI (2), kinetic data on hormone binding (3), amino acid sequences (4), and the DNA coding region sequence for the receptor protein (5). In addition, intracellular receptor distributions within uterine tissue after estrogen treatment have been reported for the mouse (6) and the rat (7). Recent studies by Auricchio and co-workers (8) have suggested that phosphorylation/dephosphorylation of the receptor protein may be important in ligand binding activity and subsequent processing. The intracellular location of the specific kinase and phosphatase enzyme has been elucidated (9). Nonetheless, a paucity of data exists with regard to the actual regulation of receptor protein activity as directly induced by the hormone. Recent reports (10, 11) have suggested the phosphorylation of tyrosines as a possible regulatory point; however, the relationship between this phenomenon and hormonal activation of receptor has not been established conclusively (12). Other reports indicate that, in the case of the estrogen receptor (ER), estradiol binding to the receptor directly influences receptor affinity for nonspecific nuclear binding sites (13), mechanistically theorized as a change in receptor conformation. However, it has not been shown that ligands of various agonistic potencies cause altered conformational changes in the receptor, as would be necessary to produce differing hormonal actions.

Here we report the description of nuclear-specific heterogeneous forms of ER isolated from mouse uterine tissue. These forms are detectable by differences in mobility under modified NaDodSO$_4$/PAGE conditions. The proportions of the receptor forms are dependent on the specific hormone administered. After estrogen treatment the receptor forms demonstrate an alteration in a temporal pattern that may play a role in the mechanism of hormone receptor action.

MATERIALS AND METHODS

Animal and Tissue Preparation. Animal and tissue preparation was identical to that previously reported (14) using ovariectomized CD-1 [ICR]BR mice from Charles River Breeding Laboratories. Animals were held for 14 days after ovariectomy before use in experimental procedures. Intraluminal injections of hormone in 3 μl of saline/ethanol vehicle were delivered by a 10-μl Hamilton syringe to each uterine horn of mice anesthetized by Nembutal. Animals were sacrificed by cervical dislocation, and the uteri were rapidly removed, frozen on dry ice, and stored at −70°C until use.

At the time of use, uteri were placed in ice-cold TEGM-P buffer [10 mM Tris·HCl/1.5 mM EDTA/10% (vol/vol) glycerol/3 mM $MgCl_2$/3 mM EGTA containing leupeptin, antipain, soybean trypsin inhibitor, and chymostatin at 50 μg of each per ml (pH 7.6 at 25°C)]. The tissue was processed as previously described (14) to give a washed 1000 × *g* pellet (nuclei) and a 105,000 × *g* supernatant (cytosol) for use in NaDodSO$_4$/PAGE and receptor binding experiments.

Steroids. 17β-[2,4,6,7-^{3}H]Estradiol (110 Ci/mmol; 1 Ci = 37 GBq) was purchased from Du Pont–New England Nuclear and brought to >98% radiochemical purity by thin-layer chromatography (14). [*ring*-^{3}H]Tamoxifen aziridine (15–30 mCi/mmol) was obtained from Amersham and used without further purification. Unlabeled steroids were purchased from Steraloids (Wilton, NH) and also were used without further purification.

Binding Assays. Cytosol receptor (ER$_c$) levels were measured using an *in vitro* estradiol saturation binding assay (14) with 7.5 nM [^{3}H]estradiol. Nuclear receptor (ER$_n$) levels were measured by a previously reported modified exchange method (14) using a 7.5 nM [^{3}H]estradiol concentration. ER$_n$ levels were normalized to 100 μg of DNA, and ER$_c$ levels were expressed per mg of total cytosolic protein.

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Abbreviations: ER, estrogen receptor; ER$_n$ and ER$_c$, nuclear and cytosolic ER.

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Sample Preparation for $NaDodSO_4$/PAGE. Nuclear samples containing 150 μg of DNA in a volume of 80 μl of TEGM-P buffer were brought to a $NaDodSO_4$ concentration of 5% (wt/vol) and the salt concentration was increased to 0.4 M NaCl. The sample was heated to 100°C for 2 min, diluted with 30 μl of deionized water, and then clarified by centrifugation at 200,000 × *g* at 30°C for 110 min. The resulting supernatant was removed and diluted with an equal volume of deionized H_2O. Finally, the sample was precipitated by the addition of 4 vol of acetone at −20°C followed by incubation at −70°C for 30 min. The protein precipitate was collected by centrifugation at 10,000 × *g* at 4°C for 10 min, and the resulting pellet was suspended in 10 μl of 10% (wt/vol) $NaDodSO_4$ plus 15 μl of 2× sample buffer [78 mM Tris·HCl/2.2% $NaDodSO_4$/11% (wt/vol) sucrose/0.002% bromophenyl blue, pH 6.8] and stored at −70°C until the day of electrophoresis.

Cytosolic samples were precipitated directly by addition of acetone and processed identically to the nuclear samples commencing at the step following the acetone precipitation. Partially purified ER_c, the isolation of which has been reported (15), was prepared in an identical fashion to cytosol with the exception that 150 μg of bovine insulin (Collaborative Research, Waltham, MA) was included as a carrier protein.

The ^{14}C-methylated protein mixture used as molecular weight markers for gels analyzed by immunochemical detection or by fluorography was obtained from Amersham. Amounts loaded per lane ranged from 7.5 nCi (total radioactivity) for gels to be analyzed by fluorography (corresponding to 0.833 nCi for each of six proteins) to 25 nCi for gels analyzed by immunochemical detection followed by autoradiography.

Coomassie brilliant blue-prestained bovine serum albumin and lactate dehydrogenase molecular mass standards were purchased from Diversified Biotech (Newton Center, MA).

$NaDodSO_4$/PAGE. Reagents for electrophoresis were obtained from Bio-Rad. $NaDodSO_4$/polyacrylamide slab gel electrophoresis was carried out according to O'Farrell (16) with the exception of using an 8% resolution gel with a 3% acrylamide stacking gel. Sample loading was maximized to 250 μg of protein, or 120 μg of DNA, per lane. All samples were treated prior to loading with 48 mM dithiothreitol, then heated at 100°C for 2 min, and exposed to 0.13 M iodoacetamide/0.07 M Tris base to block available sulfhydryl groups. Polyacrylamide gels were stained with silver according to a modified method of Morrissey as reported (17).

Immunoblotting and Immunochemical Detection. Bilateral diffusion blotting of proteins to nitrocellulose was carried out as described (17). Pure nitrocellulose of 0.20-μm pore size was purchased from Schleicher & Schuell. Immunochemical detection of ER from mouse uteri was performed as reported (15). The ER monoclonal antibody H-222 was the gift of Chris Nolan (Abbott Laboratories). Monoclonal antibody H-226 was a gift from Geoffrey Greene (Ben May Laboratories for Cancer Research, University of Chicago). ^{125}I-labeled sheep anti-rat IgG $F(ab')_2$ fragment was obtained from Amersham. Autoradiography was performed using Du Pont Cronex Quanta III intensifying screens and Kodak XAR-5 x-ray film.

Densitometric Measurements. Bands from fluorographs and autoradiographs were quantified by laser densitometry using an LKB UltraScan XL densitometer. Data were analyzed on a microcomputer by a gaussian curve-fit technique with a LKB 2400 GelScan XL version 1.0 software. All scans used a line beam laser and represent the average of three scans across the length of the bands to minimize the influence of any band distortions on the calculations. The proportions and positions of receptor banding for all gels were quantified and confirmed by laser densitometric analysis.

Protein Determination. Protein concentrations of nondenatured samples were determined by the method of Bradford (18) using rabbit gamma globulin as the reference standard. $NaDodSO_4$-denatured proteins were quantified by the Lowry assay (19), also using gamma globulin as the reference.

DNA Determination. DNA concentrations of nuclear samples were determined by the method of Labarca and Paigen (20) with modifications to increase sample stability. Five-microliter aliquots of nuclear suspension containing 4–25 μg of DNA were isolated and immediately suspended in 2.7 ml of high-salt buffer (10 mM Tris·HCl/2.2 M NaCl/1.5 mM EDTA, pH 7.6 at 25°C) in 13 × 100 mm borosilicate glass tubes. The samples could then be stored indefinitely (at least 3 weeks) at 4°C without degradation. For analysis, samples were brought to room temperature and treated with 100 μl of Hoechst 33258 reagent (10 μg/ml). Fluorescence was determined using a Perkin–Elmer D450 fluorescence spectrophotometer in the original glass test tubes, thus obviating the need for transfer to cuvettes. Calf thymus DNA (Sigma) was used as the reference standard.

RESULTS

Samples of ER_n and ER_c were analyzed by $NaDodSO_4$/PAGE. To achieve better separation of the 65-kDa ER_n doublet, an 8% uniform gel of dimensions 28 cm length by 14 cm width was used. As shown in Fig. 1*A* (lane 2), these conditions gave a clear demarcation of a doublet form with a visual band separation of ≈4 mm that could be detected as two distinct shouldered peaks by laser densitometry (Fig. 1*B*). The relative mobilities of the two bands corresponded to molecular masses of 66.4 and 65 kDa. The ER_c, however, displayed only one band (63.7 kDa; Fig. 1*A*, lanes 3 and 5) and partially purified ER_c (lane 4) also demonstrated a single band (65 kDa). The lower apparent molecular mass of the ER_c compared to partially purified ER was due to the presence of mouse serum albumin (MSA) in the preparation, which produced a downward influence on the ER band. This is most evident in comparison of the immunoreactivity of the ER (lane 5) and the pattern of Coomassie blue staining of the cytosol sample (lane 6). The mass of MSA on the gel results in this distortion of the proper banding pattern. ER_c was separated from MSA by isolating the 8S-sedimenting low-salt form by sucrose gradient centrifugation. This sample (lane 7) gave a form with a position corresponding to 65 kDa. Sample-mixing experiments with partially purified ER_c and ER_n indicated that, on mixing, the partially purified ER_c comigrates to the position of the lower ER_n band (data not shown). Thus the correct determination of relative mobilities was established by collective mixing experiments to be ≈65.0 kDa for all cytosolic-derived ER, 65.0 kDa for the lower ER_n band, and ≈66.5 kDa for the upper ER_n species. Nuclei isolated 1 hr after estradiol injection were analyzed by 0.4 M KCl extraction. The salt-resistant nuclear component was analyzed and demonstrated that the upper doublet band of the ER_n was primarily associated with this nuclear fraction (lane 8).

The appearance of the ER_n doublet in response to hormone was further investigated (Fig. 2). As quantified by laser densitometry, gels of ER_n isolated 1 hr after estradiol injection (lane 3) indicated an equal proportion of upper and lower bands (1:1 ratio). Weakly estrogenic compounds, such as estriol (lane 1) or 16α-estradiol (lane 2), produced a different proportion of ER_n than that produced by estradiol, with the lower band form being predominant by a ratio of 3:1. These differences were not due to the level of ER_n present in the samples since binding assays showed the levels for all three groups to be comparable.

The functionality of the steroid binding domain in the ER_n doublet was also examined to discern any differences in

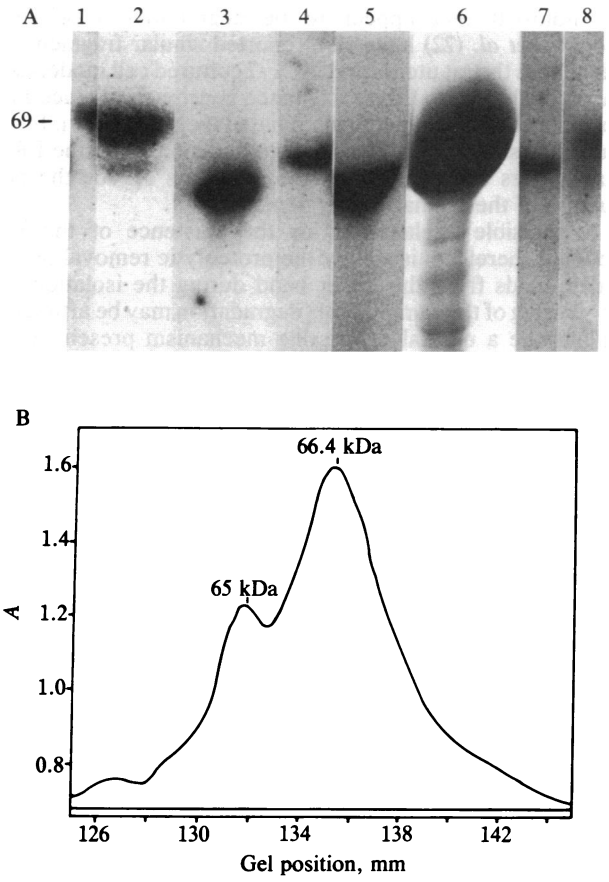

FIG. 1. Autoradiography of the ER from subcellular fractions separated by $NaDodSO_4$/PAGE. (*A*) Receptor samples from various subcellular fractions of uterine tissue from ovariectomized mice were analyzed on a uniform 8%, 14 × 28 cm, $NaDodSO_4$/polyacrylamide gel and immunochemically probed. Lanes: 1, ^{14}C-methylated protein standards (bovine serum albumin, 69 kDa); 2, ER present in nuclei containing 100 μg of DNA 1 hr after i.p. injection of estradiol; 3, ER present in 100 μg of total cytosolic protein; 4, 15 ng of partially purified cytosolic ER as quantified by a radioactive ligand binding assay; 5, ER present in 250 μg of cytosolic protein probed with monoclonal antibody H-222; 6, Coomassie blue R-250 staining of a sample identical to that of lane 5; 7, ER_c isolated as the 8S component from a sucrose gradient; 8, profile of 0.4 M KCl-resistant ER_n isolated 1 hr after i.p. injection of estradiol. (*B*) The autoradiograph shown in lane 2 of *A* was analyzed by laser densitometry.

molecular properties between the two peptides. As shown in Fig. 3*A* (lanes 1 and 2), both doublet bands bound ligand in a specific manner as determined by fluorography of ER_n

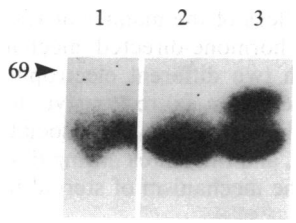

FIG. 2. Induction of the 65-kDa ER_n doublet as a function of the estrogenic potency of the bound ligand. Nuclear fractions were analyzed as in Fig. 1 following administration of various hormones. In all lanes, 18 ng of ER was loaded as quantified by radioactive ligand exchange assay. Lanes: 1 and 3, the ER_n doublet present 1 hr after i.p. injection of estriol and 17β-estradiol (10 μg/kg), respectively; 2, ER_n doublet present 30 min after i.p. injection of 16α-estradiol (20 μg/kg).

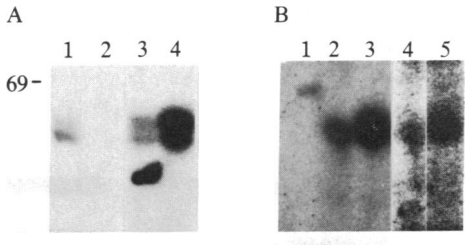

FIG. 3. Presence of functional domains in the ER_n doublet. (*A*) Steroid binding ability and specificity were demonstrated by treatment *in vivo* of uteri from ovariectomized mice with 20 nM [*ring*-^3H]tamoxifen aziridine with or without a 200-fold excess of diethylstilbestrol. Samples were processed as in Fig. 1. Lanes: 1 and 2, total ligand binding and nonspecific binding in the presence of a 200-fold excess of diethylstilbestrol; 3 and 4, autoradiograms of the samples from lanes 1 and 2 as immunochemically probed by monoclonal antibody H-222. (*B*) Detection of the DNA binding region of ER_n and ER_c. Lanes: 1, marker proteins; 2 and 3, ER_n; 4 and 5, ER_n probed with H-222 and H-226, respectively. Each nuclear sample lane represents extracted proteins (≈150 μg) corresponding to 100 μg of DNA. Cytosolic samples represent 100 μg of acetone-precipitable protein.

samples labeled with [*ring*-^3H]tamoxifen aziridine *in vivo*. However, it should be noted that the lower band bound ligand to a greater extent, since immunodetection should equal amounts of both bands in the respective sample (lanes 3 and 4).

The presence of the DNA binding domain for the ER was also investigated, and the results are shown in Fig. 3*B*. The anti-ER monoclonal antibody H-226, which recognizes an epitope near or in the DNA binding domain (21), detected both bands of the doublet. Hence, the DNA binding domain is present in both bands of the ER_n doublet (lanes 4 and 5).

To determine whether the relative amounts of the ER_n change during hormone stimulation, the receptor pattern was analyzed at different times following hormone treatment. The profile is shown in Fig. 4*A*, where different forms of ER_n with relative mobilities corresponding to molecular masses of 65 and 54 kDa are seen. These results are consistent with other investigators' studies of ER from sources such as MCF-7 cells (22) and are thought to represent a 65-kDa holoreceptor and two proteolytic fragments of lower molecular mass (15, 22). The appearance of the doublet was clearly evident and dependent on the hormonal state of the tissue, with maximal amounts of the two peptides occurring by 1 hr after injection of estradiol (Fig. 4*A*, lane 4). Analysis of the autoradiograph by laser densitometric scanning (Fig. 4*B*) confirmed this visual observation. The ratio of the amounts of the two bands changed in a temporal relation following the administration of estradiol. As previously reported† the appearance of the 65-kDa band displays a bimodal temporal pattern. The densitometric scan indicated that the initial upper/lower band ratio (1:1) was maintained until the time of the second peak at ≈9.5 hr, when there was a change to a lower/upper band ratio of ≈5:1. The ER_c from the same tissue samples did not demonstrate a 65-kDa doublet but only a single band at 64 kDa (data not shown).

DISCUSSION

The observations reported here provide evidence of a specific hormonally regulated modification of the ER in response to hormone administration *in vivo*. The response, as detected by $NaDodSO_4$/PAGE, is the formation of a closely spaced ER

†Korach, K. S., Horigome, T. & Golding, T. S., 68th Annual Endocrine Society Meeting, June 25–27, 1986, Anaheim, CA, abstr. 729.

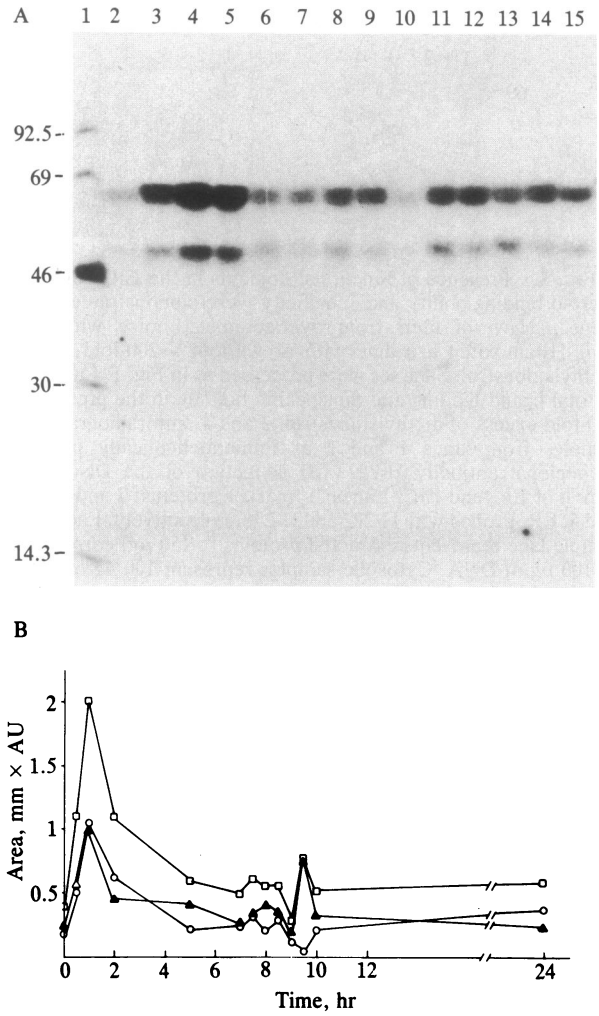

FIG. 4. Translocation profile of mouse uterine ER_n as analyzed by (9–15%) exponential gradient $NaDodSO_4$/PAGE. (*A*) Ovariectomized mice were treated i.p. with estradiol at 10 μg/kg or with saline vehicle. Animals were sacrificed at 0.5, 1, 2, 5, 7, 7.5, 8, 8.5, 9, 9.5, 10, and 24 hr. $NaDodSO_4$-soluble proteins from an amount of nuclei standardized to contain 100 μg of DNA were analyzed on a 9–15%, 12 × 14 cm, exponential gradient polyacrylamide gel. The proteins were blotted onto nitrocellulose by diffusion, probed with monoclonal antibody H-222 or with normal rat serum IgG (data not shown) followed by ^{125}I-labeled secondary antibody, and then processed for autoradiography. Lanes: 1, ^{14}C-methylated protein standards; 2 and 10, 0- and 8-hr saline controls, respectively; 3–9 and 11–15, estradiol-treated samples (0.5, 1, 2, 5, 7, 7.5, 8, 8.5, 9, 9.5, 10, and 24 hr, respectively). (*B*) Laser densitometric analysis of the autoradiogram. Areas under each peak in the 65-kDa region were quantified and plotted as a function of area of the doublet components, and total area, versus time after estrogen administration. □, Total area; ▲, lower peak area; ○, upper peak area; AU, absorbance units. This analysis is representative of three separate experiments.

doublet of 65 and 66.5 kDa that is exclusively localized to the nucleus and displays a bimodal temporal pattern following administration of estradiol. Furthermore, the relative proportions of the two bands are affected by the occupying ligand, with potent estrogens (e.g., estradiol) producing equivalent amounts of the upper and lower bands at certain time periods.

The mechanism of doublet formation is not definitively known. Two hypotheses can be considered, involving proteolysis or phosphorylation, based on known modifications to steroid receptors. With regard to the former, previous findings in this laboratory† for the mouse uterus have detailed the existence of several smaller (54 and 37 kDa) fragments of the holo-ER that appear to be degradative fragments. Monsma *et al.* (22) have also reported similar fragments of ER in both the rat uterus and MCF-7 cultured cell models and have concluded that their existence is artifactual since high concentrations of leupeptin eliminated their formation. In our studies, a similar result was evident for mouse uterine ER in that buffers containing protease inhibitors reduce the production of these smaller fragments (15).

A plausible explanation for the existence of the ER_n doublet, therefore, is simply the proteolytic removal of ≈15 amino acids from the upper band during the isolation and processing of the sample. This degradation may be artifactual or may be a normal processing mechanism present in the cells; the data, however, support the latter based on the following observations. First, the protease inhibitors that inhibited the formation of the 54- and 37-kDa fragments did not prevent the formation of the ER_n doublet. Second, the localization of the doublet is exclusively nuclear, contrary to the ubiquitous appearance of the previously mentioned smaller fragments. Third, homogenization of tissue directly in a buffer containing 0.2% $NaDodSO_4$ eliminated the formation of the smaller fragments but did not alter the formation of the 65-kDa doublet (data not shown). This result eliminates the possibility of *in vitro* artifactual degradation but has no bearing on the occurrence of *in vivo* proteolytic processing.

Alternatively, a phosphorylative mechanism could be hypothesized for the production of the ER_n doublet. Evidence of steroid receptor phosphorylation has been demonstrated for the glucocorticoid (23), androgen (24), and progesterone (25) receptors. Additionally, Aurrichio *et al.* (9) have reported the phosphorylation of the ER from calf uteri and have localized the involved phosphatase in the cytosol and the responsible kinase in the nuclei. A change in molecular mass as a steroid receptor protein is phosphorylated has been reported by Horwitz *et al.* (26) for the progesterone receptor; the existence of the upper band of the ER_n, which has a greater molecular mass than any other ER, whether nuclear or cytosolic, would be consistent as being a phosphorylated species. In addition, the localization of the doublet exclusively to the nucleus would be appropriate considering the kinase location reported by Aurrichio *et al.* (9). A possible role for this modified ER_n form is not clear; however, it appears to be associated with the salt-resistant fraction, which has been suggested to be the tightly bound chromatin sites of ER nuclear interactions (7) or the site of nuclear matrix ER (27). These results suggest that the ER_n is changed to this modified form to specifically interact with these nuclear sites.

In conclusion, the mechanism for the formation of the ER_n doublet is not clear, but molecular modification occurs that may involve phosphorylation;‡ however, other protein modifications such as glycosylation and acylation have not been ruled out. Regardless of the manner of ER_n modification, a nuclear specific hormone-directed mechanism exists that creates ER_n with two different electrophoretic mobilities. Since this phenomenon is responsive to the estrogenic potency of the bound ligand, is associated with specific nuclear fractions, and fluctuates during tissue stimulation, it is important to the mechanism of steroid hormone action.

‡Golding, T. S. & Korach, K. S., 69th Annual Endocrine Society Meeting, June 10–12, 1987, Indianapolis, IN, abstr. 160.

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