Endocrine, *vol. 22, no. 3, 211–223, December 2003* 0969–711X/03/22:211–223/$25.00

Characterization of a Membrane-Associated Estrogen Receptor in a Rat Hypothalamic Cell Line (D12)

Darlene C. Deecher, Pamela Swiggard, Donald E. Frail,[1] and Lawrence T. O'Connor[2]

Women's Health Research Institute, Wyeth Research, Collegeville, PA
[1]Pfizer, St. Louis, MO; and [2]American Medical Association, Chicago, IL

The ability of estrogens to produce rapid changes in cellular function has been firmly established. The question remains whether these changes are mediated by a modified form of the nuclear estrogen receptor (ER) that is associated with the plasma membrane (mER) or by a completely novel membrane receptor. Therefore, we characterized the biochemical properties of the nuclear and membrane-associated ERs expressed endogenously in a rat hypothalamic endothelial cell line (D12). Radioligand binding experiments using D12 membrane fractions showed that these cells exhibit properties consistent with a binding site specific for estrogens (mER). Equilibrium binding assays using [^{125}I]16-α-iodo-3,17-β-estradiol revealed saturable binding to mER, an affinity value similar to nuclear ER, with differing receptor expression levels. Competition assays revealed that 9 of 12 ER ligands tested had comparable affinities for mER and ER. For example, 17-α-estradiol and estrone had similar binding characteristics for both receptors while differences were noted for raloxifene, 17β-estradiol (E2), and genistein. Western blot and immunocytochemical analyses using antibodies specific for ERα confirmed that D12 cells expressed a membrane-associated protein with a molecular mass (67 kDa) similar to that of ERα that colocalized with caveolae-enriched membranes. A rapid increase in intracellar Ca^{2+} levels in the presence of E2 suggests that mER can mediate physiologic changes through calcium mobilization. These data support the expression of mER in these brain-derived endothelial cells that is similar to, but biochemically distinguishable from, nuclear ERα.

Key Words: Membrane estrogen receptor; estradiol; estrone; raloxifene; radioligands; MC20.

Introduction

Steroids can influence the physiologic activity of many different cell types located in a wide variety of tissues. Traditionally, their effects are thought to be mediated through binding to cognate intracellular receptors to form ligand-dependent transcription factors *(1)*. The ability of ligand-receptor complexes to modulate transcription and induce changes in cellular function is referred to as the genomic activity of steroids. Recently, steroids have also been shown to induce rapid changes in cell physiology that occur on the order of minutes rather than hours and therefore cannot be attributed solely to changes in gene transcription. This activity has been referred to as the nongenomic action of steroids *(2,3)*. Nongenomic activities have been observed with a number of different steroids including progesterone *(4,5)*, estrogens *(6–9)*, androgens *(10,11)*, glucocorticoids *(12,13)*, and mineralocorticoids *(14)*. Studies indicate that rapid signaling by steroids occurs in a wide range of tissues and through a variety of different molecular pathways. Much of the recent interest in the nongenomic activity of steroids has been focused on identifying the membrane-associated receptors that trigger initial events involved in rapid signaling. Although none of these receptors has been cloned to date, their biologic properties indicate that they have pharmacologic characteristics distinct from their nuclear counterparts and signal through traditional second-messenger pathways.

In spite of our increased understanding of the signaling pathways that mediate the nongenomic action of steroids, there still remains a question of functional consequence. Perhaps our best understanding of a physiologic role for the nongenomic action of steroids is the rapid vasodilatory effect mediated by estrogen in the cardiovascular system *(15,16)*. While estrogens have long been considered a female hormone involved primarily in reproduction, novel effects on vasodilation have prompted a reevaluation of estrogens for their role in the cardiovascular and central nervous systems. Although the mechanisms underlying these effects remain undefined, a growing body of literature indicates that nongenomic actions of estrogens may play a role in these systems. The ability of estrogens to modulate both genomic and nongenomic mechanisms results in activation of signaling

Received May 19, 2003; Revised August 27, 2003; Accepted August 27, 2003.
Author to whom all correspondence and reprint requests should be addressed: Dr. Darlene C. Deecher, Women's Health Research Institute, Wyeth Research N3114, 500 Arcola Road, Collegeville, PA 19426. E-mail: Deeched@Wyeth.com

pathways that may occur within minutes or require hours in a single cell type. This diversity of effects can only partially be explained by our current understanding of the structure and function of estrogen receptor (ER). Previous models of ER interactions can be used to understand the slower, genomic signaling pathways mediated by estrogens but fail to explain the rapid signaling effects. These rapid effects of estrogens are independent of transcription and, thus, do not fit the classic concept of nuclear localization and genomic regulation by liganded ER. However, in some systems, rapid activation of estrogen-induced signaling pathways can be blocked by the same synthetic ER antagonists that block transcriptional activation by nuclear ER *(17)*. This suggests that the receptor responsible for rapid cellular responses to estrogen may be similar to that responsible for slower genomic effects.

The existence of a putative membrane-associated ER (mER) has modified and broadened our thinking of current ER models by which steroid hormones produce both rapid signaling and downstream transcriptional events. The presence of binding sites for estrogens on the plasma membrane surface has been a topic of debate for many years since specific binding sites for estrogens were first reported in endometrial cells *(18)*. Although the molecular identity of the mER remains unknown, membrane binding sites for 17β-estradiol (E2) have been reported in a variety of tissues, such as brain *(19)*, uterus *(20)*, liver *(21)*, and bone *(9)*, with various signaling pathways implicated *(22)*. The role of these binding sites in signaling pathways and their pharmacology profiles in these various tissues have not been fully explored due to the lack of robust expression of this putative receptor.

To characterize the biochemical properties of the elusive mER, preliminary screening efforts were carried out in our laboratory on a variety of neuronal cell lines and rat brain–specific regions to identify membrane-associated estrogen radiolabeling and/or rapid E_2-induced calcium mobilization. Profiling of these various cell lines and brain region preparations identified samples that expressed nuclear ER alone, nuclear ER in combination with mER, or samples that only had mER present. Unfortunately, the preparations that contained mER alone did not express this protein at high enough concentrations to complete a biochemical characterization using reasonable measures. Thus, an immortalized cell line derived from the rat hypothalamus (D12) was chosen for our study. This cell line met defined criteria based on measurable radioligand labeling of both nuclear and mER and statistically significant calcium changes in response to E_2. Phenotyping, immunocytochemistry (ICC), and functional measures indicated that the D12 culture consists primarily of endothelial cells and that these cells express both ER and mER endogenously. Extensive characterization of mER was carried out on these cells, and the biochemical profiling is described herein.

Results

Immunocytochemical Characterization of D12 Cells

The D12 cell line was subcloned at Wyeth from an immortalized cell line derived from the hypothalamic region of an embryonic (E18) rat brain *(23)*. The most prominent colony morphology observed in D12 cultures was the clustering of cells into flattened sheets that resemble a "cobblestone matrix," reminiscent of that typically associated with endothelial cells. Immunocytochemical analyses of these cultures confirmed that the majority of cells were endothelial (>95%) based on staining for von Willebrand factor and the uptake of DiI-Ac-LDL (data not shown). The vast majority of cells also stained positive for glial fibrillary acidic protein (GFAP), a marker that has recently been used to identify endothelial cells derived from the brain *(24)*. A small subpopulation of cells in D12 cultures (<5%) appeared to be of neuronal origin based on staining for the neuronal cytoskeletal protein neurofilament M while no staining was observed for the fibroblast marker fibronectin. D12 cells in these cultures also expressed abundant levels of nuclear ER, as previously reported *(23)*. Since the vast majority of cells in D12 cultures appeared to be of an endothelial lineage, cells were subsequently grown in a specialized endothelial growth medium (Clonetics) to determine whether an activated phenotype could be induced. Incubation of cells for 24 h in the specialized medium caused them to develop an ameboid spiny phenotype, consistent with an activated endothelial cell (data not shown).

Rapid Effects of Estrogen on D12 Cells

Primary cultures of endothelial cells demonstrating a rapid response to estrogens have been published *(25)*. To determine whether D12 cells responded in a similar manner, changes in intracellular calcium were recorded by fluorimetry following exposure to 100 n*M* E_2. A twofold change (maximal change: 120 n*M* Ca^{2+}) in calcium was observed in D12 cells within 5 min after the addition of 100 n*M* E_2 (Fig. 1). Cells exposed to 10 n*M* E_2 showed no detectable calcium changes using this method. Cells exposed to 200 n*M* E_2 (data not shown) showed the same twofold change in calcium mobilization as illustrated using 100 n*M* E_2 (Fig. 1). In these assays, cells were evaluated in calcium-free buffer, indicating that changes in intracellular Ca^{2+} were caused by mobilization of intracellular calcium stores.

Isolation of mER

The ability of E_2 to induce rapid changes in intracellular Ca^{2+} levels in D12 cells indicated a nongenomic mechanism of action possibly involving a membrane-associated receptor. Therefore, binding interactions were assessed using cytosolic (S2) and membrane (P2) preparations in radioligand-binding assays. Initial determination of the binding of 200 p*M* of [^{125}I]16-α-iodo-3,17-β-estradiol ([^{125}I]16-α-iodo-E_2) was evaluated to assess the level of specific femto-

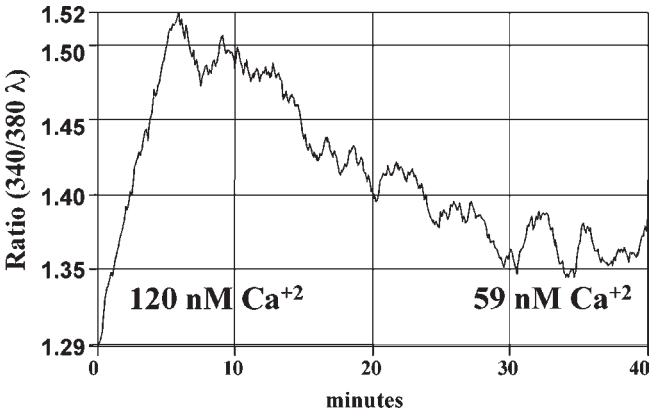


Fig. 1. Real-time graphic representation of E_2-stimulated changes in $[Ca^{2+}]i$ from FURA 2 A/M–loaded D12 cells. A rapid two-fold change in intracellular calcium levels was noted in the D12 cell line in the presence of E_2 (100 n*M*). Estrogen was administered 2 min after baseline establishment, and the change in internal $[Ca^{2+}]i$ noted here was calculated based on R_{max} (100 n*M* ionomycin) and R_{min} (2 m*M* EGTA) from a precalibration run of D12 cells. The depicted data are representative of six independent cover slips.

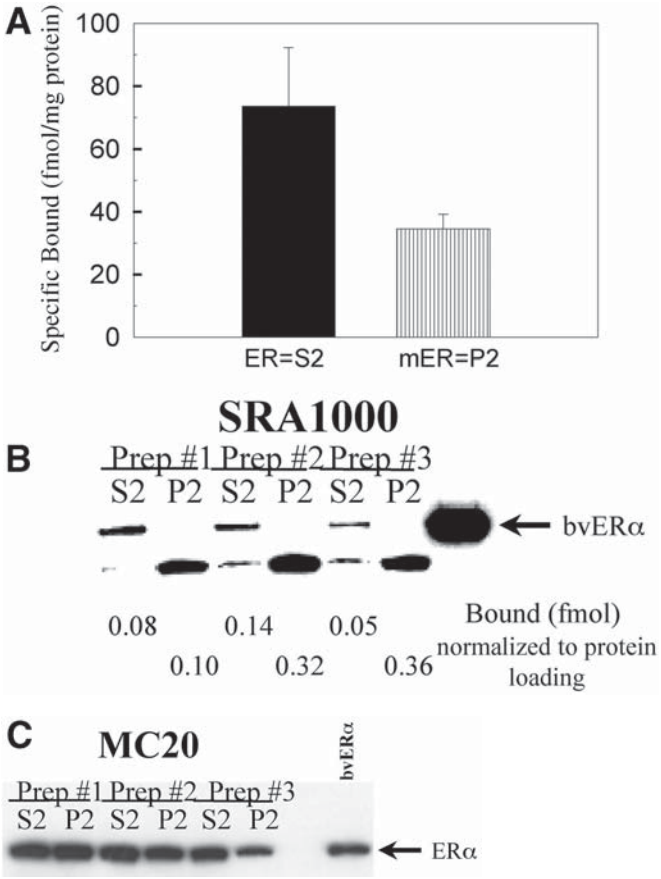


Fig. 2. (**A**) Radioligand-binding analyses of D12 cytosolic (S2) and membrane (P2) fractions reveal specific $[^{125}I]16$-α-iodo-E_2 labeling in both preparations (**B**). Data shown from Western blot analyses with a commercial ERα antibody, SRA1000, indicate that specific binding activity in P2 preparations was not due to contamination with soluble nuclear ER found in S2. The arrow indicates the molecular mass of bvERα (67 kDa) used as a positive control. Note that an unknown protein of approx 55 kDa also crossreacted with SRA1000. (**C**) Western blot analyses of these same preparations with another commercial antibody specific for ERα, MC20, indicate expression of the 67-kDa peptide in both P2 and S2 preparations. Data shown in the immunoblots represent three independent preparations.

moles per milligram of protein bound for both the membrane and soluble preparations. The results of these assays revealed specific binding activity in both membrane (P2) and cytosolic (S2) fractions. Expression levels were acceptable for further pharmacologic characterization (Fig. 2A). To ensure that the binding detected in the P2 fractions was specific for membranes and not owing to nuclear ER contamination, S2 and P2 fractions were further evaluated by immunoblots using a commercial antibody specific to the hinge region of ERα, SRA1000 (Fig. 2B). As expected, this antibody detected a protein of 67 kDa in the S2 fraction and the positive control (bvERα), whereas no corresponding immunoreactive band was noted in the P2 fraction. To ensure that appropriate protein concentrations were used to detect low levels of ER, all samples were loaded by normalizing to radioligand-binding activity based on femtomoles per milligram of protein bound. For example, the amount of protein loaded for each P2 sample (0.1–0.4 fmol of E_2-binding activity) exceeded that of the S2 (0.05–0.14 fmol of E_2-binding activity), confirming that the inability to detect ER in D12 membranes was not related to assay sensitivity. These experiments were repeated on 10 different preparations with three of these experiments depicted as examples (Fig. 2B).

Similar results were also obtained when membranes were isolated from D12 cells using sucrose gradient centrifugation. Membranes isolated from the 25% sucrose layer (crude membranes) were devoid of the 67-kDa band, whereas the cell fraction isolated from the 40% sucrose layer (enriched nuclei) possessed an immunoreactive band of the appropriate size using SRA1000 in Western blots (data not shown). Hence, for all subsequent experiments, membrane preparations (P2) were evaluated first by Western blots using SRA1000 to ensure that there was no contamination with nuclear ER.

In addition to the 67-kDA peptide, a peptide of approx 55 kDa was detected in both S2 and P2 samples using the SRA1000 antibody (Fig. 2B). Although the absolute identity of the 55-kDa protein is unknown, a crossreactive protein of similar molecular weight has been detected in other cell lysates using various ER antibodies and is thought to result from either proteolysis of native receptor *(26)* or trans-

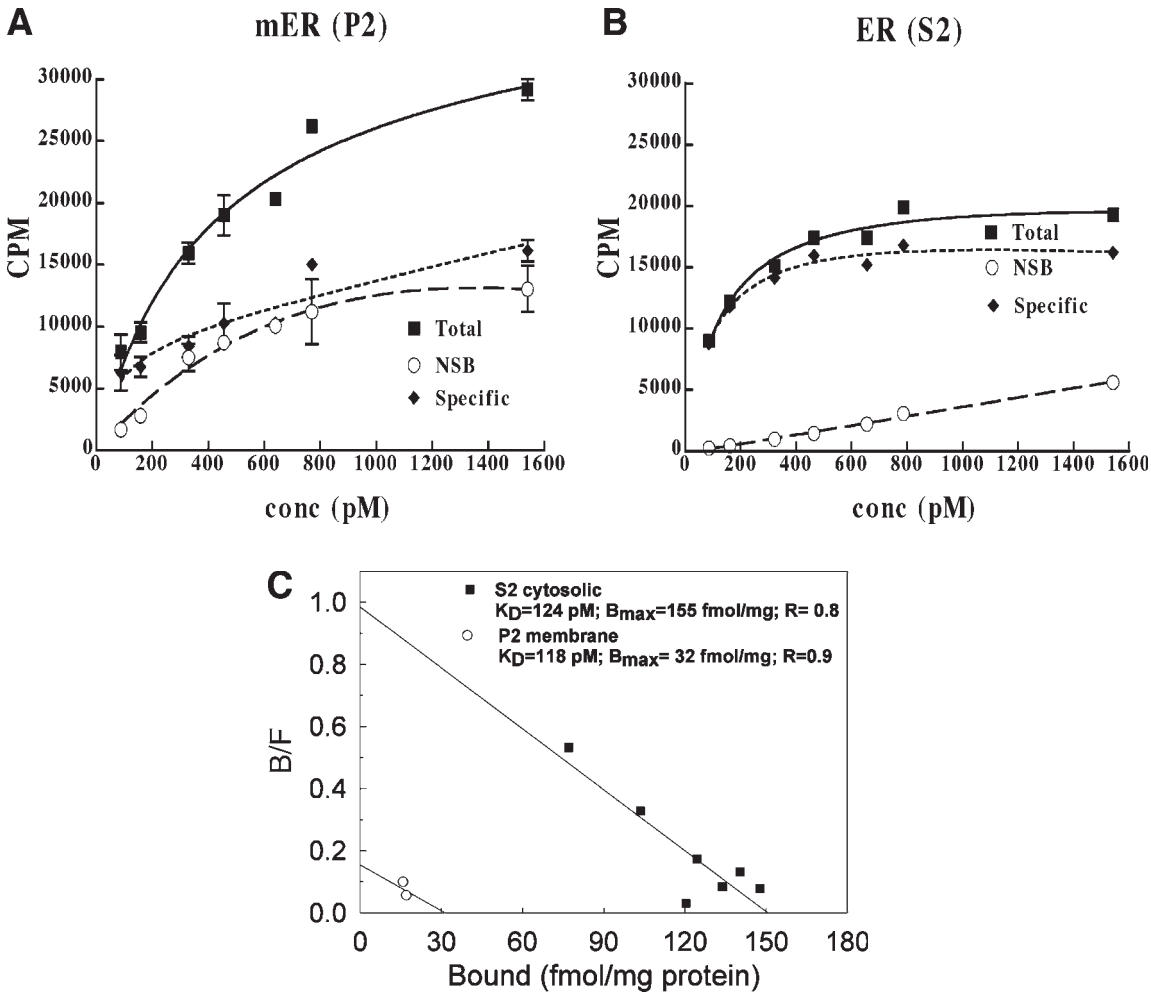


Fig. 3. Graphic representation of [^{125}I]16-α-iodo-E_2 saturation binding in P2 homogenates (**A**) and S2 preparations (**B**). The mER has similar binding affinity but lower expression levels than nuclear ER in D12 cells. Saturation isotherms are shown for total (■), nonspecific (NSB) (○), and specific (◆) binding. Values from parallel Scatchard analyses (**C**) of S2 or P2 preparations reveal that ER and mER have similar binding affinities (K_D values) for the radioligand [^{125}I]16-α-iodo-E_2 but are expressed at different receptor concentrations (B_{max} values) in D12 cells. Membrane (50 μg of protein/reaction) and cytosolic (10 μg of protein/reaction) preparations from D12 cells were incubated with increasing concentrations (10–1600 p*M*) of [^{125}I]16-α-iodo-E_2 with E_2 (1 μ*M*) included as the nonspecific determinant. Saturation data transformation generated by JMP analysis revealed a K_D value of 118 ± 43.7 p*M* and a B_{max} value of 32 ± 2.5 fmol/mg of protein for the P2 fraction, whereas a K_D value of 124 ± 17.1 p*M* and a B_{max} value of 155 ± 32.2 fmol/mg of protein were noted for the S2 fraction. This graph is one representative experiment repeated one additional time.

lation of truncated transcripts *(27)*. No correlation was observed in various P2 preparations between expression levels (immunoblots) of the 55-kDa peptide and specific [^{125}I]16-α-iodo-E_2 labeling. Therefore, this peptide crossreacts with the SRA1000 antibody but is not E_2 specific. In subsequent studies, another ERα-select antibody (MC20) recognizing a different epitope from SRA1000 was used to track the expression level of mER (Fig. 2C). This antibody has been reported by others to label putative mER *(28,29)*. Unlike SRA1000, the MC20 antibody recognized a 67-kDa peptide in both S2 and P2 preparations, indicating that mER expressed in D12 cells is antigenically similar but not identical to ERα.

Scatchard Analysis of ER in D12 Membranes and Cytosolic Fraction

To characterize the E_2-binding activity in P2 (putative mER) vs S2 (ER) in D12 cells, membrane or cytosolic preparations were incubated with increasing amounts of [^{125}I]16-α-iodo-E_2 in the absence or presence of excess unlabeled E_2 (1 μ*M*). Specific, saturable [^{125}I]16-α-iodo-E_2-binding sites were observed. The isotherm plots generated from typical equilibrium binding experiments in Fig. 3 illustrate the ratio of specific labeling of mER (Fig. 3A) vs ER (Fig. 3B). Scatchard analysis of the data revealed a single high-affinity binding site for the P2 and S2 fraction with slope values of 0.9 and 0.8, respectively (Fig. 3C). Hence, the

Table 1
Competition of Various Steroid Receptor Ligands In Radioligand-Binding Assays[a]

Ligand	ER (S2) *%I* ± SE	mER (P2) *%I* ± SE
Single concentration tested ($10^{-7}M$)		
E_2	100 ± 1	104 ± 3
Diethylstilbestrol (synthetic estrogen)	100 ± 1	100 ± 9
BPEA (antiestrogen site)	0 ± 9	12 ± 2
Dihydrotestosterone	13 ± 1	1 ± 16
Dexamethasone	5 ± 7	13 ± 4
DHEA	0 ± 4	17 ± 5
Progesterone	4 ± 1	6 ± 13
Allopregnenolone	9 ± 6	1 ± 11
	ER (S2) IC_{50} (n*M*) ± SE (*n*)	mER (P2) IC_{50} (n*M*) ± SE (*n*)
Concentration response curves (10^{-12}–10^{-6} *M*)		
E_2	0.06 ± 0.003 (3)	Nonsigmoidal (5)[b]
Genistein	28.5 ± 9.22 (4)	Nonsigmoidal (4)[b]
Raloxifene	0.11 ± 0.019 (5)	0.009 ± 0.02 (5)[b]
ICI-182780	0.084 ± 0.027 (3)	0.050 ± 0.016 (3)
4-OH-tamoxifen	0.091 ± 0.005 (4)	0.058 ± 0.018 (4)
16-α-Iodo-estradiol	0.95 ± 0.01 (4)	0.81 ± 0.008 (4)
Estriol	1.6 ± 0.07 (3)	1.19 ± 0.40 (3)
17α-Estradiol	3.03 ± 0.52 (4)	1.33 ± 0.46 (4)
Estrone	4.4 ± 0.99 (2)	2.17 ± 0.68 (2)
8,9-Δ-Dihydroestrone	8.45 ± 1.86 (2)	7.0 ± 4.24 (2)
Diethylstilbestrol (synthetic estrogen)	20.7 ± 0.19 (2)	13.5 ± 1.9 (2)
Equilelin	26.6 ± 5.30 (2)	65.7 ± 5.41 (2)

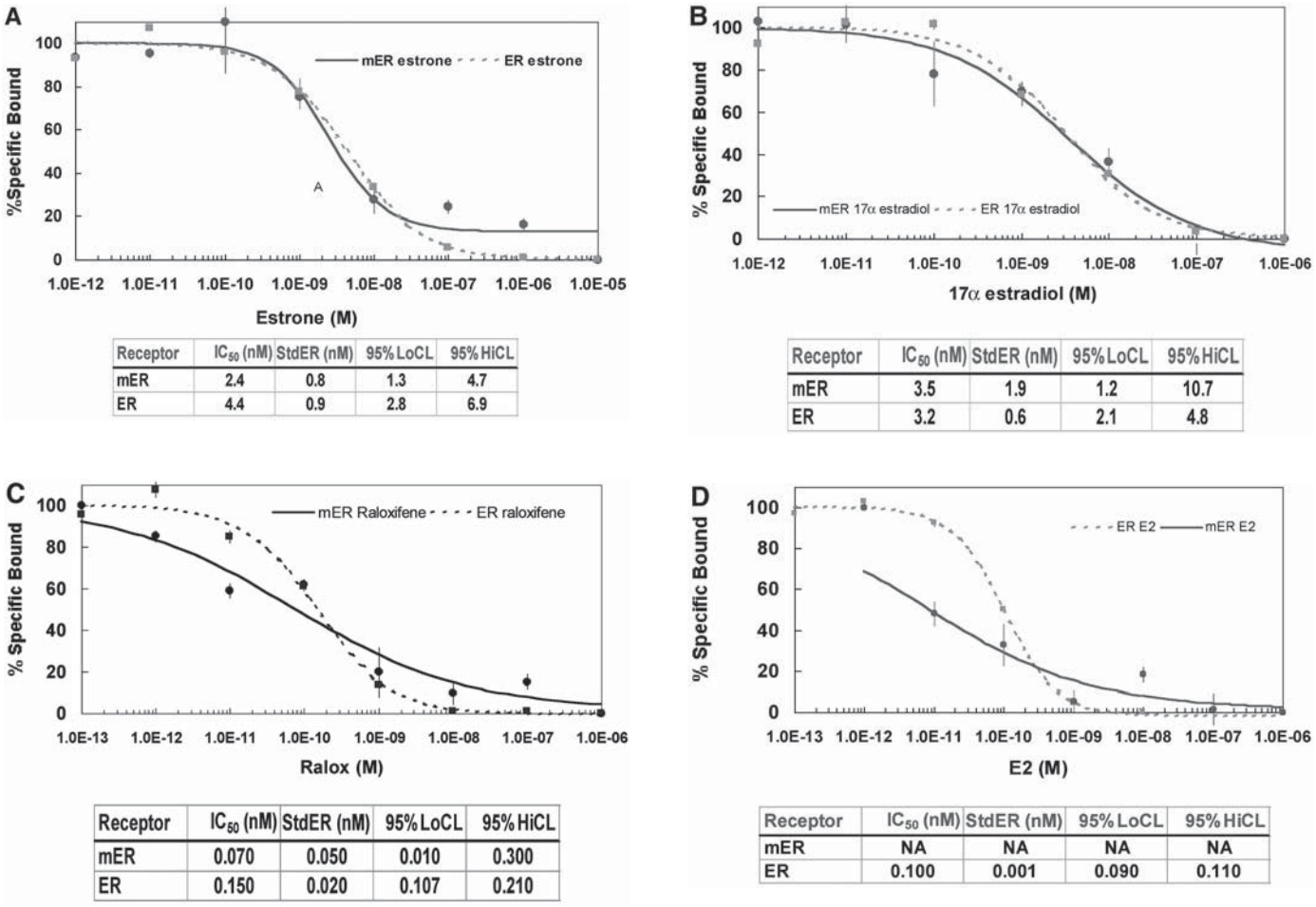
[a]Similar selectivity profiles of steroid ligands for mER and ER were noted, whereas specific estrogens depict differences in pharmacology between the two receptors. The results from single point concentration (100 n*M*) of steroids are expressed as percent inhibition (*%I*) values of radioligand bound for each ligand tested. The reported *%I* values are based on pooled raw data from separate experiments. Results from competition assays are expressed as IC_{50} (n*M*) means ± SE values corresponding to the number (*n*) of experiments listed for each compound. Each experiment was performed with triplicate determinations. Values for IC_{50} were estimated using a logistic dose response statistical program, and the weighted estimated means and SEM were used in the one-way ANOVA summary program.

[b]Statistical significance ($p < 0.05$).

binding parameters were determined using a locked slope of 1 as indicated in Materials and Methods. Analysis of the data by linear regression from two separate experiments generated K_D values of 118 ± 43.7 and 124 ± 17.1 p*M* and B_{max} values of 32 ± 2.5 and 155 ± 32.2 fmol/mg protein for membrane (P2) and cytosolic (S2) preparations, respectively. Statistical analysis of the binding parameters showed no significance between K_D values, whereas the B_{max} values were significantly different ($p < 0.05$). Since the affinity of [^{125}I]16-α-iodo-E_2 for receptors in either P2 or S2 preparations were the same, the following competition assays were done using the same radioligand concentration for both preparations.

Selectivity of Estrogens for Receptor Labeling (Membrane vs Cytosolic)

The equilibrium binding experiments provided evidence that mER may be related to the nuclear ERα based on similar estimated affinity values (K_D) for [^{125}I]16-α-iodo-E_2. To determine selectivity of steroid interactions with mER, various steroid receptor ligands were competed for either the P2 or S2 fractions using a single concentration of radioligand (200 p*M*) and percent inhibition (*%I*) values were reported (Table 1). Specificity of interaction was shown only for estrogens, indicating that these binding sites are select for this class of ligands. Once the estrogen selectivity of these membrane receptors was established, competition



Receptor	IC_{50} (nM)	StdER (nM)	95% LoCL	95% HiCL
mER	2.4	0.8	1.3	4.7
ER	4.4	0.9	2.8	6.9

Receptor	IC_{50} (nM)	StdER (nM)	95% LoCL	95% HiCL
mER	3.5	1.9	1.2	10.7
ER	3.2	0.6	2.1	4.8

Receptor	IC_{50} (nM)	StdER (nM)	95% LoCL	95% HiCL
mER	0.070	0.050	0.010	0.300
ER	0.150	0.020	0.107	0.210

Receptor	IC_{50} (nM)	StdER (nM)	95% LoCL	95% HiCL
mER	NA	NA	NA	NA
ER	0.100	0.001	0.090	0.110

Fig. 4. Graphic representation of binding characteristics of various estrogens when competed for [^{125}I]16-α-iodo-E_2 in P2 and S2 preparations from D12 cells. Data points represented are the means ± SEM from triplicate determinations of cpm bound at each test concentration. Defining the maximum (total, 100%) and minimum (nonspecific (NSB), 0%) binding normalizes each data point to percent specific bound. Total binding (100%) was measured in the presence of buffer whereas nonspecific binding (0%) was in the presence of 1 μ*M* E_2. The *%I* of the mean data point for each test concentration was calculated as follows: *%I* = {[(total mean cpm − test compound mean cpm)/(total mean cpm − NSB mean cpm)] × 100}. The represented competition curves for estrone (**A**) depict an IC_{50} value of 2.4 ± 0.8 n*M* for P2 (●) vs 4.4 ± 0.9 n*M* for S2 (■) with overlapping 95% confidence limits (CL). Concentration-response curves for 17α-estradiol (**B**) generated IC_{50} values of 3.5 ± 1.9 n*M* for P2 (●) compared with 3.2 ± 0.6 n*M* for S2 (■) with overlapping 95% CL. Curves generated from the raloxifene (**C**) competitions yielded IC_{50} values of 0.07 ± 0.05 n*M* for P2 (●) compared with 0.15 ± 0.02 n*M* for S2 (■). The IC_{50} value of E_2 (**D**) for [^{125}I]16-α-iodo-E_2 using the S2 preparation is 100.0 ± 10.1 p*M*. The IC_{50} value is not calculated from the P2 preparation due to E2's inability to fully compete off the radioligand. The graphs are from one experiment that is representative of at least two to five additional experiments.

assays were subsequently performed using [^{125}I]16-α-iodo-E_2. Twelve known estrogen ligands were tested to determine their relative affinity and binding characteristics for both receptors (Table 1). The radioligand binding assays were run simultaneously on both P2 and S2 preparations from the same cell harvest using the same radioligand concentration (200 p*M*), and each compound was competed up to five times. In general, the majority of estrogen ligands tested had similar IC_{50} values for both the ER (S2) and mER (P2) (Table 1) although there were some notable observations. For example, the mean IC_{50} values of estrone and 17α-estradiol for P2 were not statistically different from the affinities generated from S2 (Fig. 4A, estrone; Fig. 4B, 17α-estradiol). By contrast, the affinities of raloxifene (Fig. 4C) were statistically different ($p < 0.001$) for the P2 compared with the S2 preparations. Furthermore, IC_{50} values could not be determined for E_2 (Fig. 4D) and genistein (data not shown) when competing for the mER. The inability to estimate IC_{50} values for various ligands in competition experiments may be due to the shallow slope of the concentration-response curves (Fig. 4D) or because compounds at 100 n*M* did not inhibit the specific radioligand bound (Table 1). Results of these assays demonstrated that while the pharmacology of these two receptors being compared appears similar, there were notable binding characteristics that differentiate these two receptors. In addition, the inability of E_2

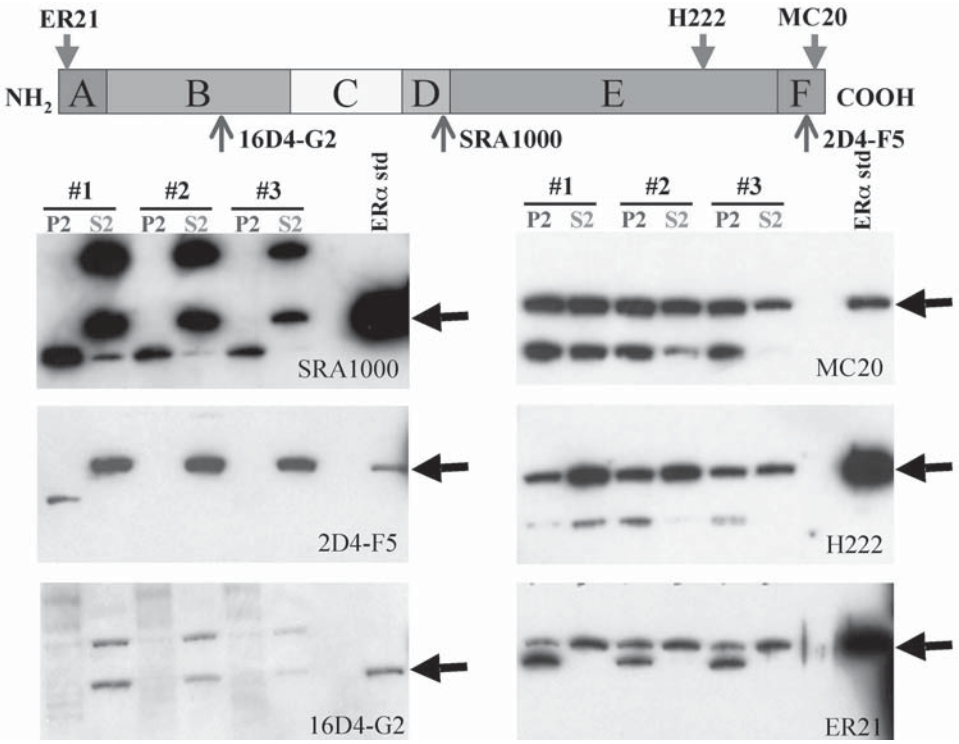


Fig. 5. Western blot analyses suggest that ER and mER are similar but not identical in amino acid sequence. (**Top**) Schematic of ERα protein indicating relative locations of epitopes to which the various ERα antibodies were generated. Functional domains of ERα are depicted including transactivation-1 domain (B), DNA-binding domain (C), hinge region (D), and ligand-binding/transactivation-2 domain (E). (**Bottom**) Preparations of S2 and P2 extracts ($n = 3$) were probed with various antibodies generated against different regions of ERα. While all antibodies recognized ERα in S2 preparations, a subset (MC20, H222, and ER21) also reacted with a membrane protein in P2 fractions with a molecular mass (67 kDa) similar to that of ERα. Three independent P2 and S2 preparations were evaluated.

to fully compete off the [^{125}I]16-α-iodo-E_2 suggests a possible noncompetitive interaction with the mER. This interaction needs to be further investigated.

Crossreactivity of ERα Antibodies with mER

Radioligand binding characterization of the S2 and P2 extracts indicated that the estrogen-binding activity in D12 membranes had properties of a receptor (saturable and estrogen selective) and had discriminating compound profiles from the nuclear ER. To gain a better understanding of the similarity of these two receptors at the amino acid level, Western blot analyses were performed on S2 and P2 fractions using antibodies that recognized different ERα epitopes (Fig. 5, top). While all of these antibodies recognized the appropriate 67-kDa ERα protein in the S2 fractions, a subset of these antibodies also recognized a similar-sized protein in P2 extracts (Fig. 5, bottom). Of the 10 different ERα antibodies assayed, only 4 were able to recognize a 67-kDa protein in both S2 and P2 fractions (Table 2). Note that the epitopes to which these antibodies were generated were located at both the amino- and carboxy-terminal portions of ERα. In general, each of these antibodies tended to crossreact with a number of other smaller and larger molecular weight peptides in S2 and P2 preparations, although no correlation was observed between expression levels and [^{125}I]16-α-iodo-E_2 binding activity. Interestingly, three of these antibodies (7A9-E1, 2D4-F5, and MC20) showed dif-

Table 2
Summary of Western Blot Analyses of D12 Preparations Using Various Antibodies Specific for ERα Demonstrate Differences in Their Ability to Recognize 67-kDa Protein in Cytosolic and Membrane Extracts

Antibody	Epitope	Domain	D12-S2	D12-P2
ER21	**1-21**	**A**	+	+
H-184	**2-185**	**A/B**	+	+
3E6-F2	22-43	A/B	+	–
16D4-G2	127-141	B	+	–
8A11-F6	148-169	B	+	–
SRA1000	287-300	D	+	–
H222	**463-528**	**E**	+	+
7A9-E1	575-589	F	+	–
2D4-F5	575-595	F	+	–
MC20	**580-599**	**F**	+	+

[a]All of these antibodies recognize the nuclear form of ER in S2 extracts, whereas only a subset recognizes mER in P2 membrane extracts. Bold signifies positive for mER.

ferences in recognizing the 67-kDa peptide in P2 fractions even though they were generated against peptides with overlapping epitopes. This apparent discrepancy may be related to the fact that unreactive antibodies were monoclonal (7A9-E1, 2D4-F5) while the reactive antibody was polyclonal (MC20). Finally, Western blot analysis of S2 and P2 fractions with a customized polyclonal antibody generated against ERβ did not reveal any staining, and the MC20 antibody did not recognize recombinant ERβ protein (data not shown).

Localization of mER to Caveolae

Much of the evidence for the rapid effects of steroids has been based on their ability to activate a variety of signaling pathways. Recent studies indicate that components of many of these pathways are associated with a specialized compartment (caveolae) within the plasma membrane for integrating transmembrane signaling events *(29–31)*. To confirm the membrane localization of mER and determine whether it was associated with caveolae, D12 cells were probed with antibodies against ERα (MC20) and the integral membrane protein caveolin-1 using both immunocytochemical and immunoblotting techniques (Fig. 6). A punctate immunofluorescent labeling pattern was observed on D12 cells for both ERα and caveolin-1 that was consistent with membrane staining (Fig. 6, top). Note that nuclear staining for ERα was negligible in these preparations because D12 cells were processed in a manner designed to preserve plasma membrane and minimize antibody penetration into the cell *(28,32)*. To provide further evidence that mER was associated with caveolae, membrane fractions enriched for caveolae were isolated from D12 cells by sucrose gradient centrifugation and analyzed by Western blot (Fig. 6, bottom). Consistent with published reports, caveolae were enriched in portions of the sucrose gradient corresponding to the 5–35% interface (Fig. 6, bottom, fraction 5). Within this same fraction, a peptide of molecular weight similar to that of ER was detected by the ERα-specific antibody, indicating that mER is associated with caveolae. In lower-gradient fractions, a similar-sized peptide consistent with soluble nuclear ER was also recognized by MC20. Signal intensities of these two peptides confirm results from radioligand-binding analyses indicating that nuclear ER is expressed at much higher concentrations in D12 cells than mER.

Discussion

A growing body of literature describing rapid effects of estrogens in various tissues suggests that mechanisms other than the classic genomic pathways play an important role in steroid signaling. The strongest body of evidence supporting a nongenomic action of estrogen exists in the cardiovascular system, where estrogens have an influence on both vasculature structure and function (for review, see ref. *3*). In addition, hormonal-based gender differences in cardiovascular disease have been noted although the mechanisms underlying these disparities remain poorly defined. Finally, recent reports indicate that a variety of cell types from the cardiovascular system rapidly respond to estrogens in functional in vitro assays *(25,33,34)*. Identifying specific cell types, such as these, that contain the necessary endogenous components needed to produce rapid responses to estrogens provides the necessary tools to elucidate pathways involved in nongenomic action. Ultimately, these cells can then be used to generate material for protein sequence identification.

One of the priorities in characterizing the nongenomic action of estrogen is to identify the receptor mediating these responses. While numerous studies provide evidence in support of a membrane-associated form of the ER, characterization at both the biochemical and pharmacologic levels has been hindered by low and variable expression in various cellular systems. In fact, a recent study demonstrated that expression levels of an mER in a rat pituitary tumor cell line can be influenced by cell density, cell passage number, and serum levels *(35)*, which we found to be true for mER in D12 cells. In the present study, calcium mobilization assays and membrane radioligand-binding assays were used as screening tools to survey cell lines for rapid calcium signaling and estrogen-specific radioligand binding. A cell line (D12) subcloned from rat hypothalamus with endothelial-like characteristics was identified using this strategy, satisfying both screening criteria. On further evaluation, this cell line was found to express adequate amounts of mER for biochemical profiling. Moreover, the fact that this cell line also expressed nuclear ER provided a convenient source of ERα for direct comparison with mER in all subsequent studies.

Pharmacologic characterization of the endogenously expressed mER confirmed saturable and estrogen-select pharmacology. Nonestrogens as well as 4-*tert*-butyl-phenoxyethyl diethylamine (BPEA), a known ligand that identifies an antiestrogen-binding site *(36)*, did not compete for mER in radioligand-binding assays, whereas raloxifene, genistein, and E_2 showed differences in binding characteristics for mER vs ER. Raloxifene competed in a concentration-dependent manner for both receptors but exhibited a statistically significant higher affinity and slope difference for mER than ER. In contrast, E_2 and genistein bound with high affinity to the nuclear ER, whereas IC_{50} values could not be estimated for mER owing to nonsigmodial binding characteristics. These observations suggest a possible noncompetitive interactive site on mER for these ligands. These data, coupled with the fact that nine known estrogen ligands bound with similar affinities to both receptors, suggest that mER is similar in pharmacology to classic ERs with a few exceptions. Perhaps the most striking difference noted was the competition studies with E_2. This compound is known to compete for ER yet it did not compete in a concentration-dependent manner for the radioligand used to characterize mER.

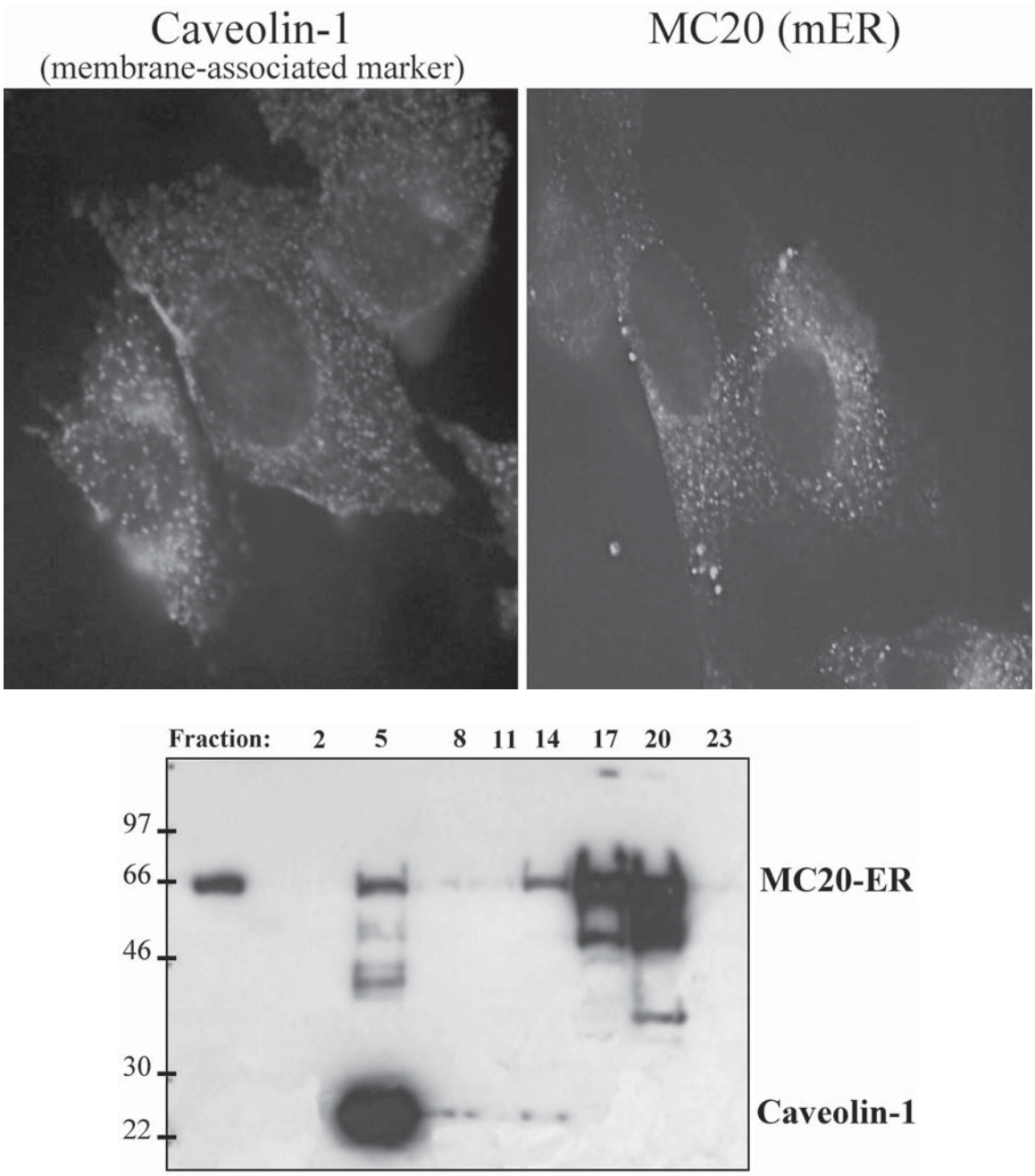


Fig. 6. (**Top**) Immunofluorescent staining of D12 cells with antibodies specific for caveolin-1 and ERα (MC20) confirm membrane localization of ER. D12 cells were processed in a manner designed to preserve plasma membrane integrity and therefore minimize nuclear staining for ERα. (**Bottom**) Membrane fractions enriched for caveolae were separated using a sucrose gradient and samples analyzed using Western blot analyses. Lane 5 shows colocalization of caveolin-1 and mER in the same fraction. Lane 1 is the bvERα standard.

Consistent with the pharmacology, mER appears to be similar but not identical in amino acid sequence. Western blot analyses utilizing a variety of antibodies that recognize discrete regions of ERα indicated similarities between these two forms of the receptor at both the carboxyl and amino termini. These regions of the ER are known to display the most heterogeneity between nuclear ERα compared with ERβ. In addition, while similarities were observed in the ligand-binding domain between mER and ERα, no cross-reactivity was detected in the hinge region. Only a single antibody specific for the hinge region of ERα was used in our studies. Thus the question remains, whether antibodies recognizing other epitopes in the hinge region would also be unreactive. Watson et al. *(37)* have recently reported that the hinge region of an mER was sensitive to ligand-induced epitope masking.

The inability of certain ERα-specific antibodies to cross-react with the 67-kDa protein in membrane preparations from D12 cells cannot be explained by differences in protein secondary or tertiary structure since denaturing and reducing conditions were used in preparing samples for Western blots. The fact that the apparent molecular weight of mER is similar to that of ERα further indicates that differences in antibody crossreactivity are unlikely to be caused by modification of the ERα gene sequence that might occur following insertion, deletion, or alternative splicing events. Therefore, the most likely explanation for dissimilarities between ER and mER is subtle but distinct differences in either amino acid sequence or posttranslational modifications of similar sequences.

The localization of mER within caveolae of the plasma membrane is consistent with other published reports *(31, 38)*. In fact, recent evidence indicates that a specific serine residue located in the E domain of nuclear ERα plays a role in association with the plasma membrane and calveolin *(39)*. Sequestration of mER in this structure in close proximity to other components of the signaling pathways would enable estrogens to induce very rapid changes in cell physiology via interactions with various signal transduction cascades. We have been unable to demonstrate in D12 cells, however, that estrogens induce any substantial changes in phosphorylation of several kinases (e.g., Akt and mitogen-activated protein kinase) that have been associated with rapid effects of estrogen in other cell systems *(22,33)*. The inability to demonstrate rapid changes in certain signal transduction pathways may be owing to the expression of nuclear ER in these cells. Previous studies of mER in cortical extracts indicated that activation of nuclear ERα inhibited phosphorylation of extracellular signal-regulated kinases (ERK) *(35)*.

Observations that mER is associated with caveolae and that the D12 cells respond rapidly to E_2 in a calcium mobilization assay are consistent with this protein existing as a membrane-associated receptor. The more important question is how this form of ER is targeted to the membrane. One possibility is that mER is a lipid-modified form of ER that has been targeted to the membrane for signal transduction. Common strategies used to target proteins to the plasma membrane include lipid modifications (e.g., palmitoylation) and/or insertion of domains that allow specific interaction with other membrane proteins. A recent study indicated that recruitment of a truncated form of the nuclear ERα to the plasma membrane in an immortalized human endothelial cell line is mediated in a manner dependent on palmitylation *(40)*.

Chemical modifications to the nuclear form of ER may induce conformational changes in the protein that could alter ligand-binding specificity. Alternatively, differences noted in the pharmacologic profiles of various estrogens for mER could be explained by its mere association with the hydrophobic membrane environment. Alternatively, the estrogen-binding activity associated with D12 membranes could potentially represent a novel steroid-binding protein or possibly the receptor for the sex hormone–binding globulin (SHBG) protein that binds estrogen along with other steroids *(41)*. Both of these possibilities seem unlikely since BPEA and dihydrotestosterone, which demonstrate high affinity for the antiestrogen site and SHBG, respectively, were inactive in our P2 preparations.

Results from our study suggest that mER in D12 cells may be related to nuclear ERα but is biochemically and immunologically distinct. Unlike ER-X, the apparent molecular weight of mER appears to be similar to that of ERα although this difference could be explained by variations in posttranslational modifications that influence migration in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels. Whether the expression of nuclear ER is a prerequisite for mER in the D12 cells is yet to be determined. Limiting the expression of nuclear ERα in D12 cells by antisense or RNA interference techniques may help to answer this question and may also unmask signal transduction pathways inhibited by activation of nuclear ER. The fact that some estrogens have different binding characteristics for mER is consistent with mERs described by others. The question remains, of course, whether the mER expressed in D12 cells is also found in nongenomic pathways existing "naturally" in endothelial cells. The D12 cells do provide, however, a valuable system to study the mER. It expresses mER endogenously and therefore does not require genetic manipulation and overproduction of exogenous nuclear ER. Cells lines such as this are more amenable than tissue sources for isolating large amounts of membrane proteins that will be required for purification and sequencing of mER. Finally, if mER does prove to be a modified form of ERα, then the fact that D12 cells express both receptors endogenously should facilitate the identification of pathways involved in its conversion.

Our current data provide evidence that an endothelial cell line subcloned from the rat hypothalamus expresses a membrane-associated form of the ER. Estrogens signaling through an mER and functioning in nongenomic pathways may be of critical importance when considering methods of drug delivery of current hormone replacement therapies because this signaling can be selectively targeted and may effect ER-mediated transcriptional outcomes. The existence of an mER in various tissues, in particular the brain, should be considered when developing estrogen-selective compounds because the interaction with mER may be important in the physiologic response in target tissue. Further characterization and sequence identification of this novel mER will lead to a better understanding of nongenomic estrogen action.

Materials and Methods

Compounds

The compounds E_2, tamoxifen, and ethinyl estradiol were purchased from Sigma-Aldrich (St. Louis, MO). Raloxifene,

diethylstilbestrol, dihydrotestosterone, dexamethasone, progesterone, allopregnenolone, and dehydroepiandrosterone (DHEA) were obtained from the Wyeth Research compound repository. Genistein was obtained from Research Biochemical (Natick, MA), and BPEA *(36)* was obtained from J. Katzenellenbogan (University of Illinois). The estrogen antagonist, ICI 182780, was purchased from Zeneca (Mereside Alderley Park, Maccleffield Cheshire, England).

Cell Culture

The D12 cell line was subcloned at Wyeth *(23)* from an immortalized rat (E18) hypothalamic cell line (RC17) obtained from Richard Robbins (Yale University). Cells were propagated in growth medium consisting of Dulbecco's modified Eagle's medium (DMEM):F12 (1:1) supplemented with 5% fetal calf serum (FCS), 1% penicillin, and 1% Gluta MAX-1 (Life Technologies, Gaithersburg, MD) at 30°C in a humidified chamber with 5% CO_2. The cell line was assessed for mER expression levels using radioligand-binding assays. Maximal mER expression was detected only after cells were passed at least 10 times in this medium, and, thus, only cultures between passages 10 and 20 were used throughout this study. For membrane preparations, 3×10^6 cells were initially plated on 150-mm culture dishes in growth medium for 24 h, and then the medium was replaced with phenol red–free DMEM:F12 medium containing 5% charcoal-stripped FCS (HyClone) overnight. The next day, membranes were isolated from cells as described later. For calcium mobilization experiments, 2×10^5 cells were plated on glass cover slips in growth medium for 24 h followed by a 24 h washout period with phenol red–free medium containing stripped serum.

Fluorescence ICC

Immunocytochemical characterization of the D12 cell line was performed with markers for endothelial cells (von Willebrand Factor VIII [1:100]; Sigma), neurons (neurofilament M [1:1000]; Chemicon, Temecula, CA), astrocytes (GFAP [1:1000]; Chemicon), and fibroblasts (fibronectin [1:200]; Chemicon). A separate assay was also used to confirm the identity of endothelial cells and was based on their ability to accumulate acetylated LDL (DiI-ac-LDL; Biomedical Technologies, Inc., Stoughton, MA). Expression of nuclear ER was established by labeling with an antibody specific for ERα (MC20; Santa Cruz Biotechnology, Santa Cruz, CA) after cells were permeabilized with 0.5% NP40 in phosphate-buffered saline (PBS) per the manufacturer's instructions. Parallel staining was done on cells with or without primary antibody to define the specific staining of each antibody.

D12 cells were washed in Dulbecco's phosphate-buffered saline (DPBS) and lightly fixed for 30 min at room temperature in fixative containing 2% paraformaldehyde, 0.15 *M* sucrose, and 0.1% glutaraldehyde in PBS, pH 7.4. This light fixation procedure was performed to preserve the integrity of the plasma membrane *(28,32)*. Following fixation, cells were washed in DPBS, incubated for 1 h in 50 m*M* NH_4Cl, and then blocked in 10% bovine serum albumin (BSA) for 1 h. Cells were incubated with an antibody against either the C-terminus region of ERα (MC20; Santa Cruz Biotechnology) or caveolin-1 (C37120; Transduction, Lexington, KY) for 3 h at room temperature, then washed in DPBS and incubated with fluorescein isothiocyanate–or Texas red sulfonyl chloride-labeled secondary antibodies for 1 h at room temperature. Cells were subsequently washed in DPBS, and digitized images were obtained by fluorescent microscopy (Nikon PM2000).

Calcium Mobilization Assay

D12 cells plated on glass cover slips were incubated for 30 min at 37°C in loading medium (phenol red–free DMEM high glucose, 0.1% BSA, and 10 μ*M* sulfinpyrazone) containing FURA2 A/M (1 μ*M*) dispersed in pluronic acid (Molecular Probes, Eugene, OR). After dye loading, the cover slips were rinsed in a 2X vol of loading medium and then equilibrated in a 2X vol of Hepes buffered solution (HBS) medium (120 m*M* NaCl, 4.75 m*M* KCl, 1 m*M* KH_2PO_4, 1.44 m*M* $MgSO_4$, 5 m*M* $NaHCO_3$, 5.5 m*M* glucose, 20 m*M* HEPES, pH 7.4). Calcium recordings were performed using a flourimeter (LS50B; Perkin Elmer, Norwalk, CT) with excitation set at 340 nm (channel 1) and 380 nm (channel 2) with fixed emission set at 509 nm. Analysis was performed using FL WinLab version 3.0 software (Perkin Elmer) following calibration using ionomycin (100 n*M*) for R_{max} and EGTA (5 m*M*) for R_{min}. Ratio data collection was completed by first establishing a 2-min baseline followed by the addition of E_2 (100 n*M*) directly into the recording chamber containing HBS medium. The concentration of intracellular calcium was calculated based on the R_{max} and R_{min} determined in the calibration run. The recording time for each experiment was 15 min.

Cell Fractionation and Membrane Isolation

Membranes were isolated from D12 cells by a variety of techniques, each evaluated for the effects of protease inhibitors, ionic strength, pH, and homogenization on eliminating contamination of nuclear ER from the membrane preparations. Based on these findings, the following optimal conditions were established to achieve highest mER labeling: Cells were collected by centrifuging at 1000*g* for 10 min at 4°C and resuspended in a binding buffer (10 m*M* Tris-HCl, 1 m*M* EDTA, 1 m*M* DTT, pH 7.2) containing 5 μg/mL of protease inhibitors including aprotinin, leupeptin, phosphoramidon, phenylmethylsulfonyl fluoride, and pepstatin. Cells were homogenized by mechanical disruption (Polytron), and the unlysed cells (P1) and debris were removed by centrifuging at 15,000*g* for 15 min at 4°C. The resulting supernatant (S1) was homogenized again, and crude membranes were isolated by centrifuging at 100,000*g* for 1 h at 4°C. Following the high-speed spin, the supernatant was

labeled S2 (cytosol) while the pellet was labeled P2 (membranes). For radioligand-binding and immunoblotting experiments, the P2 pellet was resuspended in binding buffer whereas the S2 was used directly. Since the P2 pellet contains a mixture of various cellular membranes, efforts were made to further separate each of the different cellular membrane pools by sucrose density centrifugation. For these experiments, the P2 pellet was resuspended in 3 mL of 0.25 *M* sucrose in binding buffer using a glass homogenizer. The P2 sample was added to the top of a discontinuous sucrose gradient consisting of 0, 10, 25, and 41% sucrose using J tubes for layering. Gradients were centrifuged at 35,000*g* for 1 h and then fractionated into 0.5-mL aliquots. Protein concentrations were determined with BCA reagent (Pierce).

Radioligand-Binding Assays

Initial experiments were performed using an established estrogen radioligand-binding assay *(42,43)*. Assay conditions were modified for labeling membrane proteins. Prior to performing radioligand-binding experiments, assay conditions were optimized for time, temperature, protein concentration, and establishment of bound vs free radioligand ratio concentrations for both P2 and S2 preparations (data not shown).

Equilibrium binding assays were performed on P2 or S2 preparations using 40–60 or 10–20 μg/reaction, respectively. Samples were incubated with 10–1600 p*M* [^{125}I]16-α-iodo-E_2 (New England Life Sciences, Boston, MA) for 2 h at room temperature, and unbound radioligand was removed either by charcoal precipitation (soluble ER) or by centrifugation (mER). For competition experiments, unlabeled compounds (10^{-12}–10^{-6} *M*) were added directly to membranes, and the binding reaction was initiated by adding [^{125}I]16-α-iodo-3,17-β-E_2 (200 p*M*). The specific labeling for ER and mER in these assays was 92 ± 8 and 73 ± 15%, respectively.

A three-parameter model with parameters for the dissociation constant (K_D), maximal binding capacity (B_{MAX}), and slope was fitted to evaluate a two-site saturation model. For testing the slope of the line, a nonlinear regression of bound to free was used. If the slope estimate was not significantly different from 1, which suggests a one-site model, the slope was locked to 1 and the analysis was repeated to produce a linear Scatchard plot using a customized JMP (SAS Institute, Cary, NC) application. Statistical significance between K_D and B_{MAX} values was determined using a pairwise *Z*-test.

A customized SAS-excel (SAS Institute) application was used applying a four-parameter logistic model to determine IC_{50} values. A logistic dose transformation was performed on counts per minute (CPM). The mean total bound cpm and nonspecific bound cpm were used in the analysis as the maximum (buffer wells; 100% bound) and minimum (1 μ*M* E_2 wells; 0% bound) of the competition curves, respectively. The values depicted in the top of Table 1 were determined by calculating the percent specific inhibition of binding $\%I = \{[(\text{total bound} - \text{competitor bound})/(\text{total bound} - \text{nonspecific bound})] \times 100\}$ in the presence of compound. The values shown in the bottom of Table 1 were calculated by using the weighted mean estimates and SEs of all IC_{50} values and SEs from each individual experiment. Statistical significance was determined using the summary of one-way analysis of variance (ANOVA). Statisticians in the Biometrics Department (Wyeth Research, Princeton, NJ) developed the customized JMP applications.

Western Blot Analysis

Cytosolic (S2) and membrane (P2) preparations were evaluated for ER expression by Western blots using a variety of antibodies generated against different epitopes of the ERα protein (Table 2). Samples were diluted in a Laemmli sample buffer containing 0.71 *M* β-mercaptoethanol and heated for 5 min at 95°C prior to loading on a 10% SDS-PAGE gel according to either protein levels or activity determined from the radioligand-binding assays. Proteins were then transferred to a polyvinyl difluoride membrane (Invitrogen) for immunoblotting according to the manufacturer's recommendations. Following transfer, membranes were blocked for 1 h at room temperature with blocking buffer (PBS, 5% milk, and 0.03% Tween-20) and then incubated with primary antibody diluted in blocking buffer overnight at 4°C. The various ERα antibodies used in these analyses included H-184 (Santa Cruz Biotechnology); ER-21 and H222 (provided by Geoffrey Greene, University of Chicago); 16D4-G2, 2D4-F5, and 8A11-F6 (Covance); SRA1000 (StressGen); and MC20 (Santa Cruz Biotechnology). The same calveolin-1 antibody (C37120; Transduction) used for immunofluorescence ICC was also used for immunoblotting studies. Blots were washed the following morning in TPBS (PBS containing 0.3% Tween-20) and incubated at room temperature for 2 h with a 1/20,000 dilution of the appropriate secondary antibody conjugated with horseradish peroxidase (Bio-Rad, Hercules, CA). Blots were washed sequentially in TPBS and PBS, and then immunoreactive proteins were visualized with a chemiluminescent substrate (SuperSignal; Pierce). Molecular mass standards (Amersham) and purified recombinant human ERα or ERβ expressed in baculovirus (bvERα or ERβ) were included in each gel.

Isolation of Caveolae

Membrane fractions enriched for caveolae were isolated using a detergent-free protocol *(48)*. Cells were washed in PBS and collected in 2 mL of 0.5 *M* sodium carbonate, pH 11.0. Cells were homogenized and the lysate was adjusted to 45% sucrose by the addition of 2 mL of 90% sucrose (w/v) prepared in MBS buffer (25 m*M* Mes, pH 6.5; 0.15 *M* NaCl). Samples were transferred to an ultracentrifuge tube, and 6 mL of 35% sucrose and 2 mL of 5% sucrose (both prepared in MBS and 0.25 *M* sodium carbonate buffer) were layered over the sample to form a 5–45% discontinous

sucrose gradient. Gradients were centrifuged (SW40Ti rotor) at 200,000*g* for 20 h at 4°C. After centrifugation, 0.5-mL fractions were collected from the top of the gradient and analyzed by Western blots. As reported previously, caveolae were enriched in fractions located at the interface between the 5 and 35% sucrose layers *(44)*.

Acknowledgments

We kindly thank Dr. Robert Mitchell (Wyeth) for technical input and Dr. John Katzenellenbogen (University of Illinois) for supplying the BPEA.

References

1. Beato, M. and Sánchez-Pacheco, A. (1996). *Endocr. Rev.* **17,** 587–609.
2. Wong, M., Thompson, T. L., and Moss, R. L. (1996). *Crit. Rev. Neurobiol.* **10,** 189–203.
3. Falkenstein, E., Tillmann, H. C., Christ, M., Feuring, M., and Wehling, M. (2000). *Pharmacol. Rev.* **52,** 513–555.
4. Blondeau, J. P. and Baulieu, E. E. (1984). *Biochem. J.* **219,** 785–792.
5. Luconi, M., Bonaccorsi, L., Maggi, N., et al. (1998). *J. Clin. Endocrinol. Metab.* **83,** 877–885.
6. Kelly, M. J., Moss, R. L., and Dudley, C. A. (1976). *Brain Res.* **114,** 152–157.
7. Nabekura, J., Oomura, Y., Minami, T., Mizuno, Y., and Fukuda, A. (1986). *Science* **233,** 226–228.
8. Towle, A. C. and Sze, P. Y. (1983). *J. Steroid Biochem.* **18,** 135–143.
9. Lieberherr, M., Grosse, B., Kachkache, M., and Balsan, S. (1993). *J. Bone Miner. Res.* **8,** 1365–1376.
10. Yamada, Y. (1979). *Brain Res.* **172,** 165–168.
11. Benten, W. P. M., Lieberherr, M., Giese, G., et al. (1999). *FASEB J.* **13,** 123–133.
12. Orchinik, M., Moore, F. L., and Rose, J. D. (1994). *Ann. NY Acad. Sci.* **746,** 101–112; discussion 112–114.
13. Haller, J., Halasz, J., Makara, G. B., and Kruk, M. R. (1998). *Neurosci. Biobehav. Rev.* **23,** 337–344.
14. Moura, A. M. and Worcel, M. (1984). *Hypertension* **6,** 425–430.
15. Farhat, M. Y., Abi-Younes, S., and Ramwell, P. W. (1996). *Biochem. Pharmacol.* **51,** 571–576.
16. Mendelsohn, M. E. (2002). *Am. J. Cardiol.* **89,** 12E–17E; discussion, 17E–18E.
17. Aronica, S. M., Kraus, W. L., and Katzenellenbogen, B. S. (1994). *Proc. Natl. Acad. Sci. USA* **91,** 8517–8521.
18. Pietras, R. J. and Szego, C. M. (1977). *Nature* **265,** 69–72.
19. Zheng, J. and Ramirez, V. D. (1997). *J. Steroid Biochem. Mol. Biol.* **62,** 327–336.
20. Pietras, R. J. and Szego, C. M. (1979). *J. Steroid Biochem.* **11,** 1471–1483.
21. Moats, R. K. II and Ramirez, V. D. (1998). *Biol. Reprod.* **58,** 531–538.
22. Migliaccio, A., Di Domenico, M., Castoria, G., et al. (1996). *EMBO J.* **15,** 1292–1300.
23. Fitzpatrick, S. L., Berrodin, T. J., Jenkins, S. F., Sindoni, D. M., Deecher, D. C., and Frail, D. E. (1999). *Endocrinology* **140,** 3928–3937.
24. Ghazanfari, F. A. and Stewart, R. R. (2001). *Brain Res.* **890,** 49–65.
25. Russell, K. S., Haynes, M. P., Sinha, D., Clerisme, E., and Bender, J. R. (2000). *Proc. Natl. Acad. Sci. USA* **97,** 5930–5935.
26. Ikeda, M., Ogata, F., Curtis, S. W., et al. (1993). *J. Biol. Chem.* **268,** 10296–10302.
27. Friend, K. E., Ang, L. W., and Shupnik, M. A. (1995). *Proc. Natl. Acad. Sci. USA* **92,** 4367–4371.
28. Norfleet, A. M., Thomas, M. L., Gametchu, B., and Watson, C. S. (1999). *Endocrinology* **140,** 3805–3814.
29. Toran-Allerand, C. D., Guan, X., MacLusky, N. J., et al. (2002). *J. Neurosci.* **22,** 8391–8401.
30. Toran-Allerand, C. D. (2000). *Novartis Found. Symp.* **230,** 56–69; discussion 69–73.
31. Razandi, M., Oh, P., Pedram, A., Schnitzer, J., and Levin, E. R. (2002). *Mol. Endocrinol.* **16,** 100–115.
32. Clarke, C. H., Norfleet, A. M., Clarke, M. S. F., Watson, C. S., Cunningham, K. A., and Thomas, M. L. (2000). *Neuroendocrinology* **71,** 34–42.
33. Hisamoto, K., Ohmichi, M., Kurachi, H., et al. (2001). *J. Biol. Chem.* **276,** 3459–3467.
34. Caulin-Glaser, T., Garcia-Cardena, G., Sarrel, P., Sessa, W. C., and Bender, J. R. (1997). *Circ. Res.* **81,** 885–892.
35. Campbell, C. H., Bulayeva, N., Brown, D. B., Gametchu, B., and Watson, C. S. (2002). *FASEB J.* **16,** 1917–1927.
36. Sheen, Y. Y., Simpson, D. M., and Katzenellenbogen, B. S. (1985). *Endocrinology* **117,** 561–564.
37. Watson, C. S., Campbell, C. H., and Gametchu, B. (2002). *Steroids* **67,** 429–437.
38. Schlegel, A., Wang, C. G., Pestell, R. G., and Lisanti, M. P. (2001). *Biochem. J.* **359,** 203–210.
39. Razandi, M., Alton, G., Pedram, A., Ghonshani, S., Webb, P., and Levin, E. R. (2003). *Mol. Cell. Biol.* **23,** 1633–1646.
40. Li, L., Haynes, M. P., and Bender, J. R. (2003). *Proc. Natl. Acad. Sci. USA* **100,** 4807–4812.
41. Rosner, W., Hryb, D. J., Khan, M. S., Nakhla, A. M., and Romas, N. A. (1998). *Steroids* **63,** 278–281.
42. Fitzpatrick, S. L., Funkhouser, J. M., Sindoni, D. M., et al. (1999). *Endocrinology* **140,** 2581–2591.
43. Shen, E. S., Meade, E. H., Perez, M. C., Deecher, D. C., Negrovilar, A., and Lopez, F. J. (1998). *Endocrinology* **139,** 939–948.
44. Song, K. S., Li, S. W., Okamoto, T., Quilliam, L. A., Sargiacomo, M., and Lisanti, M. P. (1996). *J. Biol. Chem.* **271,** 9690–9697.