Splice isoform estrogen receptors as integral transmembrane proteins

Kyung Hee Kim, Derek Toomre, and Jeffrey R. Bender

Departments of Internal Medicine (Cardiovascular Medicine) and Immunobiology and the Raymond and Beverly Sackler Foundation Cardiovascular Laboratory, Yale University School of Medicine, New Haven, CT 06511; Department of Cell Biology, Yale University School of Medicine, New Haven, CT 06520

ABSTRACT In addition to enhancing or repressing transcription, steroid hormone receptors rapidly transduce kinase activation signals. On ligand engagement, an N-terminus–truncated splice isoform of estrogen receptor (ER) α, ER46, triggers membrane-initiated signals, resulting in endothelial nitric oxide synthase (eNOS) activation and endothelial NO production. The orientation of ER46 at the plasma membrane is incompletely defined. With the use of epiplurin-fused ER46, total internal reflection fluorescence microscopy in live human endothelial cells illustrates that ER46 can topologically conform to a type I transmembrane protein structure. Mutation of isoleucine-386 at the center of ER46’s transmembrane hydrophobic core prevents membrane spanning, obscures the N-terminal ectodomain, and affects a marked reduction in membrane-impermeant estrogen binding with diminished rapid eNOS activation and NO production, despite maintained genomic induction of an estrogen response element–luciferase reporter. Thus there exist pools of transmembrane steroid hormone receptors that are efficient signaling molecules and potential novel therapeutic targets.

INTRODUCTION

The effects of ovarian steroid hormones, in particular estrogens, on cardiovascular disease is an ongoing subject of debate. What has become clear is that estrogen has favorable biological effects on the vascular endothelium, and that cell signaling responses to estrogen can be divided into those initiated at the plasma membrane and those confined to the nucleus. We and others have described 17β-estradiol (E2)–stimulated endothelial nitric oxide synthase (eNOS) activation, with consequent NO production and vasodilation, mediated by membrane estrogen receptors (ERs; Chen et al., 1999; Darblade et al., 2002; Guo et al., 2005; Li et al., 2007). In addition to classic ERs, ERα and ERβ, with masses predicted by their respective full-length coding regions, tissue-specific ER splice variants exist (Taylor et al., 2010). Tissue-specific alternative promoter usage and splicing generate multiple mRNA transcripts from a single ERα gene (Kos et al., 2001; Ishii et al., 2010). ER46, an ERα splice isoform, was first cloned from MCF-7 breast cancer cells more than 25 years ago (Walter et al., 1985). This splice isoform is identical to the full-length ERα (ER66), except that it lacks the N-terminal A/B domain (Figtree et al., 2003). This smaller ERα gene product has also been identified in human osteoblasts, vascular endothelial cells (ECs), and breast tumor tissues (Flouriot et al., 2000; Russell et al., 2000; Denger et al., 2001). Vascular experiments performed with the first-generation ER gene-deleted mouse surprisingly demonstrated E2-responsiveness, mediated by retained ERα splice isoforms, ER55 and ER46 (Iafrati et al., 2000; Pare et al., 2002; Pendaries et al., 2002). An N-terminus (A/B, AF-1 domain or first 173 amino acids)–deleted ERα isoform, ER46, is an efficient transducer of E2-stimulated, rapid, membrane-initiated responses in ECs (Haynes et al., 2003; Li et al., 2003). Both ERα and ER46 have been defined as high-affinity E2-binding sites in plasma membrane fractions of MCF-7 breast cancer cells (Márquez and Pietras, 2001). A recently generated ER knock-in mouse demonstrates the potential relevance...
of N-terminus-truncated ERα isoforms in the vasculature. ERαAF-1° (amino acids 2–148, AF-1 deleted) mice express a 49-kDa ERα that mediates favorable vascular responses to E2, including aortic ring basal NO production, reendothelialization of injured carotid artery segments, and prevention of atherosclerosis (Billon-Galés et al., 2009).

It has been accepted that ERs can become plasma membrane proteins through posttranslational lipid modification (palmitoylation) and/or association with other plasma membrane proteins. The EC membrane caveola has been demonstrated to be the signaling organelle at which an ER-centered, multimolecular complex resides and at which E2-stimulated eNOS activation occurs (Chambloss et al., 2000; Acconcia et al., 2005; Kim and Bender, 2005). These previous findings led to the concept of a “plasma membrane–associated ER.” Indeed, we previously suggested that E2 stimulation enhances ERα caveolar colocalization with c-Src and eNOS in ECs (Kim and Bender, 2005). As a consequence, E2-stimulated eNOS activation and endothelium-dependent vasodilation can be regulated by ERK, PI-3 kinase, and c-Src pathways (Guo et al., 2005; Li et al., 2007). In addition to this growing body of evidence for plasma membrane–associated ERs, preliminary cell imaging studies suggested that at least a subset of ERs may comprise molecules with transmembrane-spanning domains and ectodomains. There are no available structural data for ERα, although molecular modeling reveals a potential transmembrane domain (see later discussion). There is little evidence for transmembrane classic steroid hormone receptors. G protein–coupled receptor 30 was initially described as an endoplasmic reticulum transmembrane, estrogen-responsive protein, and more recent evidence suggests plasma membrane localization as well. However, its precise localization and E2 responsiveness are debated (Revankar et al., 2005; Filardo et al., 2007; Otto et al., 2008). We believe that, if indeed a transmembrane form of ERα exists in ECs, there may be as-yet-unidentified mechanisms of estrogen binding, cellular uptake, signaling, and potential for selective therapeutic targeting. In that context, we studied the topology of ERα at the plasma membrane in ECs.

RESULTS

Assessment of endothelial ERα localization by total internal reflection fluorescence microscopy

To visualize ERα at the plasma membrane, fluorophore-tagged ERα was expressed in the immortalized human EC line EA.hy926 (EA) and an elegant optical imaging technique, total internal reflection fluorescence microscopy (TIRFM), was used. ERα was expressed as a fusion protein with either enhanced yellow fluorescent protein (EYFP) or ecliptic pHluorin at the N- or C-terminus (Figure 1A). Ecliptic pHluorin, a mutant of green fluorescent protein (GFP), is reversibly fluorescent in a pH-sensitive manner, with a marked difference in fluorescence intensity between pH 7.0 and 8.0 and a total loss of fluorescence at pH < 6.0 (Miesenböck et al., 1998). All images were acquired in live cells. TIRFM images of EA cells expressing EYFP-ERα or ERα-EYFP demonstrate that the recombinant receptors localize equally to the cell surface (Figure 1, B, bottom, and C). However, the pattern for pHluorin-fused ERα is entirely dependent on the orientation of the fusion protein. With the cell medium (extracellular) pH fixed at 7.8, membrane-expressed pHluorin-ERα was brightly fluorescent, whereas ERα-pHluorin confers a much lower fluorescence level (Figure 1, B, top, and C). Using the pH indicator 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF) and the nigericin calibration technique (Kopic et al., 2010), we determined the intracellular pH to be 7.22 at the time of initial imaging, when the medium pH was 7.8. The initial fluorescence level of expressed pHluorin-ERα was 3.73-fold higher than that of expressed ERα-pHluorin, consistent with the previously described threefold difference of ecliptic pHluorin’s fluorescence intensity between pH 7.2 and 7.8 (Schulte et al., 2006). The observed fluorescence intensity difference was not a consequence of distinct intrinsic fluorescence properties of N- and C-terminal pHluorin fusion proteins (Gao et al., 2004). This striking difference in the fluorescence intensity of the two expressed recombinant proteins can be explained by a transmembrane orientation of ERα, such that the N-terminus comprises the ectodomain, whereas the C-terminus is intracellular. This representative orientation is diagrammatically displayed in Figure 1D.

Definition of endothelial ERα topology by dynamic pHluorin fluorescence

The N-terminal ectodomain orientation of ERα can be further demonstrated by taking advantage of pHluorin’s dynamic pH sensitivity. Live TIRFM images were acquired every 1 s. The absence of pHluorin photobleaching was determined before the experiments. Figure 2A displays total loss of pHluorin-ERα’s intense, baseline fluorescence when the extracellular pH is changed from 7.8 to 6.5. This is shown quantitatively [IF = Fo/F0] in Figure 2B. HCl was added at 120 s of imaging. Eclipse of pHluorin-ERα fluorescence begins within 10 s of changing the extracellular pH and is complete by 25 s (Supplemental Movie S1). This is consistent with an extracellular localization of the N-terminal domain, onto which pHluorin is fused. This dynamic fluorescence change of pHluorin-ERα is in contrast with that of ERα-pHluorin, which has a much lower baseline fluorescence when expressed in EA cells and does not change when the extracellular medium is acidified (Figure 2, A and B, and Supplemental...
Movie S2). With HCl addition to the medium, intracellular pH minimally changed (7.22 to 7.04). Further evidence for an ER46 N-terminal ectodomain is derived from reestablishing an extracellular pH of 7.4 by the addition of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-buffered Hank's balanced salt solution (HBSS) containing 50 mM NH₄Cl. Images shown were acquired at 330 s (column 3, pH 7.4 + NH₄Cl). As indicated, controls for fused ER46 include the cytoplasmic pHluorin and the type II integral membrane protein TfR. (B) Relative fluorescence quantification of TIRFM images as described in A, with (F – F₀)/F₀ values displayed from 10-s interval images over 6 min at 37°C. Data are presented as mean ± SD from three separate experiments. pH values in parentheses indicate the intracellular pH measured with BCECF at 37°C. Data are presented as mean ± SD (n = 6). (C) Sequential TIRFM live images of EA cells expressing pHluorin-ER46, with abrogation of fluorescence upon addition of HCl at 120 s (top) and reestablishment of fluorescence upon addition of NH₄Cl at 240 s. Scale bar, 20 μm.

FIGURE 2: TIRFM live imaging of extracellular pH change effect on endothelial cells expressing pHluorin-fused recombinant proteins. (A) At 44 h after transfection with plasmids encoding the indicated recombinant proteins, live EA cells in HBSS (extracellular pH 7.8) were imaged by TIRFM to establish the baseline fluorescence (column 1, pH 7.8). At 120 s, HCl was added to abruptly change the extracellular pH to 6.5, and images are shown at 180 s (column 2, pH 6.5). At 240 s, the extracellular pH was again abruptly changed to pH 7.4 with HEPES-buffered HBSS containing 50 mM NH₄Cl. Images shown were acquired at 330 s (column 3, pH 7.4 + NH₄Cl). As indicated, controls for fused ER46 include the cytoplasmic pHluorin and the type II integral membrane protein TfR. (B) Relative fluorescence quantification of TIRFM images as described in A, with (F – F₀)/F₀ values displayed from 10-s interval images over 6 min at 37°C. Data are presented as mean ± SD from three separate experiments. pH values in parentheses indicate the intracellular pH measured with BCECF at 37°C. Data are presented as mean ± SD (n = 6). (C) Sequential TIRFM live images of EA cells expressing pHluorin-ER46, with abrogation of fluorescence upon addition of HCl at 120 s (top) and reestablishment of fluorescence upon addition of NH₄Cl at 240 s. Scale bar, 20 μm.

Proteolysis of endothelial ER46 ectodomain

The PSORT II transmembrane prediction program delineates an ER46 (and ER60) model with a single transmembrane-spanning domain from Val-376 to Val-392 and an N-terminal ectodomain (Figure 3A). Proteolysis experiments were performed in an attempt to confirm ER46's transmembrane orientation. EA cells expressing EYFP-ER46 or ER46-EYFP were trypsin (0.01%) treated for 5 min at 37°C. Intracellular proteolysis does not occur at this concentration, time, and temperature. GFP and ER46 have 26
products of tryptic cleavage within prominent predicted target sites between Asn-304 and Gln-375 may not have been generated, as this region is within the ligand-binding domain and may have been protected from proteolysis by estrogen occupancy. Nonetheless, these biochemical data further support the EC expression of ER46 as a type I integral membrane protein.

**Effect of transmembrane domain mutation on endothelial ER46 topology**

The aforementioned molecular model of ER46 predicts Ile-386 at the hydrophobic center within the transmembrane-spanning domain. This isoleucine was mutated to cysteine, as this point mutation is predicted to dramatically reduce the hydrophobicity of the core domain. The EYFP-ER46-Ile386Cys mutant protein, when expressed in EA cells, efficiently localizes to the plasma membrane, similar to localization of the wild-type EYFP-ER46 fusion protein, as observed in live-cell TIRFM images (Figure 4A) and in ER immunoblots on purified plasma membrane fractions (Figure 4B). However, the ER46-Ile386Cys mutant has a different membrane orientation than wild-type ER46. Despite its membrane localization, experiments performed with pHluorin-ER46-Ile386Cys demonstrated minimal fluorescence at baseline, no change with acidification of the medium, and a modest increase in fluorescence upon addition of NH₄Cl to alkalinize the exterior (and interior) of the cell (Figure 4, C and D, and Supplemental Movie S5). The fluorescence level of pHluorin-ER46-Ile386Cys was much lower than that of pHluorin-ER46 and similar to that of ER46-pHluorin (Figure 4E). Thus, although the ER46-Ile386Cys point mutant remains plasma membrane localized, likely through palmitoylation-dependent membrane targeting, it has lost its transmembrane-spanning and N-terminal ectodomain due to a reduction in hydrophobicity at the center of its predicted transmembrane hydrophobic core.

Additional point mutants were generated to confirm the critically hydrophobic nature of the Ile-386 at the transmembrane core. A conservative substitution of Ile-386 to valine neither alters the predicted hydrophobicity nor affects the transmembrane orientation of ER46. The fluorescence of pHluorin-ER46-Ile386Val, expressed in EA cells with the extracellular pH at 7.8, is unchanged from that of pHluorin-ER46 (Figure 4E). Furthermore, none of the isoleucine-to-cysteine point mutations at positions 358 (ectodomain), 389 (another site within the TM domain), or 424 (intragel cellular domain) affected the N-terminal pHluorin fluorescence intensity (Figure 4E). Thus, as predicted in hydrophobicity calculations, Ile-386 is a critical residue in conferring the noted transmembrane orientation.

**Effect of transmembrane domain mutation on impermeant E2 binding, eNOS activation, and NO production**

The results described demonstrate that at least a subset of ER46, when expressed in ECs, can assume a type I integral membrane orientation. To address whether this orientation can be functionally relevant, a series of ligand-binding and eNOS activation experiments were performed. Figure 5A displays a greater level of impermeant E2 binding (fluorescein isothiocyanate [FITC] fluorescence) in wild-type (WT) ER46—compared with ER46-Ile386Cys—expressing cells. This difference is shown quantitatively in Figure 5B, displaying total fluorescence as the product of FITC-positive cell number and mean fluorescence intensity. As expected, given endogenous ER expression in EA cells, a significant number of ER46-Ile386Cys transfectants bound E2—bovine serum albumin (BSA). However, both the number of FITC-positive cells and the intensity of fluorescence were substantially greater in the WT ER46 transfectants. Pretreatment of (both) transfectants with the selective ER antagonist ICI 182,780 greatly reduced total fluorescence, demonstrating specificity of E2 binding (Figure 5B). The diminished E2 binding raises the possibility that the Ile-386Cys mutation renders ER46 intrinsically less functional or nonfunctional. To address this, COS-7 cells were cotransfected with expression plasmids encoding either the WT ER46 or ER46-Ile386Cys and an estrogen response element (ERE)–driven luciferase reporter. Estrogen-stimulated ERE induction and luciferase expression has been widely used to assess classic nuclear (genomic) ER responses. Transfectants expressed equal levels of the respective ER46 or
With this in mind, additional membrane-initiated signaling experiments were performed to further address the functional significance of ER46’s altered conformation with regard to its transmembrane orientation. Among the most important estrogen-stimulated rapid responses in the endothelium are eNOS activation and NO release (Kim and Bender, 2005). COS-7 cells were cotransfected with plasmids expressing eNOS and either ER46-WT or ER46-Ile386Cys (not shown). Figure 5C displays similar levels (2.58 ± 0.29-fold and 2.38 ± 0.18-fold, p = NS) of E2-stimulated luciferase activity. Additional confirmation of mutant receptor functionality was made by in vitro binding of [2,4,6,7-H(N)-estradiol (17)]-E2 to recombinant WT or Ile386Cys mutant ER46. Figure 5D demonstrates no significant difference in ligand binding and an equal level of E2 binding inhibition by the ER antagonist ICI 182,780. Scatchard analysis displayed similar ligand binding affinities (ER46-Ile386Cys 1.27-fold–greater affinity than WT ER46) and receptor numbers (Bmax; not shown). These data all confirm that the mutant receptor binds ligand at least with equal affinity and is capable of triggering a functional (transcriptional) response.

ER46-Ile386Cys–expressing cells (Figure 5F, bottom) are functional, the transmembrane orientation to their classic function as ligand-activated transcription modulators, steroid hormone receptors can transduce plasma membrane–initiated signals, triggering a variety of kinase cascades. ERs can be posttranslationally lipid modified (Li et al., 2001; Acconcia et al., 2005) and interact with other signaling molecules, such as c-Src (Castoria et al., 2003; Li et al., 2001; Li et al., 2007) and Gα (Wyckoff et al., 2001), or structural proteins, such as caveolin-1 (Razandi et al., 2003), all of which promote plasma membrane localization. We and others have demonstrated ER localization to endothelial caveolae and have accepted a model in which ERs and its splice isoforms are membrane associated within a multimolecular signallingosome. With the use of TIRF microscopy and ecliptic pHluorin fusions as pH sensors, we now unambiguously demonstrate, for the first time, that ERs can also assume a type I integral membrane protein orientation in endothelial cells. Previous antibody-based imaging studies in nonpermeabilized cells (Li et al., 2007) and recent biotinylation experiments in astrocytes (Bondar et al., 2009) demonstrated that a subset of ERs can have an extracellular ligand-binding domain, may not be sufficient for ER-mediated rapid activation.

**FIGURE 4:** Effect of ER46-Ile386Cys mutation on transmembrane topology. (A) TIRFM live images of EA cells 44 h after transfection with plasmids encoding EYFP-ER46 (left) or EYFP-ER46-Ile386Cys (right). (B) At 44 h after transfection with plasmids encoding EYFP-ER46 or EYFP-ER46-Ile386Cys, EA cell plasma membranes were isolated and immunoblotted for the indicated molecules. Anti-ERα was used to detect the wild-type and mutant fusion protein. Anti–VE-cadherin, anti-HDAC2, and anti-GM130 antibodies were used as controls for plasma membrane, nuclear, and Golgi membrane proteins, respectively. (C) TIRFM live images of EA cells expressing pHluorin-ER46 or pHluorin-ER46-Ile386Cys at baseline (pH 7.8), upon medium acidification at 180 s (pH 6.5) and upon resumption of extracellular neutral pH with the addition of NH4Cl (pH 7.4 + NH4Cl). Scale bar, 20 μm. (D) At 44 h after transfection with plasmids encoding either pHluorin-ER46 or pHluorin-ER46-Ile386Cys, live EA cells were imaged and continuous fluorescence was acquired for 6 min at 37°C. Relative fluorescence quantification at 10-s intervals is shown as (F − Fo)/Fo ± SD (n = 3 experiments). Extracellular pH changes are achieved at 120 s with the addition of HCl (pH 6.5) and 240 s with the addition of NH4Cl (pH 7.4 + NH4Cl). (E) Comparative TIRFM fluorescence levels of EA cells transfected with pHluorin-ER46, ER46-pHluorin, pHluorin-ER46-Ile386Cys, pHluorin-ER46-Ile386Val, pHluorin-ER46-Ile358Cys, pHluorin-ER46-Ile389Cys, and pHluorin-ER46-Ile424Cys at extracellular pH 7.8, with mean pixel determination of n = 10, ± SD; *p < 0.001.

**DISCUSSION**

There is widespread consensus that, in addition to their classic function as ligand-activated transcription modulators, steroid hormone receptors can transduce plasma membrane–initiated signals, triggering a variety of kinase cascades. ERs can be posttranslationally lipid modified (Li et al., 2003; Accconcia et al., 2005) and interact with other signaling molecules, such as c-Src (Castoria et al., 2001; Li et al., 2007) and Gα (Wyckoff et al., 2001), or structural proteins, such as caveolin-1 (Razandi et al., 2003), all of which

ER46-Ile386Cys–expressing cells (Figure 5E demonstrates a much stronger E2-stimulated phosphorylation of eNOS-Ser1177 in the ER46-WT–expressing (lane 4), compared with the ER46-Ile386Cys–expressing (lane 6) cells. The more robust ER46-mediated eNOS activation was confirmed in 4-amino-5-methylamino-2',7'-difluorofluorescein (DAF-FM) fluorescence (NO production) assays. Figure 5F displays an estrogen stimulation time course of DAF-FM–loaded COS-7 cells cotransfected with eNOS and either the WT or mutant ER46. Equal eNOS and ER levels were expressed in both transfectants (Figure 5H). NO accumulation begins at 3 min and steadily increases in multiple cells over the 18-min imaging time course (Figure 5F, top). There is minimal NO accumulation in ER46-Ile386Cys–expressing cells (Figure 5F, bottom; quantitative DAF-FM fluorescence shown in Figure 5G). These results demonstrate that, although both the WT and mutant receptor are functional, the transmembrane orientation of ER46 can promote signaling responses. The data suggest that, in certain settings, membrane targeting and localization, without the presence of an extracellular ligand-binding domain, may not be sufficient for ER-mediated rapid activation.
A subset of ER46 can exist as a transmembrane molecule with an N-terminal extracellular domain. The predicted (PSORT II) molecular model for such a receptor is one in which the hydrophobic Val-376–Val-392 region comprises the transmembrane-spanning domain. Indeed, when Ile386, at the center of the hydrophobic core, is replaced by cysteine, such that hydrophobicity is greatly reduced, ER46 can still be plasma membrane targeted but loses its transmembrane orientation (Figure 4). Based on our immunoprecipitation studies, the wild-type ER46, with its N-terminal ectodomain, does bind ligand. The E2-ERα ligand-binding domain cocrystal structure previously defined the phenolic hydroxyl of E2’s A ring (C3) to be located in a cavity between ERα helix 3 (Met-342 to Leu-354) and helix 6 (Trp-383 to Arg-394), making a direct hydrogen bond to Glu-353 (Brzozowski et al., 1997). On the basis of our ER46 model, helix 3 and Glu-353 are within the extracellular domain. Indeed, an ER46 Glu353Gln point mutant, predicted to be incapable of forming the direct E2 hydrogen bond, does not transduce an E2-stimulated eNOS activation response, consistent with the critical nature of this residue in extracellular E2 binding and cell activation. However, helix 6 is not within the ectodomain. This makes the formation of the previously described (crystallized) polar cavity for E2 binding unlikely when the receptor has this orientation. Because we now know that this ER isoform found at the plasma membrane can have a ligand-binding ectodomain, future structural studies will be required to define the precise molecular nature of extracellular hormone–receptor interaction.

Additional molecular modeling demonstrates that the aforementioned hydrophobic region, Val-376–Val-392, can also conform to a transmembrane-spanning domain in full-length ERα (ER66). Although not done...
in the context of transmembrane domain analysis, prior studies included mutations within the aforementioned hydrophobic region. In an analysis of coactivator interactions and transactivation competence, an expressed ERα Met388Val point mutant displays enhanced transactivation of an ERα-based reporter (Bush et al., 1996). Subcellular localization was not addressed, but it is possible that this amino acid–388 substitution resulted in a transmembrane domain mutant, thereby promoting, as a consequence, a greater level of nuclear localization. Although we previously showed that ER46 more efficiently transduces membrane-initiated, estrogen-stimulated responses than ER66 (Li et al., 2003), both isoforms can be endothelial membrane targeted and effect rapid eNOS activation. As mentioned, PSORT II analysis of ER66 defines the identical transmembrane (376–392) and an extended N-terminal ectodomain. Tryptic analysis of ER66-EYFP transfectants demonstrated a reduction in mass to 53 kDa (predicted tryptic site at Arg-363) and protection of the cytosol-localized EYFP (data not shown). Thus it appears that multiple ER isoforms can conform to the noted transmembrane structure. It is important to emphasize that, although we provide definitive evidence for a type I integral membrane ER, we consider this orientation to represent a subset pool of receptors. That is, in addition to nuclear, cytosolic, and caveolar membrane–associated pools, ERs (and likely other steroid hormone receptors) can span the plasma membrane.

We are attempting to define the biological and physiological significance of this receptor pool. Possibilities include facilitated hormone delivery to specific subcellular compartments, triggers for receptor down-regulation and/or recycling, and specialized protein–protein interactions occurring through ectodomain contacts. ERs, including non–full-length isoforms, have been shown to interact with other transmembrane proteins, such as the metabotrophic glutamate receptor 1a in astrocytes (Brzozowski et al., 1997; Dewing et al., 2007). It is intriguing to speculate about ectodomain interactions with other signaling receptors, such as ErbB2 and VEGFR2. Such interactions could play major roles and be selective therapeutic targets in oncogenesis and tumor angiogenesis. More broadly, this fourth subset of ERs allows for therapeutic targeting by molecules that remain exclusively outside the cell. As such, a new generation of selective estrogen receptor modulators, potentially useful to enhance physiology and prevent pathology, can be designed.

MATERIALS AND METHODS
Reagents
17β-Estradiol, E2-BSA-FITC, and DMSO were from Sigma-Aldrich (St. Louis, MO). DMEM, HBSS, and trypsin were from Invitrogen (Carlsbad, CA). ERα (F10), GFP (B2), which detects EYFP, actin (I19), VE-Cadherin (F8), HDAC2 (H54), and GFP(B2)-AC antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). GM130, p-eNOS, and eNOS antibodies were from BD Transduction Laboratories (Lexington, KY). DAF-FM diacetate, BCECF-AM, and Alexa 680–conjugated secondary antibody were from Molecular Probes (Invitrogen). Alexa 800–conjugated secondary antibody was from Rockland (Gilbertsville, PA). Nigericine was from EMD (San Diego, CA). Dual-Glo Luciferase Assay System was from Promega (Madison, WI). Radiolabeled [3H]-E2 was from PerkinElmer-Cetus (Waltham, MA). ICI 182,780 was from Tocris Bioscience (Ellisville, MO). ICI 182,780 was from Tocris Bioscience (Ellisville, MO). Radioactive [3H]-E2 was from PerkinElmer-Cetus (Waltham, MA). ICI 182,780 was from Tocris Bioscience (Ellisville, MO).

Construction of expression vectors
Ecliptic pHluorin-ER46 was generated by PCR amplification with primers containing BamHI and EcoRI sites for pHluorin using template ecliptic Tir-pHluorin, EcoRFV and XhoI sites for ER46 using template pCMV-tag3c-ER46, and ligating into pcDNA3.1(+). Ecliptic pH46-pHluorin was generated by PCR amplification with primers containing BamHI and EcoRI sites for ER46 using template pCMV-tag3c-ER46, EcoRFV and XhoI sites for pHluorin using template ecliptic Tir-pHluorin, and ligating into pcDNA3.1(+). pHluorin and Tir-pHluorin cDNA constructs were kind gifts from C. J. Merrifield (MRC Laboratory of Molecular Biology, Cambridge, United Kingdom) and J. E. Rothman (Yale University, New Haven, CT). EYFP-ER46 and ER46-EYFP were generated by PCR amplification with primers containing EcoRI and KpnI sites using template pCMV-tag3c-ER46, ligating into pEYFP-C3 and pEYFP-N1 (Clontech, Mountain View, CA), respectively. Transmembrane mutant ER46-Ile386Cys and other mutants (ER46-Ile386Val, ER46-Ile358Cys, ER46-Ile389Cys, and ER46-Ile424Cys) were generated by site-directed mutagenesis PCR. The numbering of ERα refers to human ERα (Swiss-Prot P03372). Constructs were all verified by sequencing.

Cell culture and transfection
The immortalized vascular EC line EA.hy926 (EA) was a kind gift from Cora-Jean Edgell (University of North Carolina, Chapel Hill, NC), maintained as described previously (Haynes et al., 2003). Transient transfection was performed with 1–2 μg of purified plasmid DNA 24 h after seeding cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s guidelines.

Live-cell TIRF imaging
Cells were imaged 44 h after transfection in glass-bottomed, 35-mm dishes (MatTek, Ashland, MA) at 37°C by TIRFM using an Olympus objective-type IX-70 inverted microscope fitted with 60x 1.45 numerical aperture TIRFM lens (Olympus, Melville, NY) and controlled by Andor iQ software (Andor Technologies, Belfast, Ireland). The 488-nm laser line from an argon laser (Melles Griot, Carlsbad, CA) was coupled to the TIRFM condenser through a single optical fiber. The calculated evanescent field depth was ~100 nm. Cells were detected with a back-illuminated Andor iXON887 EMCCD camera (512 × 512, 12-bit). For pHluorin experiments, imaging was started with the cells in HBSS, pH 7.4. After 120 s, the solution pH was changed to pH 6.5 by addition of HCl. The final concentration of HCl was 2.35 mM. After 120 s, HEPES-buffered HBSS, pH 7.4, containing NH4Cl was added to adjust extracellular pH to 7.4. The final concentration of HEPES and NH4Cl was 20 and 50 mM, respectively. The relative fluorescence level was calculated using (F − Fo)/Fo, where F is the fluorescence and Fo is the fluorescence at time 0 s. Note that (F − Fo)/Fo is the calculated fluorescence compared with fluorescence at time 0 s, so that negative values indicate fluorescence loss. For the proteolysis assay, trypsin was added to cells in phosphate-buffered saline (PBS) and incubated at 37°C. The final concentration of trypsin was 0.01%. ImageJ (National Institutes of Health, Bethesda, MD) was used to analyze raw images and to generate integrated intensity plots of areas of interest.

Intracellular pH measurement
EA cells were incubated with 10 μM BCECF-AM in HBSS (pH 7.8) for 20 min. The perfusion chamber was mounted on the stage of an inverted microscope (Olympus IX50), which was used in the epifluorescence mode with a 60x objective. Individual cells were outlined and simultaneously monitored during the course of the study. BCECF-loaded cells were excited at 440 ± 10 nm and 490 ± 10 nm, respectively, while monitoring the emission at 530 ± 10 nm every 15 s. The ratio data were used to calculate the intracellular pH using the high-K+/nigericine calibration technique (Kopic et al., 2010).
Proteolysis by trypsin
EA cells transfected with plasmids encoding EYFP, EYFP-ER46, or ER46-EYFP were incubated with trypsin (0.05%) at 4°C for 30 min, and the digestion was stopped by soybean trypsin inhibitor (0.2 mg/ml). After 5 min, the cells were washed with ice-cold PBS and harvested in HEPES buffer, pH 7.6, containing 1% Triton.

Plasma membrane isolation
Plasma membranes were isolated from EA cells transfected with plasmids encoding EYFP-ER46 or EYFP-ER46-Ile386Cys by a cationic colloidal silica isolation technique as previously described (Jacobson et al., 1992).

E2-BSA-FITC binding
EA cells were maintained in E2-deprivation media (phenol-free DMEM containing 10% gelding horse serum for 24 h, followed by addition of E2-BSA-FITC [100 ng/ml], and incubated at 4°C for 15 min, after which they were washed with Dulbecco’s PBS, fixed with 2.5% paraformaldehyde, and imaged by TIRFM (Taguchi et al., 2004). For quantification, 20 cells per experimental group were assessed by two blinded observers for FITC-positive fluorescence.

ERE-luciferase assay
COS-7 cells were transfected with three expression plasmids encoding a Renilla luciferase, an inducible firefly luciferase driven by estrogen response element (ERE-Luc), and either ER46 or ER46-Ile386Cys for 24 h, followed by incubation with DMSO or E2 (30 nM) for 24 h. Firefly luciferase activity was measured in cell lysates using the Dual-Glo Luciferase Assay System normalized to Renilla luciferase. The assay was performed according to the manufacturer’s instruction.

NO detection
COS-7 cells were transfected with plasmids encoding ER46 or ER46-Ile386Cys, and eNOS. After 20 h in E2-deprivation medium, transfected cells were loaded with DAF-FM diacetate. DAF-FM diacetate is cell permeable and retained after hydrolysis by intracellular esterases to generate active DAF-FM. DAF-FM fluoroses upon interaction with NO (forming DAF-FM triazole; Sheng et al., 2005). After 30-min loading at 37°C and medium washes, cells were excited at 488 nm and TIRFM imaging was begun, with fluorescence detection at 515 nm (37°C).

Immunoblot analysis
Cells were lysed in HEPES buffer, pH 7.6, containing 1% Triton (20 mM HEPES, 200 mM NaCl, 1 mM EDTA, 1 mM NaF, 1 mM Na3VO4, 1 mM dithiothreitol, 1% Triton X-100, and Complete Protease Inhibitor cocktail tablet [Roche, Indianapolis, IN]). Proteins were subjected to SDS–PAGE, transferred onto nitrocellulose membrane, and immunoblotted with the indicated antibodies. Detection was achieved using Alexa 680 or 800–conjugated secondary antibody by the Li-Cor (Lincoln, NE) system.

Hormone-binding assay
COS-7 cells were transfected with plasmids encoding GFP-ER46 or GFP-ER46-Ile386Cys. After 44 h, recombinant GFP-ER46 and GFP-ER46-Ile386Cys were immunoprecipitated from cell lysates with anti-GFP(B2)-agarose at 4°C for 3 h. Immunoprecipitated GFP-ER46 and GFP-ER46-Ile386Cys were incubated with [3H]-E2 in the presence or absence of a 200-fold excess of radiodinet E2 at 4°C for 2 h, and unbound E2 was removed. For the competition assay, ICI 182,780 (1 μM) was added.

Statistical analysis
Student’s t test was used for statistical analysis.

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