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The *Octopus vulgaris* Estrogen Receptor Is a Constitutive Transcriptional Activator: Evolutionary and Functional Implications

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Steroid hormones such as estrogens and androgens are important regulators of reproduction, physiology, and development in a variety of animal taxa, including vertebrates and mollusks. Steroid hormone receptors, which mediate the classic cellular responses to these hormones, were thought to be vertebrate specific, which left the molecular mechanisms of steroid action in invertebrates unresolved. Recently an estrogen receptor (ER) ortholog was isolated from the sea hare *Aplysia californica*, but the functional significance of the receptor was unclear because estrogens and other steroids are not known to be important in that species. Furthermore, the *Aplysia* ER was found to be a constitutive transcriptional activator, but it was unclear whether the estrogen independence of the ER was an *Aplysia*-specific novelty or a more ancient character general to the mollusks. Here we report on the isolation and functional characterization of the first ER ortholog from an invertebrate in which estrogens are produced and play an apparent role, the cephalopod *Octopus vulgaris*. We show that the *Octopus* ER is a strong constitutive transcriptional activator from canonical estrogen response elements. The receptor does not bind estradiol and is unresponsive to estrogens and other vertebrate steroid hormones. These characteristics are similar to those observed with the *Aplysia* ER and support the hypothesis that the evolving ER gained constitutive activity deep in the mollusk lineage. The apparent reproductive role of estrogens in *Octopus* and other mollusks is unlikely to be mediated by the ER and may take place through an ancient, non-ER-mediated pathway. (*Endocrinology* 147: 3861–3869, 2006)

STEROID HORMONE RECEPTORS (SRs) play crucial roles in regulating reproduction, development, and metabolism in vertebrates (1). They act as high-affinity molecular mediators between steroid hormone ligands, specific target sequences in genomic DNA, and coregulator proteins that activate or repress transcription of nearby genes. SRs have a modular structure in which two highly conserved domains, the DNA-binding and ligand-binding domains (DBD and LBD, respectively), are particularly important to ligand-activated transcription. In the classical mode of action of steroid hormone receptors, the receptor is in an inactive conformation in the absence of a hormone ligand. When the hormone is present, it binds tightly and specifically to the pocket of the LBD, changing its conformation and facilitating dimerization and nuclear transport (2). The receptor DBD binds to a specific response element, a 15-bp sequence of genomic DNA in the control region of target genes (3). In this active conformation, stabilized by ligand, surfaces of the LBD are exposed for tight interactions with coactivator proteins that modify chromatin or otherwise affect transcription. Expression of the target gene is then selectively up- or down-regulated (4). In this way, steroid hormones stimulate coordinated cascades of gene expression that underlie such functions as secondary sexual differentiation, female reproductive cycling, long-term response to stress, and adaptation to changing osmolarity throughout the vertebrates.

Steroid hormones are also found in invertebrates, including mollusks, in which there is evidence that they play an endocrine role (5–10). In the cephalopod *Octopus vulgaris*, for example, 17β-estradiol (E2) and progesterone are found in oviduct and ovarian tissues, and the concentration of these hormones in females correlates with phases of the reproductive cycle (7, 8). In addition, specific high-affinity estradiol binding and immunoreactivity to antihuman estrogen receptor (ER) antibodies have been detected in *O. vulgaris* female reproductive tract (7). In the bivalve mollusk, *Mytilus edulis,* E2 has been detected in gonads, pedal ganglia, and hemolymph (11, 12). In explants of hemocytes and pedal ganglia of this species, very low doses of estradiol produce strong and rapid cell-signaling effects (12, 13). There is evidence that synthetic pollutants that act as estrogens in vertebrates cause endocrine disruption in gastropods (14). Numerous other steroids have also been found in a variety of mollusks and may have functional roles (15, 16).

The molecular mechanisms of steroid action in mollusks remain unknown, however. Steroid receptors were long thought to be vertebrate-specific novelties, based on their complete absence from the fully sequenced genomes of insects and nematodes, but a recent study identified an ER ortholog in the mollusk *Aplysia californica* (17). Functional studies showed that the *Aplysia* ER does not bind E2 and is

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Abbreviations: AF, Activation function; DBD, DNA-binding domain; E2, 17β-estradiol; ER, estrogen receptor; ERE, estrogen response element; ERR, ER-related receptor; ERRE, estrogen-related response element; GRE, glucocorticoid response element; HAP, hydroxyapatite; LBD, ligand-binding domain; LRH, liver receptor-homolog; RACE, rapid amplification of cDNA ends; SF, steroidogenic factor; SR, steroid hormone receptor; TEGDK, buffer of Tris-HCl, EDTA, glycerol, dithiothreitol, and KCl; UAS, upstream activating sequence.

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a powerful constitutive transcriptional activator, even in the absence of E2 or any other added hormones (17). To understand the evolution of this feature, the ancestor of all SRs was reconstructed, synthesized, and functionally characterized. This receptor was found to specifically bind E2 and activate transcription only when estrogens were added, a result consistent with the ancestor's high sequence similarity to vertebrate ERs, particularly at critical sites in the ligand-binding pocket (17, 18). The constitutive activity of the *Aplysia* ER was therefore inferred to be a derived state. Several other nuclear receptors are also constitutively active, including the liver receptor-homolog (LRH)-1 of rodents and the ER-related receptors (ERRs), the receptors most closely related to the steroid receptors (19). Both of these receptor groups have evolved structural modifications that stabilize them in the active conformation, even in the absence of a ligand (20–23).

The loss of ligand regulation in the *Aplysia* ER left two important questions unanswered. First, is constitutive activity specific to the *Aplysia* lineage or is it a general feature of protostome ERs? Second, what is the functional significance of the *Aplysia* ER's constitutive activity? Virtually nothing is known about sea hare endocrinology, and there is no evidence that estrogen or other steroids are present or play biological roles in *Aplysia.* To illuminate the role of ERs in estrogen-sensitive mollusks and its importance to receptor evolution, we studied the molecular characteristics of the ER of *O. vulgaris*, a cephalopod distantly related to *Aplysia*, in which there is evidence that estrogens are likely to be of endocrine importance. We sought to determine whether an estrogen receptor is present in this species and, if so, whether it is a ligand-dependent or constitutive transcriptional activator.

Materials and Methods

Isolation of ER

RNA was extracted from the ovary and oviduct of *O. vulgaris*, using the EZNA mollusk RNA kit (Omega Bio-Tek, Doraville, GA), and then reverse transcribed using an oligo-dT primer and ThermoScript reverse transcriptase (Invitrogen, Carlsbad, CA). The resultant cDNA was used as template in nested degenerate PCR, using Taq polymerase and a slow ramp strategy with nested primers designed against the DBDs of vertebrate ERs. Rapid amplification of cDNA ends (RACE) was conducted using the SMART RACE cDNA amplification kit (CLONTECH, Mountain View, CA) with gene-specific primers designed from the degenerate PCR amplicon sequence. All products were cloned into pCR2.1-TOPO (Invitrogen) and sequenced from multiple clones, using a majority rule method to correct sequencing errors. A single full-length transcript of the open reading frame was then amplified from start codon to stop codon. An AF-2 mutant *Octopus* ER was constructed by site-directed mutagenesis using the QuikChange kit (Stratagene, La Jolla, CA), with primers that change the LITEML motif in helix 12 to LITQML and thereby abolish the interaction with nuclear receptor coactivators (24).

Characterization of ER expression

Total RNA was extracted from brain, liver, kidney, gill, muscle, branchial heart, testis, ovary, oviduct, and oviducal gland of *O. vulgaris* and reverse transcribed as described above. Primers to amplify a 606-bp fragment of *O. vulgaris* actin were designed from existing cDNA sequence (GenBank accession AB053937.1). Primers were also designed to amplify a 636-bp fragment of the ER; both sets of primers have annealing temperatures of approximately 65 C. Separate PCRs using Phusion polymerase (New England Biolabs, Beverly, MA) with actin and ER primers were carried out for each tissue; the same amount of cDNA template was used in both amplifications, all of which were cycled as follows: initial denaturing at 98 C for 30 sec, followed by 30 cycles of 98 C denaturing for 10 sec, 68 C annealing for 20 sec, and 72 C extension for 20 sec, with a final extension of 5 min at 72 C. The quantity of cDNA from each tissue was adjusted to yield approximately equal actin amplification across tissues.

Phylogenetic and sequence analysis

The predicted protein sequence of the *O. vulgaris* ER open reading frame was inferred and aligned to a large database of steroid receptor and related nuclear receptor protein sequences (supplemental Table S1, published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org). Alignments were prepared using ClustalX (version 1.81; see Ref. 46), assuming the Gonnet protein matrix and a geometric series of gap-change costs (1, 2, 4, 8, 16). Alignments that failed to correctly align the highly conserved DNA-binding domain and the AF-2 core of helix 12 in the LBD were deemed trivial and discarded. The remaining alignments (costs 4, 8, and 16) were concatenated into a single master data matrix for phylogenetic analysis. The effect of this elision strategy (25) is to upweight positions that align consistently across alignment costs, whereas downweighting positions that are alignment ambiguous without discarding their information altogether. The nonconserved N-terminal domain, which is too divergent to align, was excluded from the analysis.

Phylogenies were inferred using both nonparametric and parametric methods. Parsimony analysis was conducted in PAUP*4.0b10 using a heuristic search strategy of 100 iterations of random stepwise addition followed by tree bisection and reconnection branch swapping. A step matrix derived from the empirical Gonnet protein matrix (26) was used to weight amino acid changes by the inverse of their probability. Support was inferred using nonparametric bootstrapping (100 bootstrap data sets, Gonnet-weighted, 10 replicates of random addition with TBR per data set).

Bayesian Markov Chain Monte Carlo phylogenetic analysis was conducted using MrBayes software (version 3.0b4). We assumed a gamma distribution of among-site rate variation [prior for alpha parameter uniform on (0.05,10)], uniform priors for trees, and uniform on (0, 5) priors for branch lengths. The model of protein evolution was treated as a variable and integrated out using the MCMC chain. Trees and parameter values were sampled from the posterior probability distribution by metropolis-coupled MCMC in three independent runs beginning from random trees, each of which included four chains, one of them heated. The first 50,000 generations, a point well past stationarity, were discarded as burn-in. The protein sequence of the *Octopus* ER was also compared with the reconstructed sequence of the ancestral steroid receptor (GenBank AAQ98789) (17).

Cell culture and reporter activation

The DBD of the *O. vulgaris* ER and the human ERα (a gift of B. Katzenellenbogen, University of Illinois) was directionally cloned by restriction digestion/ligation into the expressible fusion vector pCMV-AD (Stratagene). LBDs (including the hinge) were cloned into pSG5-Gal4-DBD (a gift of D. Furlow, University of California, Davis, CA). The full-length transcript of the *O. vulgaris* ER was cloned into the pCDNA3 vector, with a Kozak sequence added to increase expression efficiency (27). A full-length transcript of human ERα in pCDNA3 was a gift of B. Darimont (University of Oregon).

CHO-K1 cells were maintained in 100-mm plates in phenol-red-free αMEM (Invitrogen) with 10% dextran-charcoal stripped fetal bovine serum (Hyclone, Logan, UT) and passaged with trypsin (Invitrogen) at 85–95% confluence. Reporter assays were conducted by passage into 96-well plates. For DBD reporter assays, 4 ng of receptor plasmid per well was transfected with 2 ng of the 4-EREc38-luc reporter plasmid (a gift of C. Klinge, University of Louisville) (3), using Lipofectamine and Plus as the transfection reagents (Invitrogen). After 4 h, the transfection mixture was replaced with medium supplemented with stripped serum. For LBD reporter assays, 0.1–5 ng of receptor plasmid was transfected as above with 100 ng of the pFR-luc reporter plasmid (Promega, Madison, WI). On the following day, transfected cells were treated with varying concentrations of hormones (ranging from 1 pM to 1 μM) diluted in medium with stripped serum and incubated for 24 h. For full-length

receptor assays, 4 ng of receptor plasmid was transfected with 2 ng of the 4-EREc38-luc reporter plasmid. In all assays, 0.1 ng of the *Renilla* luciferase plasmid phRltK (Promega) was cotransfected as a normalization plasmid. After incubation, cultures were lysed and assayed for reporter activation using the Dual-Glo luciferase assay kit (Promega). Firefly luciferase activity was normalized for transfection efficiency against *Renilla* luciferase activity. All assays were conducted in triplicate and repeated multiple times. Dose-response relationships were estimated by nonlinear regression using Prism4 software (GraphPad, San Diego, CA).

EMSA

CHO-K1 cells were transfected with 4 μg of pcDNA3 (negative control) or *Octopus* ER in pcDNA3 using Lipofectamine and Plus reagents as described above. Cells were harvested by trypsinization, pelleted, and resuspended in 200 μl ice-cold TEGDK buffer [10 mM Tris-HCl, 1 mm EDTA, 0.4 M KCl, 10% (vol/vol) glycerol, 1 mm dithiothreitol] with 1% protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). Cells were lysed by four freeze-thaw cycles and spun down at 10,000 $\times$ *g* for 20 min at 4 C. Total protein in extract was quantified using the protein assay (Bio-Rad Laboratories, Hercules, CA).

For EMSA, 10 μg of total protein were preincubated with EMSA binding buffer (Panomics, Redwood City, CA) and 1 μg poly-d(I-C) for 5 min at room temperature. Ten nanograms of biotinylated estrogen response element (ERE) probe (Panomics, 5′-GTCCAAAGTCAGGTCACAGTGACCTGATCAAAGTT-3′), with or without an excess of unlabeled competitor DNA, were added and incubated at 18 C for 30 min. Specific binding of the ER to the labeled ERE was assessed by introducing unlabeled ERE in a 132-fold molar excess. We also assessed the ability of unlabeled glucocorticoid response elements (GREs; 5′-GTCCAAGTCAGAACACAGTGTTCTGATCAAAGTT-3′) and estrogen-related response elements (ERREs; 5′-AGTGGCGATTTGTCAAGGTCACACAGTTAG-3′) to compete for ER binding to the labeled ERE, again with competitor in 132-fold molar excess. GREs and ERREs were synthesized as single-stranded oligonucleotides and annealed by boiling for 5 min in buffer (10 mM Tris, 1 mm EDTA, 50 mM NaCl) followed by gradual cooling. After incubation, reaction products were separated on a 5% native polyacrylamide gel in 1× Tris-borate EDTA buffer. The gel was run in an ice-water bath for 40 min at 120 V and transferred to a Biodyne B nylon membrane (Pall, Ann Arbor, MI) for 29 min at 300 mA. Chemiluminescent detection of biotinylated DNA was performed using the Panomics EMSA kit according to the manufacturer's directions.

Ligand binding assays

CHO-K1 cells were grown to approximately 90% confluence on two 100-mm plates and transfected with 4 μg of human ERα or *Octopus* ER in pcDNA3 using 30 μl Lipofectamine and 20 μl Plus reagent per plate and incubated for 4 h. Cells were harvested by trypsinization, spun down, resuspended in 7 ml ice-cold TEGDK buffer [10 mM Tris-HCl (pH 7.4), 1 mm EDTA, 0.4 M KCl, 10% (vol/vol) glycerol, 1 mm dithiothreitol], and homogenized on ice in a ground-glass tissue grinder. Homogenate was spun down at 100,000 $\times$ *g* for 1 h at 4 C. Supernatant was divided into 200-μl aliquots and incubated overnight at 4 C in triplicate with varied concentrations of 2,4,6,7-^{3}H-estradiol (NEN Life Science Products/PerkinElmer, Boston, MA) for total binding or with labeled estradiol plus a 200-fold molar excess of unlabeled estradiol for nonspecific binding. Samples were incubated for 15 min at 4 C with 200 μl of a 50% slurry of hydroxyapatite (HAP; Bio-Rad) in TEGDK buffer with vortexing every 5 min, after which the HAP was subjected to three repetitions of spinning down (12,000 $\times$ *g* for 30 sec), resuspension, and washing in 1 ml of cold TEGDK. Bound ligand was extracted overnight from the washed HAP in 1 ml ethanol. The following day, 500 μl of the suspension was added to 5 ml scintillation fluid and counted on a liquid scintillation counter. Specific binding was calculated as total minus nonspecific bindings; binding constants were estimated using Prism software (GraphPad). All experiments were conducted in triplicate and repeated multiple times.

Results

Using degenerate primers designed to match highly conserved motifs in the DBDs of vertebrate ERs, we amplified, cloned, and sequenced a single-gene fragment from *O. vulgaris* ovary and oviduct. We used RACE to obtain the full-length cDNA. From this sequence, we designed new primers and amplified the full-length open reading frame of this receptor. This cDNA is 1467 bp long, with a predicted protein length of 489 amino acids. The sequence contains the five recognizable steroid receptor domains, in the expected order: the N-terminal region, DBD, hinge, LBD, and C-terminal extension (Fig. 1). The C-terminal extension domain is typical of SRs and is not found in the other nuclear receptor groups most closely related to them: the ERRs and the steroidogenic factor SF-1/LRH-1 group. In the DBD, the *Octopus* receptor has highest protein sequence identity to the aplysia ER (94%) and the human ERα and ERβ (86%) and much lower similarity to other steroid receptors (such as the androgen, progestin, and corticoid receptors) and the ERRs (Table 1). The same pattern is apparent for the LBDs, although this domain is less conserved overall (Table 1). In addition, in the P-box of the DBD, a short highly conserved motif that confers specificity for response elements (28), the *Octopus* receptor has the signature sequence of estrogen receptors, which differs from those of the other SRs or ERRs (Table 1).

To determine whether the *Octopus* receptor is an ortholog of the ERs, we included it in a phylogenetic analysis with 77 steroid receptor and closely related nuclear receptor genes. The analysis was conducted using both a parametric method (Bayesian Markov Chain Monte Carlo analysis, which uses a probabilistic model of sequence evolution) and a nonparametric technique (maximum parsimony, which assumes only that shared derived amino acid states give evidence of common ancestry). Both analyses indicated that the *Octopus* receptor is most closely related to the *A. californica* ER, and these two genes cluster as a sister group to the vertebrate ERs (Fig. 2). Support for these relationships is very strong: in the Bayesian analysis, the grouping of the *Octopus* ER with the *Aplysia* ER has posterior probability 100%, as does the grouping of the mollusk ERs with the vertebrate ERs. Parsimony bootstraps for these same nodes were 100 and 98%, respectively. These analyses indicate that the gene we isolated is the *Octopus* ortholog of ER.

Expression of the ER is not restricted to reproductive tissues. To provide an initial characterization of the distribution of the *Octopus* ER, we extracted RNA from brain, liver, kidney, gill, muscle, and branchial heart of both male and female *Octopus* as well as testis, ovary, oviduct, and oviducal gland. We used reverse transcription and PCR to determine whether ER is expressed in these tissues. We observed some ER expression in all tissues, with the highest expression in ovary (Fig. 3).

To determine the intrinsic functions of the *Octopus* ER protein, we deployed several molecular assays. Using a reporter gene activation assay, we found that the functions of the *Octopus* ER DBD are similar to those of other ERs. We expressed the *Octopus* ER DBD fused to the constitutively active nuclear factor-κB activation domain in CHO-K1 cells along with a luciferase reporter driven by four canonical EREs. Luciferase expression mediated by the *Octopus* ER DBD was significantly elevated over background and was equivalent to that driven by the human ERα DBD (Fig. 4A).

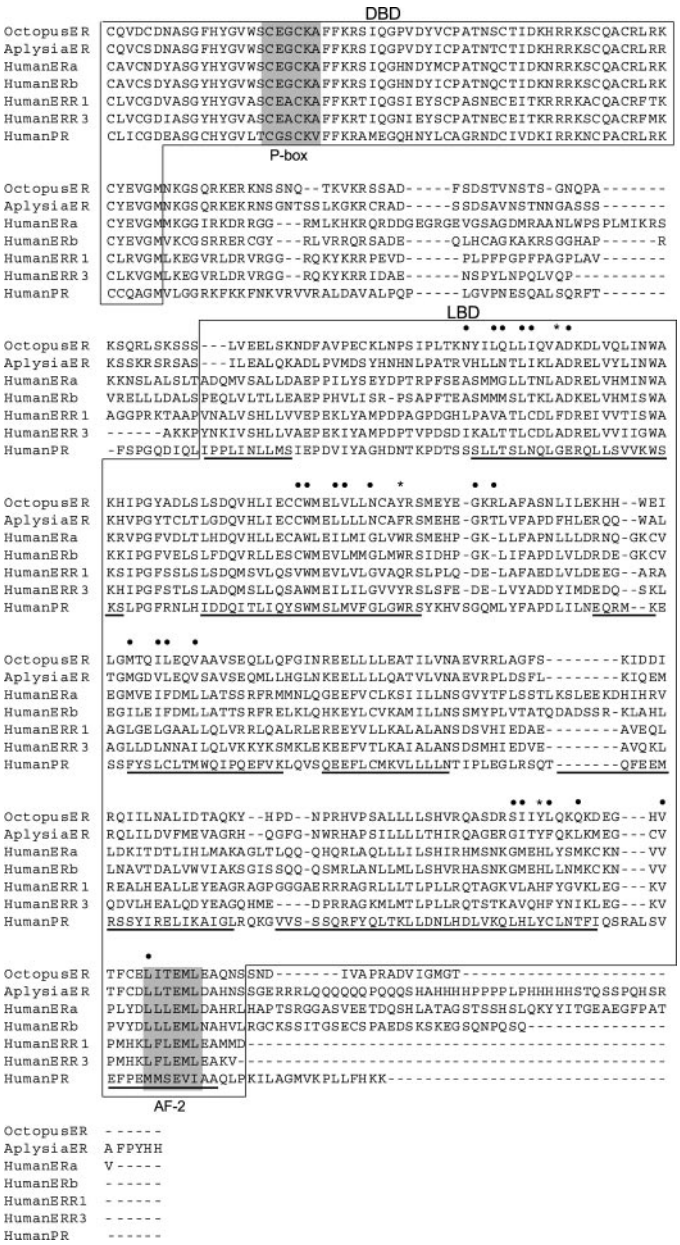

FIG. 1. Protein sequence of the *Octopus* ER. The aligned sequences of the *Octopus* ER and selected other steroid and related receptors are shown. The DBD and LBD are marked with *black boxes*. The N-terminal domain, which is inadequately conserved to align, is not shown. Residues important for DNA response element recognition (the P-box) is shaded *gray*, as is the AF-2 region, which mediates the LBD's contact with transcriptional coactivators. Residues composing the helices of the human ERα-LBD are *underlined*. Residues that line the ligand cavity in the human ERα crystal structure are marked with *filled circles*, and those that form hydrogen bonds with estradiol are noted with *asterisks* (30, 31).

This result indicates that the *Octopus* ER DBD can activate transcription from an ERE.

To test whether the *Octopus* ER DBD interacts directly and specifically with EREs, we used an EMSA. We found that the *Octopus* ER specifically binds to a labeled ERE. This binding can be eliminated by competition with an excess of unlabeled ERE but not by a more than 100-fold molar excess of a GRE

TABLE 1. Percent of aligned amino acids that are identical between the *Octopus* ER and other steroid receptors in the DBD and LBD

	DBD	LBD	P-box
Octopus ER	100	100	CEGCKA
Aplysia ER	94	57	CEGCKA
Human ERα	86	33	CEGCKA
Human ERβ	86	32	CEGCKA
Human AR	58	20	CGSCKV
Human PR	56	21	CGSCKV
Human GR	57	23	CGSCKV
Human MR	57	21	CGSCKV
Human ERRα	68	28	CEACKA
Human ERRβ	71	29	CEACKA
Human ERRγ	69	29	CEACKA

The *Octopus* ER is most similar to the *Aplysia* ER and the vertebrate estrogen receptors. The amino acid sequence of the highly conserved P-box in the DBD is also shown. AR, Androgen receptor; PR, progesterone receptor; GR, glucocorticoid receptor; MR, mineralocorticoid receptor.

(Fig. 4B). The *Octopus* ER also shows some affinity for the monomeric ERRE sequence recognized by estrogen-related receptors because its binding to labeled ERE can be partially competed with a greater than 100-fold excess of ERRE. These results are similar to those observed for the mouse ERα, which also binds to and activates from both ERE and ERRE (29). Because the *Aplysia* ER DBD also mediates transcription from canonical EREs, we conclude the classic DNA recognition function of vertebrate ERs are conserved in mollusks.

Based on the presence and apparent functional role of estradiol in *Octopus* (7, 8), we hypothesized that the transcriptional activity of the *Octopus* ER would be estrogen dependent. We prepared a fusion construct of the Gal4-DBD

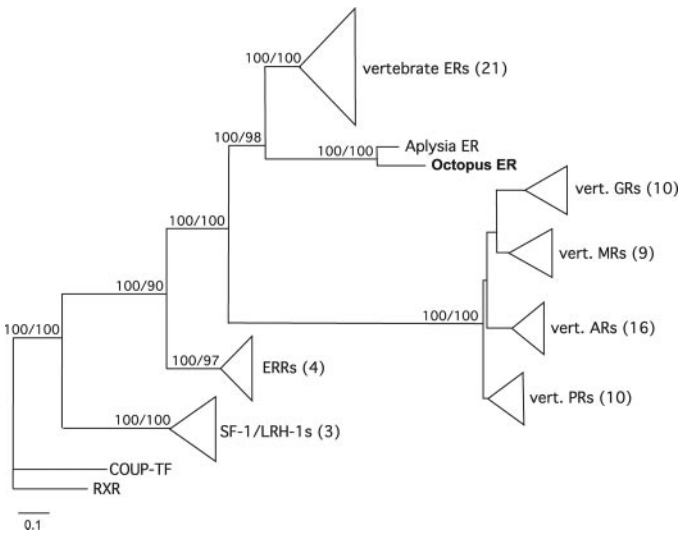

FIG. 2. The *Octopus* ER is an ortholog of the vertebrate ERs. A reduced version is shown of a phylogeny of 78 steroid and closely related receptors; the same tree was inferred using both Bayesian and maximum parsimony methods. The number of sequences in each clade is in *parentheses*. Node labels show support for each node as Bayesian posterior probabilities followed by MP bootstrap support, both as percentages. Branch lengths were inferred by the Bayesian analysis. GR, Glucocorticoid receptor; MR, mineralocorticoid receptor; AR, androgen receptor; PR, progesterone receptor; COUP-TF, chicken ovalbumin upstream promoter-transcription factor; RXR, retinoid X receptor.

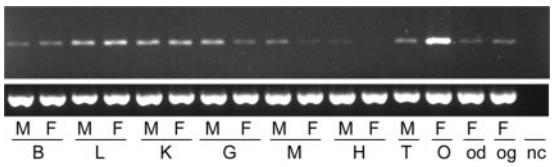

FIG. 3. The *Octopus* ER is widely expressed. RT-PCR was used to survey tissue-specific expression of the ER gene in male (M) and female (F) *Octopus*. The *upper panel* shows amplification with ER-specific primers using cDNA from brain (B), liver (L), kidney (K), gill (G), muscle (M), branchial heart (H), testis (T), ovary (O), oviduct (od), and oviducal gland (og); in the negative control (nc) reaction, no cDNA template was added. Actin (*lower panel*) was used to normalize cDNA loading.

with the *Octopus* ER LBD (including the hinge and C-terminal extension) and expressed it in CHO-K1 cells with a upstream activating sequence (UAS)-driven reporter, using charcoal-stripped serum to eliminate the potential for spurious ligand activation. As expected, there was no activation by the human ERα above background in the absence of hormone, and estradiol treatment at 1 μM produced a 25-fold increase in reporter activation. In contrast, the *Octopus* ER LBD was constitutively active, activating transcription 20-fold above background when no ligand was added; estradiol had no further effect on reporter expression (Fig. 5A). We also treated cells with varied concentrations of estradiol from 10^{-12} to 10^{-6} M: the human ERα displayed the expected dose-dependent increase in reporter expression, but the *Octopus* ER was again constitutively active and unresponsive to estrogen at all doses. (Fig. 5B). The *Octopus* ER LBD was insensitive to other steroid hormones as well: we tested a broad panel of other estrogens, progesterone, androgens, corticosteroids, and estrogenic xenobiotics, but none had a significant effect on the *Octopus* ER's constitutive transcriptional activation (Fig. 5A).

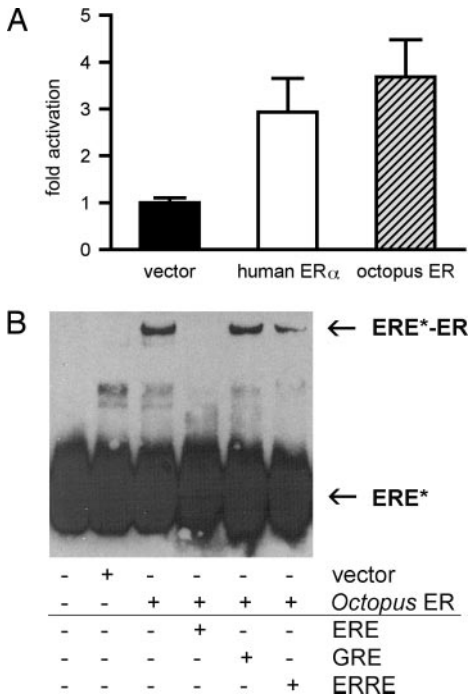

FIG. 4. Activation and binding of *Octopus* ER at EREs. A, The *Octopus* ER DBD activates transcription from a canonical ERE. Receptor DBDs were expressed in CHO-K1 cells as a fusion protein with the constitutively active nuclear factor-κB activation domain along with an ERE-driven luciferase reporter. Fold activation indicates luciferase activity relative to the vector-only control, which contains no DBD. Mean $\pm$ SE of three replicates is shown. B, The *Octopus* ER DBD specifically binds EREs. The full-length *Octopus* receptor were expressed in CHO-K1 cells, and cell extracts were tested for binding to biotinylated ERE (all lanes). Unlabeled ERE, GRE, or ERRE were used as competitor DNA at a 132-fold molar ratio to the labeled ERE. Negative controls: the first lane shows a biotinylated ERE with no cell extract added, and the second lane shows biotinylated ERE with extract of cells transfected with the empty vector.

The *Octopus* ER's constitutive activity is not an artifact of saturating assay conditions: when the quantity of the *Octopus* ER LBD fusion construct was increased while holding all other assay conditions constant, even higher levels of reporter expression could be elicited from this system (Fig. 5C). Nor was it an artifact of the use of an LBD fusion protein: we found that the full-length *Octopus* ER protein also constitutively activates an ERE-driven luciferase reporter and does not respond to estradiol treatment. (Fig. 5D).

To determine whether the *Octopus* ER's insensitivity to estradiol is an artifact of using a heterologous mammalian expression system, we conducted competitive radioligand-binding studies in a cell-free system. The human ERα bound tritiated estradiol tightly and specifically, but the *Octopus* ER showed no evidence for any specific estradiol binding, even at concentrations that saturate the human ERα (Fig. 6). The lack of estradiol binding is unlikely to be due to a lack of ER protein because receptor proteins for this experiment were expressed using the same system as in the reporter assays; the *Octopus* ER's very strong effect on transcription in that assay indicates robust expression. The receptor's unresponsiveness to estradiol in trans-activation assays therefore appears to be due to an intrinsic inability to bind the ligand.

The constitutive activity of the *Octopus* ER also does not appear to be an artifact of the heterologous system. Most nuclear receptors LBDs, including constitutively active nuclear receptors, up-regulate transcription by recruiting coactivators to the activation function (AF)-2 region on helix 12 (4). In ligand-dependent nuclear receptors, the AF-2 region is made available to coactivators only when a ligand has bound to the receptor, but constitutively active nuclear receptors, like the ERRs and mouse LRH1, assume an active conformation with an accessible AF-2 in the absence of ligand. To determine whether the assay system was producing a novel or fortuitous form of transcriptional activation, we mutagenized a single amino acid (E465Q) in the highly conserved AF-2 motif to which steroid receptor coactivators bind (24). Constitutive activity of the *Octopus* ER was completely abolished by this mutation (Fig. 7). The mechanism of *Octopus* ER constitutive activation is therefore similar to that observed for other constitutive nuclear receptors and is unlikely to be an artifact of a fortuitous binding partner present in mammalian cells.

To understand the mechanistic basis for the *Octopus* ER's constitutive activity, we compared the amino acids at positions that mediate contact with estradiol in the human ERα (30, 31) with those in the *Octopus* and *Aplysia* ERs. Very low conservation was apparent, with 17 of the 26 residues differing between *Octopus* and human (Fig. 8). Some of these are

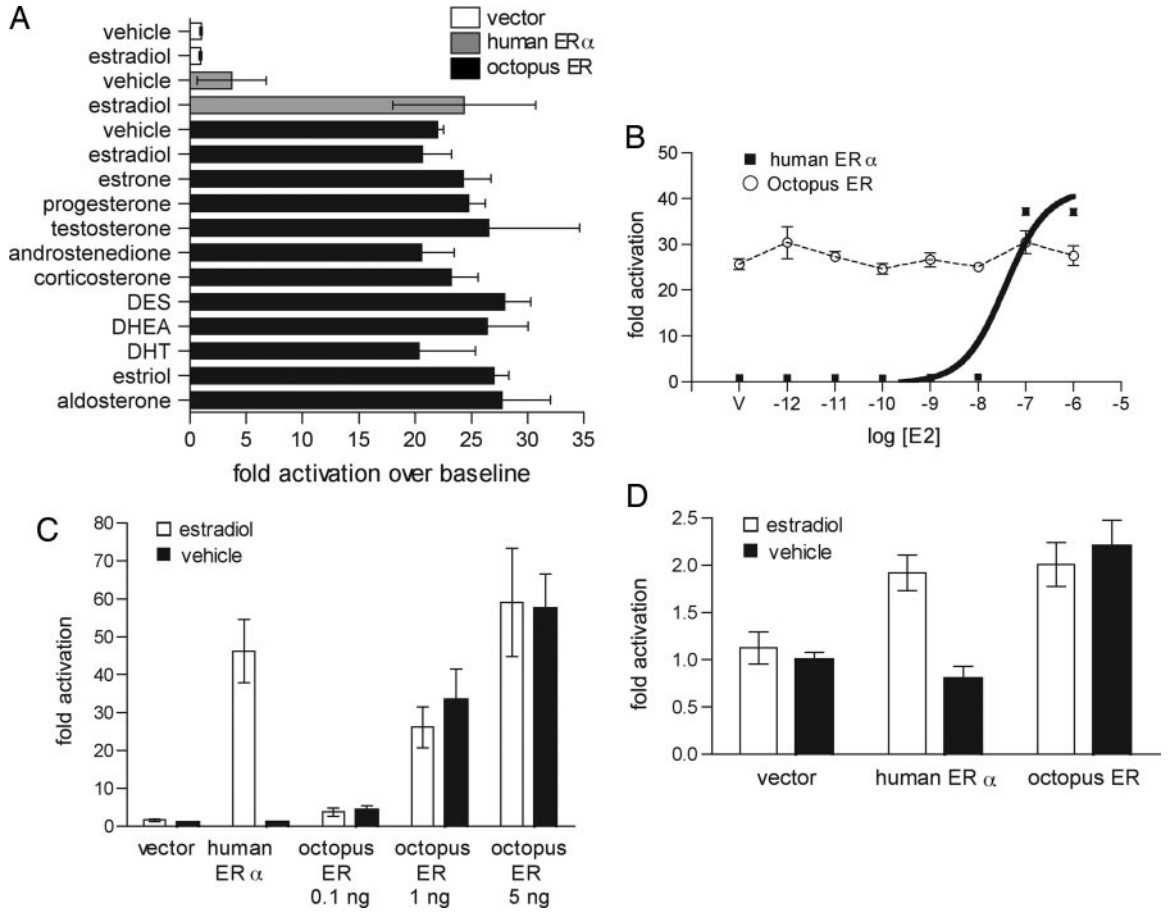

FIG. 5. The *Octopus* ER is a constitutive transcriptional activator that does not respond to steroid hormones. For A–C, receptor LBDs were expressed in CHO-K1 cells as fusion proteins with a Gal4-DBD along with a UAS-driven luciferase reporter. For all figures, fold activation indicates luciferase activity relative to the vector-only control, which contains no added LBD. Mean ± SE of three replicates is shown. A, The *Octopus* ER LBD is constitutively active and does not respond to steroid hormones. Cells were treated with 1 μM hormone or vehicle alone (ethanol). DES, Diethylstilbestrol; DHEA, dehydroepiandrosterone; DHT, dihydrotestosterone. B, The *Octopus* ER LBD does not respond to increasing concentrations of estradiol. Transfected cells were treated with vehicle only (V) or estradiol. C, The receptor assay system is not saturated by receptor activity. Maximal luciferase expression is not reached at the conditions used for the assays in A and B (1 ng receptor). Cells were treated with vehicle only (ethanol) or 1 μM estradiol. D, The full-length *Octopus* ER is a constitutive activator. Full-length ERs were expressed in CHO-K1 cells along with an ERE-driven luciferase reporter. Cells were treated with 1 μM estradiol or vehicle only.

radical replacements, including L391C, M528Q, T347I, S521G, M522I, and H524Y (based on human ERα numbering). The replacement of the histidine at position 524 of the human ERα with the much bulkier and less polar tyrosine in the mollusks is of particular interest: H524 plays a crucial role in ligand recognition by the human ERα, forming a hydrogen bond with the 17-hydroxyl moiety of estradiol, and mutations at this position are known to severely compromise estradiol binding (32). We also compared the ligand pocket residues in the *Octopus* and human ERs with those in the inferred sequence of the ancestral steroid receptor, the ancient gene from which all present-day steroid receptors evolved by duplication. This receptor has been shown experimentally to bind and activate transcription in the presence of estradiol (17). In the human ERα, 22 of the 26 ligand-pocket residues are identical with the ancestor, and none of the replacements are radical. In contrast, the *Octopus* ER is highly derived, with only 12 of 26 amino acids identical with the ancestor, and six of the replacements are radical. There are three positions in the crystal structure that form hydrogen bonds with estradiol and discriminate among ligands (31); all of these are conserved between the ancestral and human receptors, but two (including H524Y) have derived states in the *Octopus* ER.

Discussion

The *Octopus* ER is phylogenetically clustered with other estrogen receptors, but it differs functionally from the ligand-dependent ERs of vertebrates. Like all previously identified ERs, the *Octopus* ER binds to and activates transcription from classic estrogen response elements. The functions of the LBD, however, are not conserved: unlike the vertebrate ERs, the *Octopus* ER does not specifically bind estradiol, nor does it increase its transcriptional activation when treated with estradiol. Rather, it is a strong constitutive activator in the absence of any added ligand. Furthermore, the *Octopus* ER is not regulated by other vertebrate steroid hormones, and it has a molecular mode of action that is like other constitutively active nuclear receptors. Our experiments provide

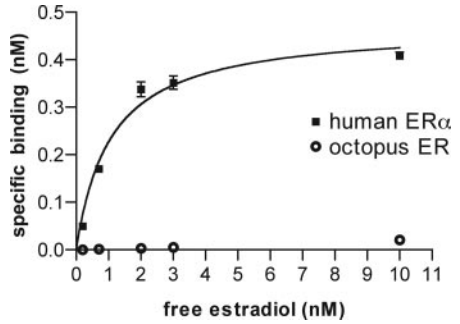

FIG. 6. The *Octopus* ER does not specifically bind estradiol. Extracts of CHO-K1 cells expressing full-length receptor transcripts were extracted and incubated with a range of concentrations of ^{3}H-estradiol with or without a 200-fold excess of cold estradiol (nonspecific and total binding, respectively). V, Ethanol vehicle only. Graph shows the mean ± SE of three replicates for specific binding, calculated as the difference between total and nonspecific binding.

strong evidence that the *Octopus* ER's constitutive activity and unresponsiveness to estrogens is likely to be authentic and not an experimental artifact. Our finding that the *Octopus* ER does not bind estradiol is consistent with the prior observation that ER immunoreactivity does not colocate with estradiol binding; in *O. vulgaris* ovary, the former is limited to the nucleus, but the latter was detected only in the cytosolic fraction (10). We cannot rule out the possibility that there may be an endogenous ligand (or some xenobiotics) that can repress the constitutive activity of the *Octopus* ER, as is the case for some ERRs (33, 34), but we have seen no evidence for this scenario in our experiments.

We found that the *Octopus* ER is widely expressed in both sexes, with the highest transcript levels in ovary. These results are consistent with a role in female reproduction but do not rule out other functions as well. Our experiments were limited to a single time point. Further studies are necessary to determine whether ER expression is temporally up-regulated in some tissues during specific stages of the *Octopus* reproductive cycle.

Our findings indicate that the constitutive activity previously observed with the sea hare ER is not *Aplysia* or even gastropod-specific; rather, constitutive ER activity is common to the mollusks. Together with previous knowledge about the estrogen responsiveness of the ancestral steroid receptor, this result supports the hypothesis that the ER evolved constitutive activity deep in the mollusk or protostome lineage. Specifically, the ancestral steroid receptor has been shown experimentally to bind estradiol and activate transcription in a dose-dependent fashion in the presence of estrogens (17). Furthermore, analysis of the ancestral ligand-binding pocket showed that the residues that mediate contact with estradiol are strongly conserved between the ancestral receptor and present-day estrogen-activated receptors (18). In contrast, the sequences of the mollusk ERs are much less similar to those of the ancestral receptor (as shown by the much longer branch lengths leading to this group in Fig. 2) and in the ligand-pocket in particular. An alternate scenario is that ligand independence is ancestral, with ligand-activation gained independently in the vertebrate ERs and in the lineage leading to the other vertebrate steroid receptors; however, the experimental and bioinformatic evidence to date support a loss of ancestral ligand dependence in the lineage leading to the mollusk or protostome ERs. Characterizing ERs from other lophotrochozoan phyla will allow further tests of this hypothesis and help determine more precisely when the evolutionary shift in receptor function occurred.

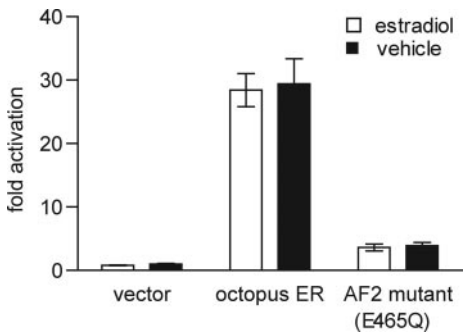

FIG. 7. A mutation in the *Octopus* ER AF-2 region abolishes constitutive activity. Wild-type and mutagenized *Octopus* ER LBD were expressed in CHO-K1 cells with a UAS-luciferase reporter. Cells were treated with 1 μM estradiol or vehicle only (ethanol). Graphs show means and SEM of three replicates. Fold activation is relative to the vector only, without added LBD.

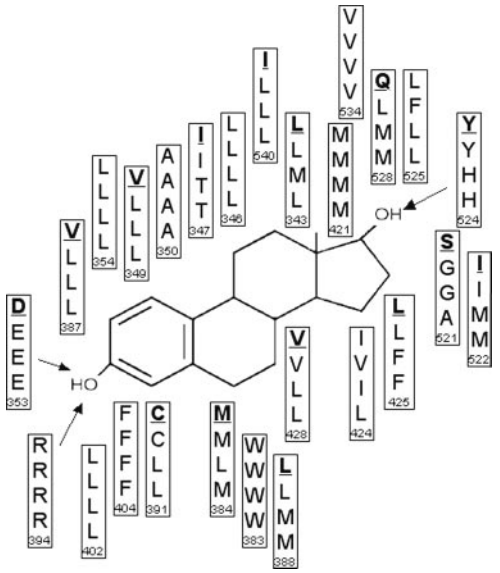

FIG. 8. Divergence of the *Octopus* ER ligand-binding pocket. 17β-Estradiol is shown surrounded by the residues at positions known to line the ligand-binding pocket in the human ERα (30, 31). The amino acid at each site is shown for *Octopus* ER, *Aplysia* ER, the ancestral steroid receptor (18), and human ERα (*top to bottom*). Sites are *numbered* according to the human sequence. Residues in which the *Octopus* ER differs from the human ERα are *bold* and *underlined*.

It has been argued that the ancestral nuclear receptor was probably a constitutively active orphan receptor and that liganded receptors emerged repeatedly from this ancestor, in parallel, during the course of receptor evolution (35). The evolution of ligand-independent constitutive activity in the mollusk ERs from an ancestral liganded receptor provides a counterexample to this model. Furthermore, this is not the first example of an evolutionary shift from a liganded to constitutive receptor. Structural and experimental studies indicate that the LRH-1 proteins of rodents are constitutive activators that have an empty ligand-binding pocket but are stable in the active conformation, in contrast to the human LRH-1 and the SF-1

proteins of humans and rodents, which require ligand binding for full transcriptional activity (22). The loss of ligand dependence in the rodent LRH-1 is due primarily to a single derived amino acid replacement, which creates a novel salt bridge within the receptor that excludes the ligand and stabilizes the receptor in the transcriptionally active conformation (22).

In addition, there are many examples of steroid receptor mutations that confer ligand-independent constitutive activity (36–40). At least one of these, at position 537 of the human ERα, occurs during the evolution of mammary tumor cells in humans (41). Intriguingly, the *Octopus* ER is not conserved with the human ERα or the ancestral steroid receptor at two of these positions, L536F and Y537C (based on human numbering). These replacements may contribute to the mechanism by which constitutive activity was likely to have been gained in the mollusk ERs. In addition, the loss of H524 from the ancestor may be important for the loss of estradiol binding in the mollusk ERs. The frequency and structural simplicity by which liganded receptors have evolved constitutive activation provides some evidence against the ancestral orphan receptor theory and favors the view that constitutive activation has evolved several times in parallel from a ligand-dependent nuclear receptor ancestor.

The apparent decoupling of the *Octopus* ER from estrogen signaling raises questions about the physiological and/or developmental functions of this receptor, which will require further investigation. Other constitutively active nuclear receptors play important roles in diverse organismal processes and are regulated by factors other than ligands, just as classic transcription factors are. For example, unliganded nuclear receptors may be regulated at the transcriptional level, by posttranscriptional modifications such as phosphorylation and sumoylation or the presence/absence of other receptor-interacting proteins, such as transcriptional coactivators (28, 42–45). We propose that similar mechanisms are likely to regulate the spatial and temporal role of the ERs in the biological processes of mollusks, which remain to be defined.

It is surprising that the *Octopus ER* does not respond to estradiol, given the circumstantial evidence for estrogen signaling in that species. In addition to studies that have identified estradiol and progesterone in *Octopus* tissue and demonstrated a correlation of hormone titers with reproductive status, there is also evidence for specific E2 binding in *Octopus* reproductive tissues (7, 8, 10). These studies provide only indirect evidence that E2 is a functional hormone; its presence does not exclude the possibility that it might be produced as an intermediate or metabolite of some other active hormone, and the binding observed in tissue extracts could be due to some other protein, *e.g.* an enzyme, not involved in endocrine signaling. Our experiments show that the *Octopus* ER does not bind or respond to estradiol, so if E2 does play an authentic signaling role in *Octopus* or other mollusks, the ER is not likely to be the mediator of these effects. There are numerous alternative pathways of steroid action, which trigger rapid, nongenomic signaling cascades that do not require transcriptional activation through the classic receptor (47). For example, some rapid effects of progesterone on vertebrate oocytes appear to be mediated by a seven-helix transmembrane protein (48, 49). In human cells, a G protein-coupled transmembrane receptor has been discovered that specifically binds estradiol and triggers ligand-dependent activation of adenylyl cyclase and phosphatidylinositol 3-kinase (50, 51). In the mollusk *M. edulis*, treatment with estradiol causes a very fast, concentration-dependent increase in nitric oxide production (12). Because the ER does not appear to mediate estrogen signaling in mollusks, these kinds of nongenomic mechanisms via alternative receptors are likely candidates for this role. If the relevant membrane receptors in mollusks are related to those in vertebrates, their conservation would indicate that nongenomic estrogen signaling is an extremely ancient mechanism for mediating steroid hormone effects in animals, one at least as old as the protostome-deuterostome divergence more than 600 million years ago.

An ancient nongenomic mechanism for estrogen signaling would suggest a plausible scenario for the evolution of the interaction between estrogens and the ancestral steroid receptor. Nuclear receptor phylogenies indicate that the steroid receptor clade was generated by duplication of a more ancient receptor related to the extant ERR and/or SF-1 family (19); the ancestral steroid receptor then diverged and increased its affinity for estrogens (17, 18). For natural selection to have played a role in this process, estrogens must have been present before the receptor evolved affinity for them, which is likely only if estrogens already had other functions in the cell. If non-ER-mediated pathways of estrogen signaling are ancient, however, then selection to maintain these functions would have stabilized the production of the hormone before the ancestral ER evolved. Such a scenario is similar to the process by which the androgen and progestin receptors, which descended from the estrogen-responsive ancestral steroid receptor by gene duplication, recruited intermediates in the synthesis of estrogens for new functional roles as signaling ligands. This process is called ligand exploitation because it involves duplicated receptors coopting steroids that had other, older functions as novel binding partners (17, 18). We hypothesize that the ancestral SR's relationship with estrogen may also have evolved by exploitation of a more ancient ligand, estrogen, whose original function was signaling through nongenomic pathways. Reports that estradiol is produced by cnidarians, basal metazoa in which there is no evidence for any steroid receptor genes, are consistent with this view (52, 53). Testing this proposal will require additional information on the pathways that mediate the role of estrogens in protostomes and basal metazoa.

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