Research paper

A second estrogen receptor from Japanese lamprey (*Lethenteron japonicum*) does not have activities for estrogen binding and transcription

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ABSTRACT

Estrogens regulate many physiological responses in vertebrates by binding to the estrogen receptor (ER), a ligand-activated transcription factor. To understand the evolution of vertebrate ERs and to investigate how estrogen acts in a jawless vertebrate, we used degenerate primer sets and PCR to isolate DNA fragments encoding two distinct ER subtypes, Esr1a and Esr1b from the Japanese lamprey, *Lethenteron japonicum*. Phylogenetic analysis indicates that these two ERs are the result of lineage-specific gene duplication within the jawless fishes, different from the previous duplication event of Esr1 (ERα) and Esr2 (ERβ) within the jawed vertebrates. Reporter gene assays show that lamprey Esr1a displays both constitutive and estrogen-dependent activation of gene transcription. Domain swapping experiments indicate that constitutive activity resides in the A/B domain of lamprey Esr1a. Unexpectedly, lamprey Esr1b does not bind estradiol and is not stimulated by other estrogens, androgens or corticosteroids. A 3D model of lamprey Esr1b suggests that although estradiol fits into the steroid binding site, some stabilizing contacts between the ligand and side chains that are found in human Esr1 and Esr2 are missing in lamprey Esr1b.

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1. Introduction

In the last few years, there has been significant progress towards understanding the evolution of steroid hormone receptors (Bertrand et al., 2004; Bridgham et al., 2008; Markov et al., 2009). These receptors, including the estrogen, androgen, progesterone, mineralocorticoid and glucocorticoid receptors, belong to the nuclear receptor family of transcription factors that mediate the physiological actions of their cognate steroids (Bridgham et al., 2010; Gronemeyer et al., 2004; Huang et al., 2010). Phylogenetic analyses indicate that the vertebrate ERs are in a separate clade from the other steroid receptors (SRs), which includes the receptors for androgen (AR), progestogens (PR), corticosteroids (GR and MR) (Baker et al., 2015; Bridgham et al., 2008; Markov et al., 2009; Thornton, 2001).

An important advance in understanding the early events in the evolution of steroid receptors was the cloning by Thornton (2001) of an ER, PR and corticoid receptor (CR) from Atlantic sea lamprey (*Petromyzon marinus*), a jawless vertebrate (agnatha), which along with hagfish are the earliest branching extant vertebrates. This ER is activated by estradiol (Paris et al., 2008).

Two distinct clades of ERs, Esr1 (ERα) and Esr2 (ERβ) have been found in amphibians, reptiles, birds and mammals, and some teleost fishes possess an additional estrogen receptor (Hawkins et al., 2000), as a result of a whole genome duplication that occurred at the base of teleost fishes (Bertrand et al., 2007; Hoegg et al., 2004; Meyer and Van de Peer, 2005; Postlethwait et al., 2004). The amino acid sequences of the steroid-binding domains on human Esr1 and Esr2 are about 60% identical (Kuiper et al., 1997, 1996).

The early events in evolution of Esr1 and Esr2 are not fully understood; that is, when did the gene duplication leading to these two ERs occur, and how does it relate to the diversity of ERs in the agnathans? In addition, the molecular mechanisms of estrogen action in jawless fish remain poorly understood (Bryan et al., 2008). Indeed, serum steroids in lamprey are unusual in containing 15α-hydroxy-estradiol (15α-OH-E2) and other 15α-hydroxylated steroid (Bryan et al., 2008; Lowartz et al., 2003). However, E2 appears to be the physiological estrogen in lamprey (Bryan et al., 2008; Mesa et al., 2010; Sower et al., 2011; Sower and Baron, 2011). To further understand the evolution of vertebrate steroid hormone receptors and to provide tools to study the molecular endocrinology of jawless fish, we isolated full length cDNA clones encoding two ER homologs from the Japanese lamprey, *Lethenteron japonicum*. These are Esr1a, which corresponds to the first ER cloned from Atlantic sea lamprey (Thornton, 2001), and Esr1b, which corresponds to an uncharacterized partial ER sequence [Ensembl accession ENSPMAP00000009392] in the genome of *P. marinus* (Smith et al., 2012, 2013), the sequence of which became available, while were completing the research reported here. We find that E2 and other vertebrate estrogens are transcriptional activators of Esr1a. Interestingly, lamprey Esr1a displays both constitutive and estrogen-dependent activation of gene transcription. Domain swapping experiments indicate that constitutive activity resides in the A/B domain of lamprey Esr1a. Unexpectedly, lamprey Esr1b does not bind E2 and is not activated by other estrogens, dihydrotestosterone (DHT), progesterone (P4) or corticosterone (B).

2. Materials and methods

2.1. Chemical reagents

We obtained chemicals from Sigma-Aldrich Corp.; 17β-estradiol (E2), estrone (E1), estriol (E3), 17α-ethinylestradiol (EE2), diethylstilbestrol (DES), 5α-dihydrotestosterone (DHT), corticosterone (B), progesterone (P4), testosterone (T), bisphenol A (BPA). All chemicals were dissolved in dimethylsulfoxide (DMSO), except for binding assays, which used ethanol solvent. The concentration of DMSO in the culture medium did not exceed 0.1%.

2.2. Estrogen receptor sequence discovery

Extraction of total RNA was carried out as described previously (Katsu et al., 2006) from Japanese lamprey (*Lethenteron japonicum*) frozen liver. cDNA was constructed from total RNA reverse-transcribed using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA) and oligo(deoxythymidine) [oligo(dT)] primer.

The full coding sequences of Japanese lamprey ERs were obtained using PCR and degenerate primers based on the Atlantic sea lamprey ER sequence (AY028456). Two conserved amino acid regions in the DNA binding domain (GYHYGVW) and the ligand-binding domain (NKGM/IEHL) of Atlantic sea lamprey ER were selected and degenerate oligonucleotides were used as primers for polymerase chain reaction (PCR). After amplification, an additional primer set corresponding to two amino acid sequences,

CEGCKAF and NKGM/IEHL, was used for the second PCR. The amplified DNA fragments were subcloned with TA-cloning plasmid pCR2.1 vector (Invitrogen), sequenced using a BigDye terminator Cycle Sequencing-kit (PE Biosystems, Foster City, CA) with T7 and M13-Reverse primers, and analyzed on the ABI PRISM 377 automatic sequencer. The 5′- and 3′-ends of the ERs were amplified by rapid amplification of the cDNA end (RACE) using a SMART RACE cDNA Amplification kit (BD Biosciences Clontech, Palo Alto, CA). Full-length transcript of the open reading frames of isolated ERs was then amplified using the primer set at the 5′ untranslated region and 3′ untranslated region.

2.3. Tissue-specific expression profiles

The tissue specific expression profile of Japanese lamprey ERs was determined using RT-PCR. For this, 2 μg total RNA isolated from specific tissues were reverse-transcribed using SuperScript III reverse transcriptase (Invitrogen) and oligo(deoxythymidine)[oligo(dT)] primer. The following primer sets were used for PCR: lamprey GAP-RT1 (control gene), 5′-CGTGCTGCCGTGCAAAAGGAA GAC-3′; and lamprey GAP-RT2, 5′-GGCCGGGATGATGTTCTGA GAAGC-3′ for lamprey glyceraldehyde-3-phosphate dehydrogenase (GAPDH, GenBank accession No. AB300852); lamprey Esr1a-RT1, 5′-AGCCGTTTCCGTGAGCTGCATCTC-3′; and lamprey Esr1a-RT2, 5′-T CGCTGGGTATGCACCCTGAACTC-3′ for lamprey Esr1a; lamprey Esr1b-RT1, 5′-AAGACGCAGCTCCTGGAGAACTCC-3′; and lamprey Esr1b-RT2, 5′-TCCCCTTGTTAGAGGCTCTGTAGC-3′ for lamprey Esr1b. Thirty cycles of amplification were carried out under the following conditions: denaturation at 94 °C for 30 s, annealing at 59 °C for 30 s, and extension at 72 °C for 1 min. At completion of the PCR, fragments were resolved on 1.5% agarose gels.

2.4. Transactivation and binding assays

HEK293 and CHO-K1 cells were used to perform reporter gene assay. These mammalian cell lines may lack co-regulators that are found in lamprey cells and are important in the transcriptional activity of lamprey ER. Transfection and reporter assays were carried out as described previously (Katsu et al., 2010). All transfections were performed at least three times, employing triplicate sample points in each experiment. The values shown are mean ± SEM from three separate experiments, and dose-response data and EC50 were analyzed using GraphPad Prism (Graph Pad Software, Inc., San Diego, CA).

The full-coding regions of lamprey ERs and human Esr1 (huEsr1: P03372) were amplified by PCR, and cloned into pcDNA3.1 vector (Invitrogen). An estrogen-regulated reporter vector containing four estrogen-responsive elements (4xERE, 5′-AGGTCAcagTGACCTcatgaAGGTCAcagTGACCTcatgaAGGTCAcagTG-ACCTcatgaAGGTCAcagTGACCT-3′), named pGL3-4xERE was constructed as described previously (Katsu et al., 2006) and a reporter plasmid having four GREs (4xGRE, 5′-GGTACAtccTGTTCT-catgaGGTACAtccTGTTCTcatgaGGTACAtccTGTTCTcatgaGGTACAtcc-TGTTCT-3′), named pGL4.23-4xGRE, was constructed by subcloning of oligonucleotides having 4xGRE into the *Nhe*I-*Hind*III site of pGL4.23 vector (Promega, Madison, WI). The ERE for lamprey ER is not known and it may differ from the ERE that we use in our assays. The DBD of amphioxus ERR and lamprey ERs was amplified by PCR, and cloned into the expressible fusion vector pACT (Promega). LBDs (including the hinge region) were cloned into pBIND vector. To construct the chimeric lamprey/human expression plasmids, four fragments corresponding to the A/B domain (lamprey Esr1a amino acids 1–245, human Esr1 amino acids 1–184), and C to F domains (lamprey Esr1a amino acids 246–740, human Esr1 amino acids 185–595) were amplified by

PCR, and ligated of lamprey and human fragments to each other to create two chimeric ER cDNAs. All constructs were verified by sequencing.

Ligand-binding assays were used to assess ability of lamprey ERs to bind estradiol, as previously described (Keay et al., 2006). Briefly, extracts of CHO-K1 cells expressing full-length Esr1a and Esr1b transcripts were incubated with a range of concentrations of ^{3}H-estradiol with or without a 200-fold excess of unlabeled estradiol to determine total and nonspecific binding, respectively. Bound and free hormone were separated using a hydroxyapatite slurry, and bound hormone was subsequently quantified radiometrically. Specific binding at each concentration was calculated as total binding minus nonspecific binding; binding constants were estimated using Prism software (GraphPad). All experiments were conducted in triplicate and repeated multiple times.

2.5. Phylogenetic analysis

The predicted protein sequences of the lamprey ERs were inferred from the nucleotide sequence and aligned to a database of 190 steroid and closely related receptors [Supplemental Table S1]. Alignments were prepared using MUSCLE (Edgar, 2004) implemented in Geneious Pro v5.3 (http://www.geneious.com/) using the default parameters. Initial alignments of full-length and available partial protein sequences were created in order to identify the boundaries of the conserved regions. Following manual curation of the alignment, the variable portions of the N-terminal, hinge, and C-terminal regions that could not be un-ambiguously aligned were removed from the dataset. The remaining regions were concatenated for use in the phylogenetic analyses.

Prior to phylogenetic reconstruction a model-fitting analysis using ProtTest 3 (Darriba et al., 2011) was performed to identify

the model of protein evolution that best fit the dataset. The best model was found to be the Jones-Taylor-Thornton (JTT) (Jones et al., 1992) model with a four-category discrete gamma distribution of among-site rate variation and a proportion of invariant sites with all model parameters, including the equilibrium amino acid frequencies, estimated from the dataset and optimized by maximum likelihood (JTT + IGF). Phylogenetic analyses were performed using maximum likelihood implemented in PhyML v3.0 (Guindon et al., 2010). Branch support was assessed by obtaining the approximate likelihood ratio value for each node, defined as the estimated ratio of the likelihood of the most likely tree to that of the best tree without the node of interest (Anisimova and Gascuel, 2006). Nodes with a likelihood ratio of less than 2 were treated as unresolved.

2.6. Construction of a 3D model of lamprey Esr1b

We used the homology option in Insight II software to construct a 3D model of lamprey Esr1b. We used human Esr2 complexed with E2 [PDB:3OLS] (Mocklinghoff et al., 2010) and human Esr1 complexed with E2 [PDB:1G50] as templates for constructing the 3D model of lamprey Esr1b. The structure of a short segment of five amino acids in human Esr2 is not found in the crystal structure or other Esr2 structures in the PDB. We used human Esr1 to model this segment. The sequences of the steroid-binding domain of lamprey Esr1b and human Ers2 are 46% identical, and there are 68% positives after including conservative replacements with only gaps at five positions in the sequence (Figs. 1 and 2). We inserted E2 into the apo-3D model lamprey Esr1b by overlapping lamprey ER2 with human Esr2 and extracting E2 from human Esr2 and inserting E2 into lamprey Esr1b using the Biopolymer option in Insight II.

We refined the structure of lamprey Esr1b with E2 using Discover 3 in Insight II. For this energy minimization step, Discover
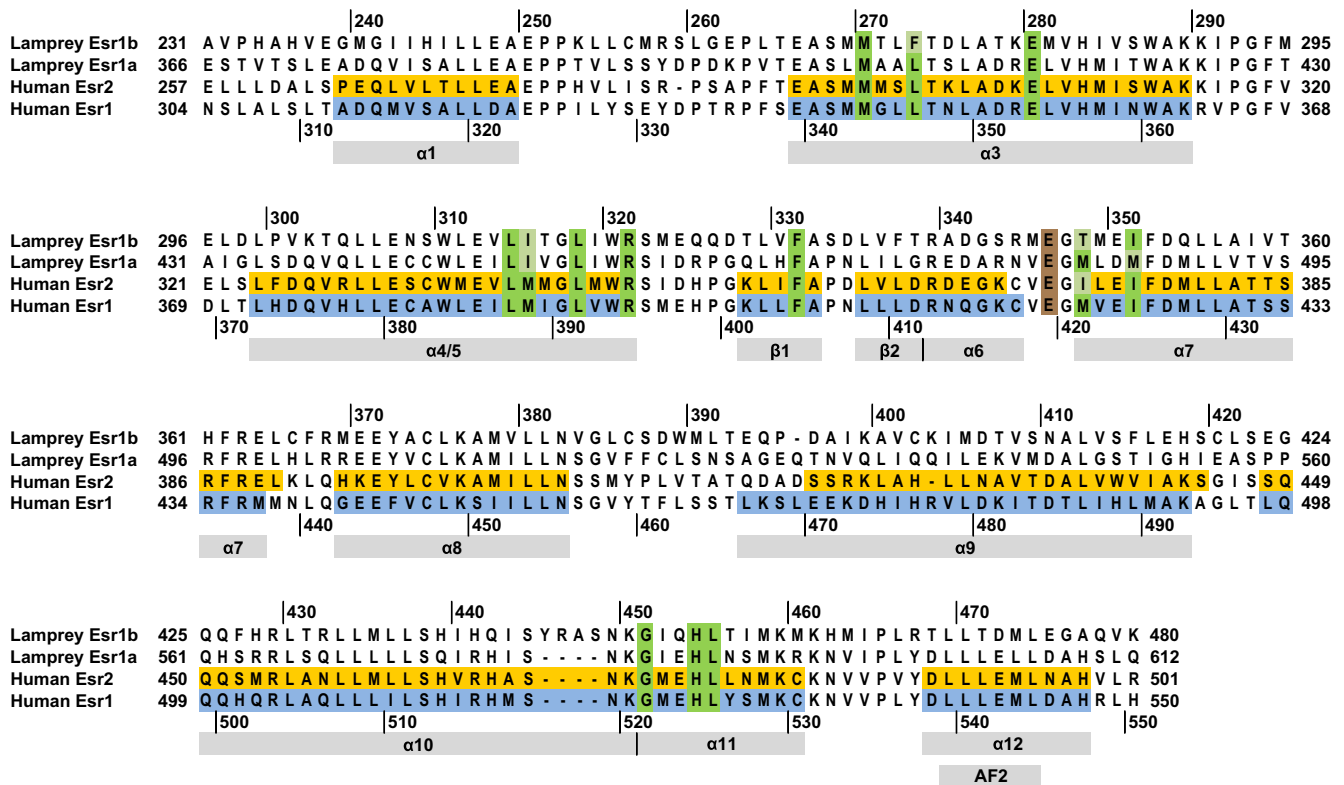

Fig. 1. Ligand binding domain sequence alignment of Japanese lamprey Esr1a and Esr1b with human Esr1 and Esr2. α-helices and β-strands from the crystal structures of Esr1 and Esr2 are shaded in each sequence and notated below the alignment. Residues in human Esr1 involved in binding of estradiol are shown in green. Glu-419, which stabilizes His-524 in human Esr1, is shaded in brown. The numbering of amino acids in lamprey Esr1b and human Esr1 is shown at the top and bottom, respectively, of the alignment.

3 was run for 10,000 iterations, using the CVFF force field and a distant dependent dielectric constant of 2. We also constructed a 3D model of Esr1b in which residues (YRAS) between 445 and 448 were removed. These four residues comprise an insert that is absent in Esr1a.

3. Results

When we began this research only one ER was known from Atlantic sea lamprey, *P. marinus* (Thornton, 2001). To clone ERs in a related species, the Japanese lamprey, we used degenerate primers designed from Atlantic sea lamprey ER to screen cDNA synthesized from Japanese lamprey liver RNA for ER orthologs. One DNA fragment (clone L2) was obtained after sequential PCR. This DNA fragment is identical at 97% to Atlantic sea lamprey ER (Thornton, 2001). The 5′ and 3′ ends corresponding to clone L2 were obtained by 5′RACE and 3′RACE. A full-length cDNA clone, derived from clone L2, was obtained by RT-PCR from Japanese lamprey liver mRNA, and designated as lamprey Esr1a (Fig. 1, GenBank accession number AB626148). Japanese lamprey Esr1a has strong sequence similarity to human Esr1 and Esr2 (Figs. 1 and 2A) and

Fig. 2. Comparison of functional domains on Japanese lamprey Esr1a and Esr1b with Atlantic lamprey Esr1a, human Esr1 and human Esr2. (A) Comparison of the Japanese lamprey Esr1a protein with sea lamprey (*P. marinus*) Esr1a and human ERs. The functional A/B to F domains are schematically represented with the numbers of amino acid residues indicated. The percentage of amino acid identity is depicted. (B) Comparison of the Japanese lamprey Esr1b protein with Japanese lamprey Esr1a, sea lamprey Esr1a and human ERs. The functional A/B to F domains are schematically represented with the numbers of amino acid residues indicated. The percentage of amino acid identity is depicted. Only partial sequences of Atlantic lamprey Esr1a and Esr1b have been completed. (C) Comparison of the DBD and LBD in Japanese lamprey Esr1b with Agnatha ERs and human steroid receptors. (D) Sequences in the P-box and AF-2 core region.

to Atlantic sea lamprey ER (Figs. 1 and 2A) (Thornton, 2001). Within the DBD on Japanese lamprey Esr1a, the P-box, a six-residue motif that mediates recognition of specific response elements by estrogen and other steroid hormone receptors (Thornton et al., 2003; Zilliacus et al., 1995), is identical to that of the human ERs (Fig. 2C). In lamprey Esr1a, the AF-2 activation domain, a small region in the LBD that mediates ligand-regulated interactions with coactivators (Lonard and O'Malley, 2005; Rosenfeld et al., 2006), is nearly identical to that of the ERs, but not to those in other steroid hormone receptors (Fig. 2D).

3.1. Isolation of a second estrogen receptor from Japanese lamprey

As mentioned above, when we began this research only one ER had been found in Atlantic sea lamprey (Thornton, 2001), leaving unknown whether lampreys contained a second ER. To investigate the existence of a second Japanese lamprey ER, we used a sequential PCR strategy to search for a second ER in cDNA from Japanese lamprey ovary RNA. Two ER-like DNA fragments were isolated. One was similar to lamprey Esr1a, and the other (clone D8) was similar to, but not identical to lamprey Esr1a. This second sequence was designated lamprey Esr1b (GenBank accession number AB626149). BLAST analysis (Altschul et al., 1997) indicated that lamprey Esr1b was similar to human ERs (Figs. 1 and 2B) and to the partial sequence of a second ER in the Atlantic sea lamprey genome [Supplemental Fig. S1]. Similar to lamprey Esr1a, the P-box, is identical to that of the human ERs, and the AF-2 activation domain is nearly identical to that of the human ERs (Fig. 2D).

3.2. Phylogenetic analysis of Japanese lamprey ER1 and ER2

Maximum likelihood phylogenetic analysis of a large number of diverse steroid receptor protein sequences supports a monophyletic clade consisting of Esr1 and Esr2 from jawed vertebrates (Fig. 3). This clade excludes the agnathan ERs and is reasonably well supported (approximate likelihood ratio = 15.3). This result indicates that the gene duplication that produced Esr1 and Esr2 from an unduplicated ancestral gene occurred after the split of agnathans from gnathostomes. Agnathan Esr1a and Esr1b are therefore not specific orthologs of Esr1 and Esr2, respectively.

The phylogeny indicates that *L. japonicum* Esr1a is orthologous to the previously-identified ER of the lamprey *P. marinus*. Japanese lamprey (*L. japonicum*) Esr1b clusters strongly with the ER of the hagfish *Myxine glutinosa*, suggesting a duplication of the ancestral ER deep in the agnathan lineage. The sequence data are not sufficient to clearly resolve the relationship between the two clades of agnathan ERs. The inferred phylogeny is consistent with a duplication yielding Esr1a and Esr1b within the agnathans, after their divergence from jawed vertebrates. The relevant node is weakly supported, however, so the phylogeny alone does not rule out the possibility of an additional duplication before the gnathostome-agnathan divergence to produce two ancient estrogen receptors, followed by total loss of the second gene from jawed vertebrates. This scenario, however, is considerably less parsimonious than the hypothesis of an agnathan-specific duplication leading to Esr1a and Esr1b in lamprey and hagfish. These results do not depend strongly on the statistical model chosen for phylogenetic analysis: phylogenetic analysis using the second-best model of evolution (JTT-GF) returned an identical topology and similar support values for the placement of the agnathan and gnathostome ERs. The evidence therefore favors, albeit indecisively, a single ER in the ancestral vertebrate, with an agnathan-specific duplication that produced Esr1a and Esr1b and a gnathostome-specific duplication that produced Esr1 and Esr2.
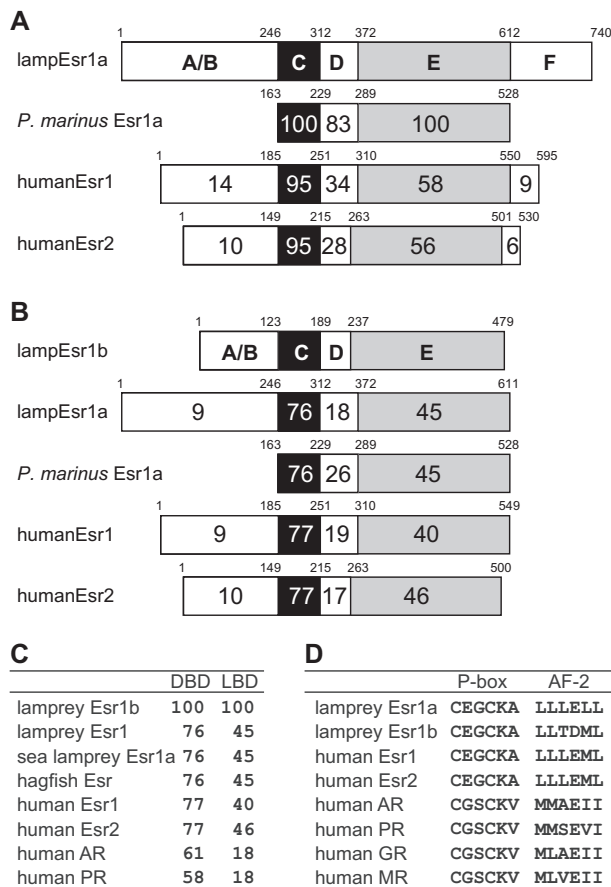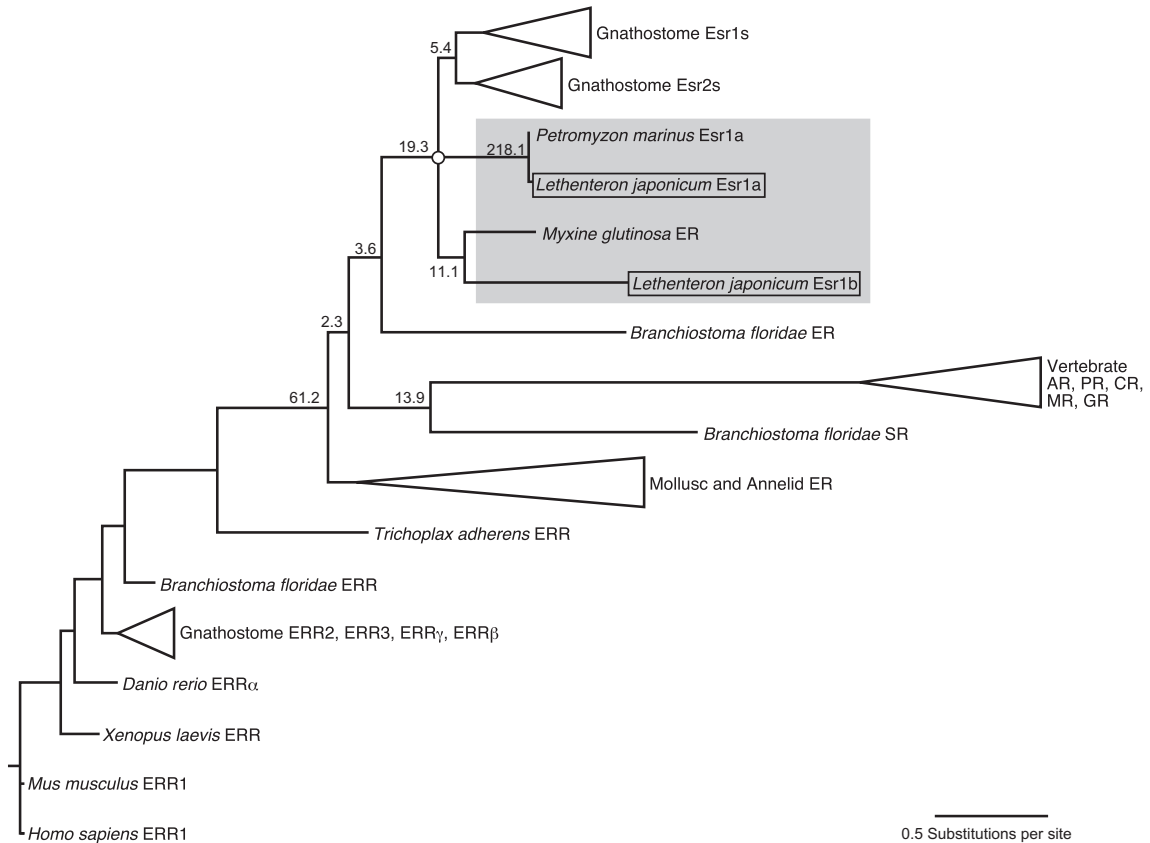
Fig. 3. The two lamprey ERs result from an independent gene duplication event specific to the agnathan lineage. A reduced maximum likelihood phylogenetic reconstruction of the steroid receptor protein sequences is shown. Each collapsed clade (triangles) contains many sequences. Genes identified in the present study are boxed. The gray box surrounds the known agnathan ERs. Node labels show support for each clade as the approximate likelihood ratio statistic, which is defined as twice the natural logarithm of the ratio of the likelihood of the best tree with the clade of interest to that of the best tree without that clade. The precise relationship between the gnathostome Esr1/Esr2 clade and the agnathan ERs was not resolved and is presented as a polytomy (circle).

3.3. Expression of Esr1a and Esr1b in Japanese lamprey

Lamprey Esr1a shows strong expression in female gut, liver and heart and male liver and gut (Fig. 4) and weaker expression in male heart and detectable expression in male gonad. In comparison, Japanese lamprey Esr1b shows strong expression in female lamprey heart, liver and gut and male heart and gut (Fig. 4). There also is weaker expression in male gonad and weak but detectable expression in female gonad and male liver.
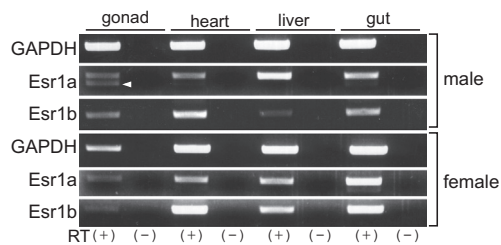

Fig. 4. Expression of Esr1a and Esr1b in male and female Japanese lamprey. Expression of Japanese lamprey Esr1a and Esr1b in different lamprey organs. Total RNA was prepared from gonad, heart, liver, and gut of both female and male adult lamprey. GAPDH was used as a positive control. Messenger RNA of ERs was detected by reverse transcription-PCR. Arrowhead indicates non-specific band.

3.4. Ligand-dependent and ligand-independent transactivation of Japanese lamprey Esr1a

We found that nM concentrations of E2 and a non-physiological concentration of 5α-dihydrotestosterone (DHT) can activate transcription by lamprey Esr1a (Fig. 5A). E2 has a half-maximal response (EC50) of 32 pM for lamprey Esr1a (Table 1). Like human ER, lamprey Esr1a responds to natural vertebrate E2, E1 and E3, as well as, synthetic estrogens, EE2 and DES (Fig. 5B and Table 1).

During the transcriptional analysis experiments, we observed that lamprey Esr1a activated gene transcription in the absence of E2 or other ligands (Fig. 6A); that is, lamprey Esr1a had partial constitutive activity, which has not been reported for either human Esr1 or Esr2 or for Atlantic sea lamprey ER. To begin to elucidate the basis for constitutive activity of Japanese lamprey Esr1a, we fused the ligand-binding domain (LBD) of lamprey Esr1a with a GAL4 DNA-binding domain (GAL4-DBD). Using the GAL4 system, we found that E2 activated transactivation of GAL4-DBD fused with lamprey Esr1a-LBD as found for human Esr1; however, the GAL4-DBD fused lamprey Esr1a-LBD lacks constitutive activity (Fig. 6B), suggesting that the A/B domain was important in constitutive activity of Esr1a. We compared the protein expression level of lamprey Esr1a and human Esr1 using immuno-blot analysis with N-terminal FLAG-tag recombinant proteins. Immuno-blot analysis showed the protein levels of lamprey Esr1a and human Esr1 were substantially similar, indicating that there was no different expression level between lamprey Esr1a and human Esr1 in our heterologous expression system [Supplemental Fig. S2].
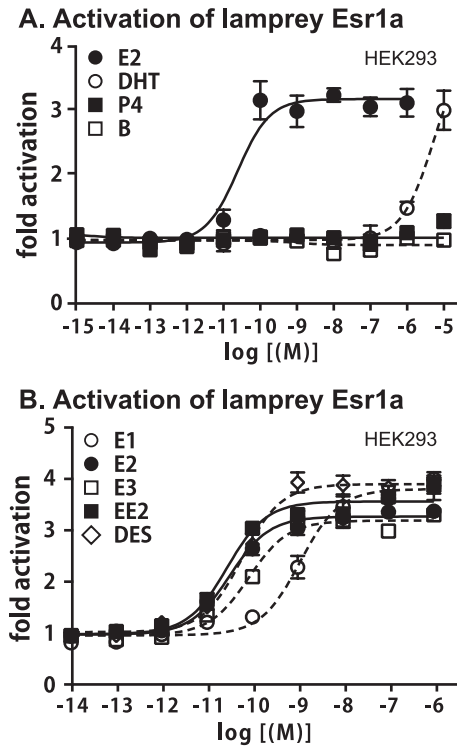
A. Activation of lamprey Esr1a

B. Activation of lamprey Esr1a

Fig. 5. Transcriptional activities of Japanese lamprey Esr1a. (A) Concentration-response profile for transcriptional activation of Japanese lamprey Esr1a activation in HEK293 cells by 17β-estradiol (E2), 5α-dihydrotestosterone (DHT), progesterone (P4) and corticosterone (B). (B) Concentration-response profile for lamprey Esr1a activation in HEK293 cells by E1 (estrone), E2, estriol (E3), 17α-ethinylestradiol (EE2) and diethylstilbestrol (DES). Results are expressed as means ± SEM, n = 3. The Y-axis indicates fold-induction compared to the activity of vehicle (DMSO) treatment alone.

Table 1
Gene transcriptional activities of estrogen mediated by Japanese lamprey Esr1a.

Estrogen	EC50 (M)	95% CI[a] (M)	RP[b] (%)
E1	1.1×10^{-9}	7.3×10^{-10} to 1.7×10^{-9}	2.9
E2	3.2×10^{-11}	2.2×10^{-11} to 4.7×10^{-11}	100
E3	8.3×10^{-11}	5.7×10^{-11} to 1.2×10^{-10}	38.6
EE2	2.8×10^{-11}	1.8×10^{-11} to 4.2×10^{-11}	114.0
DES	6.0×10^{-11}	4.1×10^{-11} to 8.8×10^{-11}	53.3

E1: estrone, E2: 17β-estradiol, E3: estriol, EE2: ethynyl estradiol, DES: diethylstilbestrol.
 [a] 95% CI: 95% confidence intervals of EC50.
 [b] RP: relative potency = (EC50 E2/EC50 other estrogens) × 100.

Next, we constructed chimeric ERs in which the A/B domains were swapped between Japanese lamprey Esr1a and human Esr1. That is, human Esr1 had its A/B domain replaced by the A/B domain of lamprey Esr1a, and lamprey Esr1 had its A/B domain replaced by the human Esr1 A/B domain. We found that a chimeric human Esr1 with the lamprey Esr1a A/B domain had constitutive activity, while lamprey Esr1a with the A/B domain of human Esr1 did not have constitutive activity (Fig. 6C). These results indicate that transcriptional activity in lamprey Esr1 in the absence of E2 resides in its A/B domain.

3.5. Estrogens are not transcriptional activators of Japanese lamprey Esr1b

We expressed lamprey Esr1a or lamprey Esr1b in HEK293 cells with a luciferase reporter driven by four canonical EREs. Japanese
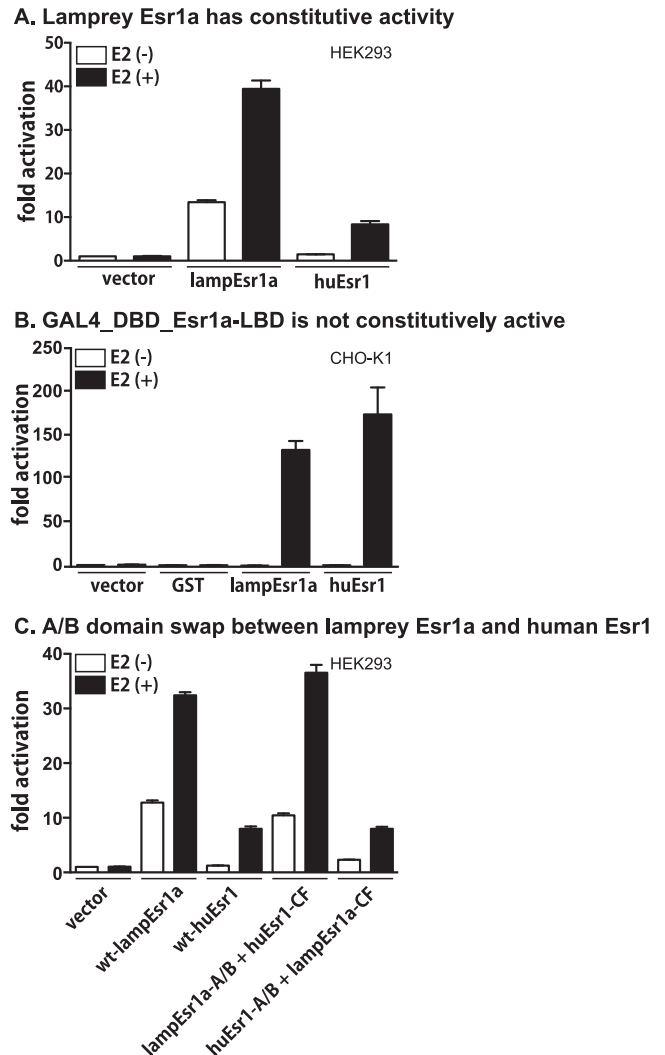
A. Lamprey Esr1a has constitutive activity

B. GAL4_DBD_Esr1a-LBD is not constitutively active

C. A/B domain swap between lamprey Esr1a and human Esr1

Fig. 6. Japanese lamprey Esr1a has constitutive activity. (A) Full-length lamprey Esr1a (lampEsr1a) and human Esr1 (huEsr1) were expressed in HEK293 cells with an ERE-luciferase reporter. Cells were treated with 10^{-8} M E2 or vehicle alone (DMSO). (B) Receptor LBDs were expressed on CHO-K1 cells as fusion proteins with GAL4-DBD along with a GAL4-binding site driven luciferase reporter. Cells were treated with 10^{-8} M E2 or vehicle alone (DMSO). (C) Functional analysis of chimeric ERs. A/B domain of lamprey Esr1a and human Esr1 were replaced each other. Wild type lamprey Esr1a (wt-lampEsr1a); Wild type human Esr1(wt-huEsr1); Human Esr1 replaced with A/B domain of lamprey Esr1a (lampEsr1a-A/B + huEsr1-CF); Lamprey Esr1a replaced with A/B domain of human Esr1(huEsr1-A/B + lampEsr1a-CF). All constructs were expressed in HEK293 cells with an ERE-luciferase reporter. Cells were treated with 10^{-8} M E2 or vehicle alone (DMSO). Results are expressed as means ± SEM, n = 3. The Y-axis indicates fold-induction compared to the activity of vehicle (DMSO) treatment alone.

lamprey Esr1b in the presence of E2 did not activate luciferase gene expression, even at 10^{-7} M E2 (Fig. 7A). In contrast, luciferase expression mediated by the lamprey Esr1a was significantly elevated over background, and maximal expression was found at 10^{-9} M E2 (Figs. 4 and 7A). Next, we examined the sensitivity of Japanese lamprey Esr1b to other steroid hormones using the same reporter assay. We also found that lamprey Esr1b was not activated by DHT, P4 or B (Fig. 7B). We also investigated activation of lamprey Esr1b, Esr1a and human Esr1 by bisphenol A (BPA). Although BPA could activate human Esr1 and lamprey Esr1a, there was no activation of lamprey Esr1b at 1 μM [Supplemental Fig. S3].

To determine if lamprey Esr1b could bind E2, we studied binding of E2 to lamprey Esr1b with competitive radio-ligand-binding studies in a cell-free system. Both human Esr1 and lamprey Esr1a
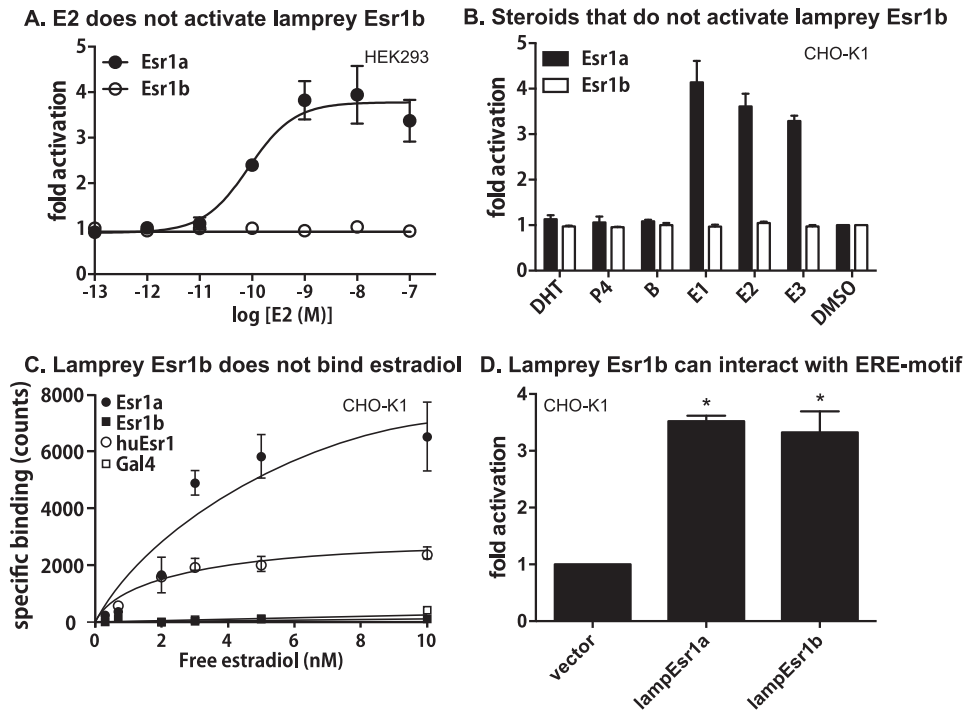
A. E2 does not activate lamprey Esr1b

B. Steroids that do not activate lamprey Esr1b

C. Lamprey Esr1b does not bind estradiol

D. Lamprey Esr1b can interact with ERE-motif

Fig. 7. Estrogens and other vertebrate steroids are not transcriptional activators of Japanese lamprey Esr1b. (A) Full-length Japanese lamprey Esr1a and Esr1b were expressed in HEK293 cells with an ERE-luciferase reporter. Cells were treated with E2 or vehicle alone (DMSO; E2(-)). Results are expressed as means ± SEM, n = 3. The Y-axis indicates fold-induction compared to the activity of control vector with vehicle (DMSO) alone. (B) Full-length Japanese lamprey Esr1a and Esr1b were expressed in HEK293 cells with an ERE-luciferase reporter. Cells were treated with 10^{-8} M steroid hormone (E1, E2, E3, P4, DHT, B) or vehicle alone (DMSO). (C) Ligand-binding assay. Extracts of CHO-K1 cells expressing full-length Esr1a and Esr1b transcripts were 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). (D) Interaction of lamprey Esr1bwith EREs. Receptor DBDs were expressed in CHO-K1 cells as a fusion protein with the herpes simplex virus VP16 activation domain along with an ERE-driven luciferase reporter. 1, control vector; 2, lamprey Esr1a; 3, lamprey Esr1b. Fold activation indicates luciferase activity relative to the vector-only control, which contains no DBD. Mean ± SEM of three replicates is shown.

bound tritiated E2 tightly and specifically, but lamprey Esr1b showed no evidence for any specific E2 binding at concentrations that saturate the human Esr1 and lamprey Esr1a (Fig. 7C). The Kds of E2 for lamprey Esr1a and human Esr1 were 2.0 ± 0.4 nM and 5.9 ± 2 nM, respectively (Kds ± SEM). Taken together, these data indicate that Esr1b's lack of transcriptional activity in the presence of E2 is due, in whole or part, to its inability to bind that hormone.

To determine if lamprey Esr1b could bind to the ERE, we expressed the DBD of lamprey Esr1b or Esr1a fused to the constitutively active herpes simplex virus VP16 activation domain in CHO-K1 cells with a luciferase reporter driven by four canonical EREs. Luciferase expression mediated by the DBDs on lamprey Esr1a and Esr1b was significantly elevated over background (Fig. 7D). This result indicates that the DBD of lamprey Esr1b can interact with ERE sequence motifs.

3.6. Construction of 3D model of Japanese lamprey Esr1b

We next considered the structural basis for lamprey Esr1b's inability to bind or be activated by steroids. Possible causes include a ligand binding site that is too small for E2 (Greschik et al., 2002; Kallen et al., 2004; Baker and Chandsawangbhuwana, 2007) or the lack of key stabilizing contacts between E2 and side chains in the lamprey Esr1b ligand binding pocket. To investigate these possibilities, we constructed a 3D model of lamprey Esr1b complexed with E2, which we compared to the crystal structure of human Esr2 with E2 (Mocklinghoff et al., 2010). The 3D model indicates that lamprey Esr1b (Fig. 8A) maintains residues corresponding to those involved in key stabilizing interactions with the ligand in human Esr2 (Figs. 1 and 8B). The 3D model of lamprey Esr1b with

E2 did not uncover any steric clashes between E2 and lamprey Esr1b, as has been found for ligands in the hormone binding site in human estrogen related receptors (ERRs) (Greschik et al., 2002; Kallen et al., 2004) and octopus ER (Baker and Chandsawangbhuwana, 2007). In fact, as shown in Fig. 8, analysis of E2 in lamprey Esr1b and human Esr2 indicates that overall E2 fits nicely into lamprey Esr1b, as found for human Esr1 (Baker, 2014; Baker et al., 2009; Brzozowski et al., 1997; Shiau et al., 1998; Tanenbaum et al., 1998), human Esr2 (Baker, 2014; Baker and Chandsawangbhuwana, 2007; Mocklinghoff et al., 2010) and Atlantic sea lamprey ER (Baker and Chandsawangbhuwana, 2007).

However, our 3D model of lamprey Esr1b also identifies a loss of key interactions between the D ring of E2 and Gly-451 and His-454. The backbone oxygen of Gly-451 is 8.1 Å from C16, and Nδ1 on His-454 is 3.6 Å from the C17 hydroxyl (Fig. 8A). In human Esr2, Gly-476 is 3.8 Å from C16 and Nδ1 on His-477 is 3.1 Å from the C17 hydroxyl (Fig. 8B). In the crystal structure of human Esr1 complexed with E2 (Brzozowski et al., 1997; Tanenbaum et al., 1998) the corresponding Gly-521 is 3.4 Å from C16 and Nδ1 on His-454 is 2.8 Å from the C17 hydroxyl [Supplemental Fig. S4]. The loss in lamprey Esr1b of the hydrogen bond between His-454 and the 17β-hydroxyl on E2 is especially important because this hydrogen bond is conserved in human Esr1 (Brzozowski et al., 1997; Shiau et al., 1998) and Esr2 (Mocklinghoff et al., 2010).

A 4-residue insertion (Tyr-Arg-Ala-Ser) after residue 444 in lamprey Esr1b near the end of α-helix 10 (Fig. 1) may contribute to the altered interaction between E2 and Gly-451 and His-454 (Fig. 9). This insertion, which is not absent in human Esr1 and Esr2 sequences, is just N-terminal to residues that form stabilizing polar contacts with the D-ring of the hormone in Esr2 (Gly-472, Leu-476 and His-475). Though lamprey Esr1b maintains the
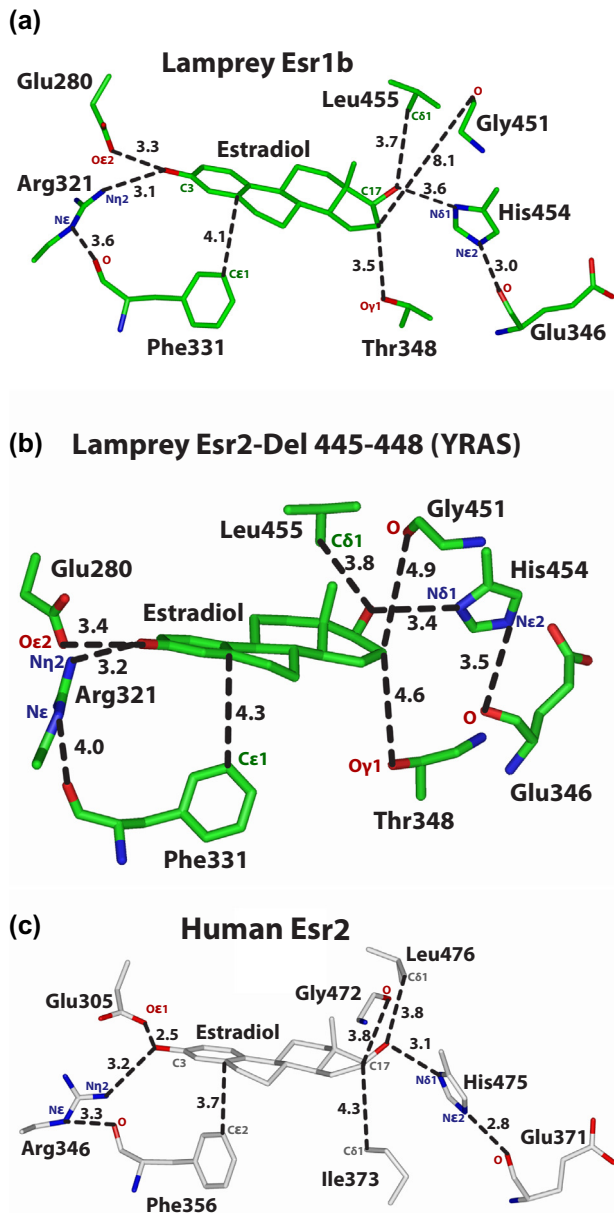
(a)

(b) Lamprey Esr2-Del 445-448 (YRAS)

(c)

Fig. 8. Interaction of E2 with human Esr2 and the 3D model Japanese lamprey Esr1b and Esr1b (Del 445–448). (A) Interaction between E2 and the 3D model of Japanese lamprey Esr1b. Lamprey Esr1b has stabilizing interactions with the A ring of E2 similar to those in human Esr2 and human Esr1. His-454 is 3.6 Å from the C17-hydroxyl on E2. The corresponding distances in human Esr2 (Fig. 8B) and human Esr1 [Supplemental Fig. S4] are 3.1 Å and 2.8 Å, respectively. The backbone oxygen on Gly-451 is 8.1 Å from C16. (B) Interaction between E2 and human Esr2. Nδ1 on His-475 is 3.1 Å from C17 hydroxyl on E2. The backbone oxygen on Gly-472 is 3.8 Å from C16. (C) Interaction between E2 and lamprey Esr1b (Del 445–448). Amino acids are labeled as in wild-type Esr1b to facilitate comparisons. After deletion of residues 445–448 the backbone oxygen on Gly-451 moves to 4.9 Å from C16. His-454 moves to 3.4 Å from the C17-hydroxyl on E2.

corresponding important residues (Gly-451, Leu-455 and His-454), the 3D model indicates a loss of key interactions between the D ring of E2 and Gly-451 and His-454 (Figs. 8A and 9B). The 3D model of lamprey Esr1b also indicates that there are weaker stabilizing contacts between Glu-280 and the C3 hydroxyl and Phe-331 and the A ring on E2. We also constructed a 3D model of lamprey Esr1b in which the YRAS insertion was removed. As seen in Fig. 8C, after removal of this insertion, Gly-451 is 4.9 Å from E2, and thus too far for a van der Waals contact. Other residues also have weaker
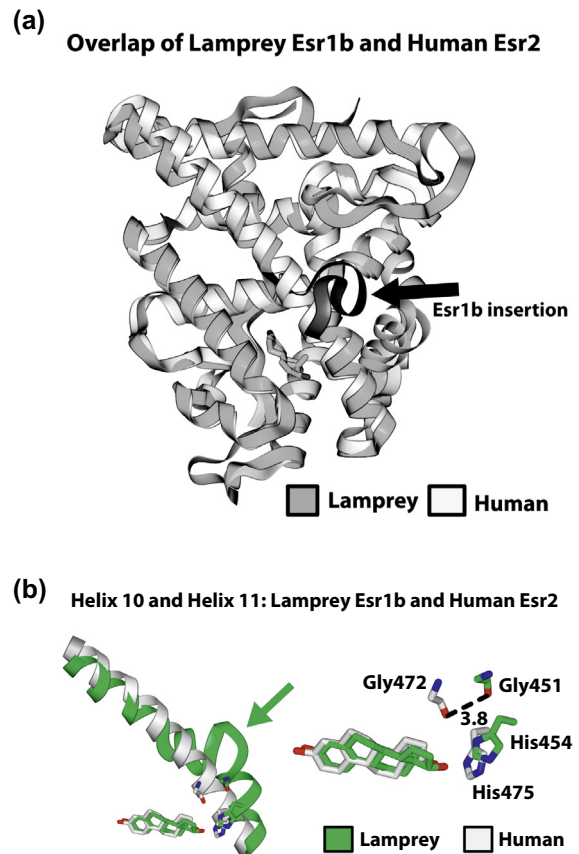
(a)

Overlap of Lamprey Esr1b and Human Esr2

(b)

Helix 10 and Helix 11: Lamprey Esr1b and Human Esr2

Fig. 9. Lamprey Esr1b contains an insertion that alters the conformation of Gly-451 and His-454. (A) Global alignment of the 3D model of lamprey Esr1b and human Esr2. Lamprey Esr1b contains a loop of four amino acids near the end of α-helix 10. This loop distorts the conformation of Gly-451 and His-454. Lamprey Esr1b is shown in green. The extra loop in lamprey Esr1b is shown in dark green. Human Esr2 is shown in gray. (B) Alignment of α-helix 10 and 11 in lamprey Esr1b and human Esr2. Gly-451 in lamprey Esr1b is displaced 3.8 Å compared to Gly-472 in human Esr2.

interactions with E2 than are found between E2 and human Esr1 and Esr2.

4. Discussion

In jawed vertebrates, such as human, other terrestrial vertebrates, and jawed fishes, two ERs, Esr1 and Esr2, mediate estrogen action (Deroo and Korach, 2006; Hawkins et al., 2000; Heldring et al., 2007; Katsu et al., 2008; Kuiper et al., 1997; Prins and Korach, 2008; Warner and Gustafsson, 2010). Teleost fish also contain a third receptor, Esr2b, but this appears to result from a lineage-specific duplication of Esr2 (Hawkins et al., 2000; Katsu et al., 2008). Thus, the common ancestor of teleost fish and tetrapods possessed Esr1 and Esr2. Here, we report that the Japanese lamprey, *L. japonicum* contains two distinct ERs, Esr1a and Esr1b. The presence of two ERs in *L. japonicum* is in agreement with the presence of an ER, corresponding to Esr1a, in Atlantic sea lamprey, *P. marinus* (Thornton, 2001) and a partial sequence of a second ER, corresponding to Esr1b, in the genome of *P. marinus*. The transcriptional properties of *P. marinus* Esr1b have not been characterized.

Phylogenetic analysis using maximum likelihood and a densely-sampled sequence alignment indicates that the Esr1 and Esr2 of jawed vertebrates were produced by a gene duplication after the agnathan-gnathostome divergence (Fig. 3). The presence of two ERs in jawless fishes appears to be due to an independent gene

duplication event specific to the agnathan lineage. Japanese lamprey Esr1a appears to be orthologous to the *P. marinus* ER, and Japanese lamprey Esr1b appears to be orthologous to the hagfish ER, but the relationship between these duplicate genes is not well supported. Evidence has been accumulating that the extant agnathans are monophyletic (Kuraku et al., 1999, 2009; Force et al., 2002), thus the most parsimonious explanation of the inferred phylogeny is that the agnathan ERs are the result of a gene duplication deep in the lineage leading to extant agnathans. This finding is consistent with previous work on other gene families, such as the Hox cluster (Force et al., 2002; Stadler et al., 2004; Kuraku et al., 2009) and thyroid hormone receptor 1 (TR1) and TR2 (Escriva et al., 2002), which also were subject to lineage-specific duplications within agnathans. Alternative explanations consistent with the phylogeny, such as a paraphyletic relationship between the two agnathan ER clades, would require additional gene duplications and losses in agnathans and jawed vertebrates to explain the present distribution of ERs.

Reporter gene assays in HEK293 cells revealed that lamprey Esr1a has a similar response to E1, E2, E3, DES and EE2 (Fig. 5) as found in human Esr1 (Kuiper et al., 1997) and other vertebrate ERs (Katsu et al., 2008, 2010), including Atlantic sea lamprey ER (Paris et al., 2008). However, unlike vertebrate ERs, lamprey Esr1a also has constitutive activity, which appears to reside in the A/B domain (Fig. 6).

Whether lamprey Esr1b has a ligand is unclear, because it was not activated by or able to bind steroids or BPA in our experiments. The loss of ligand sensitivity in *L. japonicum* Esr1b from a presumed ligand-binding ancestor could be due to, in part, to a 4-residue insertion (Tyr-Arg-Ala-Ser) located within helix 10, just N-terminal to Gly-451, Leu-455 and His-454, which contact the D-ring of E2 (Figs. 1 and 8). A similar insertion (Tyr-Ile-Ala-Ser) is found in *P. marinus* Esr1b, but not in any other Esr1 or Esr2 in GenBank. Our 3D model of lamprey Esr1b indicates that it maintains the majority of the residues involved in binding estradiol in human Esr2. However, the 4-residue insertion appears to shift key nearby residues in the putative ligand-binding pocket (Figs. 8 and 9). However, a 3D model of lamprey Esr1b in which the 4 residue segment is removed did not restore key contacts with E2. This suggests that the basis for lack of binding of E2 to lamprey Esr1b is complex, with contributions due to changes in other segments in addition to the four residue insertion.

Japanese lamprey Esr1a and Esr1b exhibit overlapping tissue expression profiles with expression differences (Fig. 4). In this study, we could not determine the quantities of Esr1a and Esr1b mRNAs by quantitative-PCR, due to limited samples of lamprey. Nevertheless, expression of Esr1b in several tissues supports a functional role for this receptor. Further research is necessary elucidate the basis for the novel physiological properties of Esr1a and Esr1b in Japanese lamprey and Atlantic sea lamprey.

Disclosure statement

The authors have nothing to disclose.

Acknowledgments

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ygcen.2016.07.014.

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