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UNIVERSITY OF CALIFORNIA, SAN DIEGO

The Study on Evolution of Functions and Structures of Steroid Receptors

A thesis submitted in partial satisfaction of the requirements for the degree
Master of Science

in

Biology

by

Kayla Yoonjoo Uh

Committee in charge:

Professor Michael Baker, Chair
Professor Scott Rifkin, Co-Chair
Professor Lakshmi Chilukuri

2012

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Co-Chair

Chair

University of California, San Diego

2012

DEDICATION

I would like to pay special thank to Michael Baker and all of the members of the Baker lab. Dr.Baker have influenced tremendously on my academic career of researcher and the work I have done could not have been done without him. I am truly grateful to his advice and teaching.

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ABSTRACT OF THE THESIS

The Study on Evolution of Functions and Structures of Steroid Receptors

by

Kayla Yoonjoo Uh

Master of Science in Biology

University of California, San Diego, 2012

Professor Michael Baker, Chair

The steroid receptors, a subgroup of nuclear receptors, regulate many physiological functions like homeostasis, growth, hormone, cancer, etc. The serum of lamprey contains two corticosteroids, 11-deoxycortisol and deoxycorticosterone (DOC). Only 11-deoxycortisol has high affinity for the CR in lamprey gill cytosol and we constructed 3D model of lamprey CR complexed

with 11-deoxycortisol and DOC. The 3D models reveal Leu-220 and Met-299 contacts with 17 α -hydroxyl on 11-deoxycortisol and van der Waals contact between helix 3 and helix 5. The contact between helix 3 and helix 5 is lost in human MR and we propose that this interaction was present in the CR and lost during the evolution of the MR and GR. The study of sea lamprey extended our interest to analyze the evolution of a 59 residue segment containing helices 3, 4 and 5 in vertebrate PRs and found that glycine corresponding to Gly-722 on helix 3 in human PR first appears in platypus. The replacement of cysteine to glycine that first appears in platypus was important in the evolution of mammals. Lastly, structure analysis of coactivator-ligand binding domain in ER α and ER β was done to provide detailed explanation of coactivator binding and to see whether coactivator binding is conserved across ER α and ER β . The Ile-1 contact with shallow depression was lost in ER β . Furthermore, sequence analysis of helices 3 through 5 and helices 11 through 12 showed high sequence conservation within mammals and marsupials and decreased sequence conservation from birds, amphibians, and fish.

Chapter 1. Introduction

Nuclear Receptors. Nuclear hormone receptors (NRs) are transcription factors that are present in metazoans. NRs regulate many physiological activities including development, reproduction, differentiation, hormone, and homeostasis. NRs are ligand-activated transcription factors thus responsible for close relationship between the ligand and transcriptional response.

NRs can be classified into three general categories based on the chemical similarity of their ligands. Steroid receptors bind hydrophobic steroid hormones like estradiol, cortisol, progesterone, aldosterone and testosterone. Other NRs, which bind thyroid hormone, vitamin D, ecdysone, retinoic acid and its trans and 9-cis isoforms form heterodimers with retinoid X receptor (RXR). Lastly, there is a third group of receptors for which ligands either do not exist or yet to be identified that are collectively known as orphan receptors. This group includes potentially important receptors such as steroidogenic factor 1, nerve growth factor-induced receptor, and X-linked orphan receptor DAX-1 (Robertson et al.1997 and Enmark and Gustafsson 1996).

The general signaling pathway of NRs has been reviewed previously and the pathway involves binding of the ligand to the receptor, a conformational change of the receptor, and transcriptional activation or repression of the genes through binding to various coactivator or corepressor proteins (Weatherman et al. 1999).

NRs are composed of common domain structure that includes highly conserved DNA-binding domain (DBD, also known as C domain), variable N-terminal region (A/B domain), a non-conserved hinge (D domain) and a moderately conserved ligand-binding domain (LBD, E domain) (Escriva et al. 2000). DNA binding domain is crucial for recognition of DNA sequences and ligand binding domain plays important role in hormone binding (Kumar and Thomson 1999).

Steroids Receptors. This report primarily focuses on study of evolution of function and structure of steroid hormone receptors, a subgroup of the larger family of nuclear receptors. Previous phylogenetic analyses indicate that current mammalian steroid receptors derived from ancestral estrogen receptor (ER) and ancestral 3-keto-steroid-receptor formed from gene duplication of the ER. An estrogen receptor first appears in amphioxus, which is a chordate just before the origin of vertebrates. The androgen receptor (AR), glucocorticoid receptor (GR), and mineralcorticoid receptor (MR) were created by series of duplications from common ancestral 3-keto-steroid-receptor (Thornton 2001).

The ligand binding domain of a nuclear receptor shares a common architecture, with 12 alpha helices and the beta turns arranged around central hydrophobic core (Zhang et al. 2005). Upon ligand binding, a conformational change of nuclear receptor takes place. The repositioned helix 12 aligns with helix 3 and helix 5 to form a pocket where transcriptional coactivators bind (Zhang et al. 2005). Previous studies have been done on the molecular

interactions that mediate these molecular events and the residues within the ligand binding domain of the steroid receptor have been identified and provided a structural basis for the steroid specificity of the receptors (Zhang et al. 2005). For steroid receptors, in particular, the volume of ligand binding pocket is larger than the volume of the corresponding ligands and the rigidity of the ligands does not allow adaptability (Moras and Gronemeyer 1998). Thus, the selectivity toward ligand is influenced more heavily with specific key residue interactions with specific steroid functional groups rather than multiple hydrophobic contacts like other receptors from nuclear receptor family (Moras and Gronemeyer 1998).

Sea Lamprey. Amongst many organisms, a sea lamprey was chosen as an initial subject of research because they are the most basal extant group of vertebrates and are thought to have existed without large change for more than 500 million years (Nikitina et al. 2009). The evolutionary position of lampreys and the easy access to adult lamprey and embryos make lamprey an excellent model to study the early vertebrate evolution (Nikitina et al. 2009). Lamprey contains an estrogen receptor, progesterone receptor, and corticosterone receptor, which is the ancestor of mineralcorticod receptor and glucocorticoid receptor. Previously, an analysis of the 3D structure of lamprey ER with various estrogens and lamprey PR with various progestin was performed. The study revealed that many structural interactions were conserved between estrogens in lamprey ER and human ER, and between progestin in lamprey PR and

human PR (Baker et al. 2011). Furthermore, both lamprey ER and lamprey PR contained unique features that play important role in binding of ligands to its receptors. The findings of these studies motivated us to analyze the binding of steroids to lamprey CR, which is the ancestor of the mineralcorticoid receptor and glucocorticoid receptor. In addition, data from previous report on steroid binding to lamprey CR motivated us to study the 11-deoxycortisol and deoxycorticosterone (DOC) binding to lamprey CR and human MR. Study done by Close et al. found that 11-deoxycortisol and DOC were the two endogenous corticosteroids in lamprey serum and *in vitro* binding studies of native CR in lamprey gill cytosol revealed that only 11-deoxycortisol had high affinity for lamprey CR (Close et al. 2010). We also studied binding of 11-deoxycortisol and DOC to human MR because lamprey CR is the ancestor of the MR and GR. Here we present the structure analysis of the binding of 11-deoxycortisol to the CR and discovery of unexpected contact between helix 3 and helix 5 present only in lamprey CR.

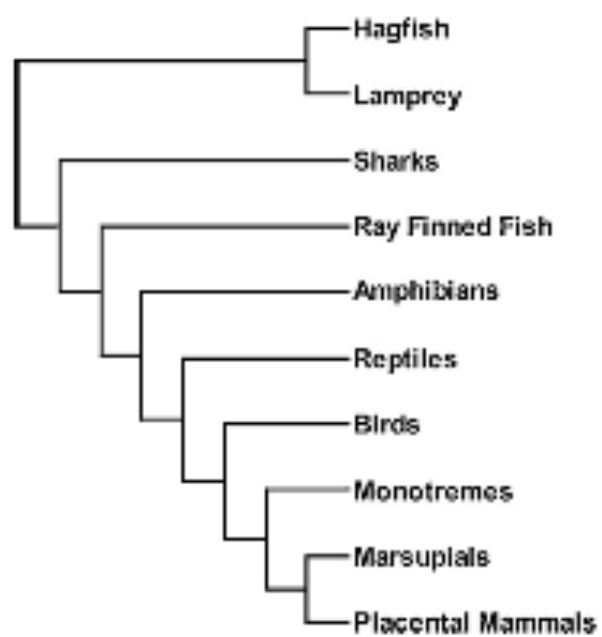


Figure 1-1. Vertebrate phylogeny. The phylogeny of vertebrate is shown. The phylogeny tree shows evolution from lamprey to placental mammals.

Progesterone Receptors. Such interesting findings from study of lamprey CR and human MR lead us to study the analysis of helix 3 and helix 5 contacts in vertebrate progesterone receptors. Previous studies have shown the importance of contact between helix 3 and helix 5. Geller et al. demonstrated that the distance between Ala-773 from helix 3 and Ser-810 from helix 5 in human MR is important as mutations of either residue alter the response of human MR to progesterone, cortisone, other steroids, and spironolactone (Geller et al. 2000). Likewise, mutations at corresponding site in human PR also alter the response of human PR to progesterone (Zhang et al. 2008). Here we investigate the evolution of the interaction between helix 3 and helix 5 on vertebrate PRs from the vertebrate phylogeny (Figure 1-1).

Chapter 2. 3D structure Analysis of Lamprey CR complexed with 11-deoxycortisol and deoxycorticosterone



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3D models of lamprey corticoid receptor complexed with 11-deoxycortisol and deoxycorticosterone

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ABSTRACT

The serum of Atlantic sea lamprey, a basal vertebrate, contains two corticosteroids, 11-deoxycortisol and deoxycorticosterone. Only 11-deoxycortisol has high affinity [$K_d \sim 3$ nM] for the corticoid receptor [CR] in lamprey gill cytosol. To investigate the binding of 11-deoxycortisol to the CR, we constructed 3D models of lamprey CR complexed with 11-deoxycortisol and deoxycorticosterone. These 3D models reveal that Leu-220 and Met-299 in lamprey CR have contacts with the 17 α -hydroxyl on 11-deoxycortisol. Lamprey CR is the ancestor of the mineralocorticoid receptor [MR] and glucocorticoid receptor [GR]. Unlike human MR and human GR, the 3D model of lamprey CR finds a van der Waals contact between Cys-227 in helix 3 and Met-264 in helix 5. Mutant human MR and GR containing a van der Waals contact between helix 3 and helix 5 display enhanced responses to progesterone and glucocorticoids, respectively. We propose that this interaction was present in the CR and lost during the evolution of the MR and GR, leading to changes in their response to progesterone and corticosteroids, respectively.

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1. Introduction

Ancestors of sea lamprey evolved over 500 million years ago, which places lamprey at the base of the vertebrate line. Lamprey's position as a basal vertebrate has motivated studies of lamprey to understand the evolution of vertebrates [1–3]. Sea lamprey (*Petromyzon marinus*) contains orthologs of the corticoid receptor [CR], progesterone receptor [PR] and estrogen receptor [ER] [4], which makes lamprey an important animal for understanding the evolution of vertebrate adrenal and sex steroid receptors [5–14].

Previously, we constructed 3D models of lamprey ER with various estrogens [15] and lamprey PR with various progestins [16]. These 3D models revealed that many structural interactions were conserved between estrogens in lamprey ER and human ER, and between progestins in lamprey PR and human PR. However, both lamprey ER and PR also contained some unique features that appeared to be important in the binding of 15 α -hydroxy-estradiol to lamprey ER and 15 α -hydroxy-progesterone to lamprey PR. The success of these previous studies motivated us to examine the binding of steroids to lamprey CR, which is the ancestor of the mineralocorticoid receptor [MR] and glucocorticoid receptor [GR] [4,7,17].

In addition to our interest in understanding how steroids bind to lamprey CR, we also were motivated by intriguing data from

two reports on steroid binding to lamprey CR. In the first report, 100 nM of cortisol, corticosterone, 11-deoxycortisol, 11-deoxycorticosterone (DOC) or aldosterone activated transcription in mammalian cells of the ligand-binding domain of lamprey CR fused to a GAL4-DNA-binding domain [7]. At concentrations below 10 nM, only aldosterone, corticosterone and DOC were transcriptionally active.

In the second report, Close et al. [18] found that 11-deoxycortisol and DOC were the two endogenous corticosteroids in lamprey serum. Neither cortisol nor corticosterone were present in lamprey serum. Further *in vitro* binding studies of native CR in lamprey gill cytosol revealed that only 11-deoxycortisol had high affinity [$K_d \sim 3$ nM] for lamprey CR. DOC, a mineralocorticoid, bound to CR with a substantially lower affinity than 11-deoxycortisol [18]. Moreover, neither cortisol, corticosterone nor progesterone bound to lamprey CR. These data and the evidence that the synthesis of 11-deoxycortisol in lamprey was stimulated by stress and that 11-deoxycortisol down-regulated synthesis of sex steroids and up-regulated gill Na⁺K⁺-ATPase prompted Close et al. [18] to propose that 11-deoxycortisol is the endogenous corticosteroid in sea lamprey.

With these reports [7,18] in mind, we constructed 3D models of lamprey CR complexed with 11-deoxycortisol and with DOC, the other corticosteroid in lamprey serum. We also constructed a 3D model of human MR with 11-deoxycortisol, which along with the crystal structure of the human MR with DOC [19], were compared with the 3D models of lamprey CR with 11-deoxycortisol and DOC. Analysis of our 3D models of lamprey CR and human

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MR with 11-deoxycortisol indicates that its 17 α -hydroxyl has van der Waals contacts with Leu-220 and Met-299 on lamprey CR and with the corresponding Leu-766 and Met-845 in human MR. In contrast, DOC does not contact either Leu-220 in lamprey CR or Leu-766 in human MR.

During our analysis of the 3D model of lamprey CR, we uncovered an unexpected van der Waals contact between Cys-227 in helix 3 and Met-264 in helix 5 in lamprey CR. This van der Waals contact is absent between the corresponding residues in helix 3 and helix 5 in human MR [Ala-773, Ser-810] [20] and human GR [Gly-567, Met-604] [21]. The pathophysiological importance of this van der Waals contact was demonstrated by Geller et al. [20], who found that a Ser-810 to Leu mutation in human MR results in an interaction between Leu-810 and Ala-773, which changes progesterone from a mineralocorticoid antagonist to an agonist. Subsequently, Zhang et al. [21] showed that for the GR, mutation of Met-604 to leucine in helix 5 leads to a van der Waals contact with Gly-567 and an increased response to glucocorticoids. The presence of this helix 3–helix 5 interaction in the 3D model of lamprey CR and its absence in human MR and human GR leads us to propose that this interaction was present early in vertebrate evolution and lost later in the evolution of the MR and GR with important consequences for their response to 3keto-steroids.

2. Experimental

2.1. Construction of 3D models

The 3D structure of human MR co-crystallized with corticosterone [PDB: 2A3I] [22] was used as a template for constructing the 3D model of lamprey CR [Genbank: AAK20930]. The sequences of the steroid-binding domain of lamprey CR and human MR are 67% identical, and after including conservative replacements [e.g. arginine/lysine, aspartic acid/glutamic acid], there are 76% positives with no gaps (Fig. 1). This strong similarity between lamprey

CR and its template gives us confidence in the accuracy of our lamprey 3D model [23]. We used the Homology option in Insight II to construct a 3D model of lamprey CR in the PDB format.

After we obtained the apo-3D model of lamprey CR, we inserted corticosterone into lamprey CR from human MR, by overlapping lamprey CR with human MR and extracting corticosterone from human MR for insertion into lamprey CR using the biopolymer option in Insight II. Corticosterone in lamprey CR and human MR was converted to 11-deoxycortisol by adding the 17 α -hydroxyl and removing the 11 β -hydroxyl. DOC was constructed from corticosterone by removal of the 11 β -hydroxyl. We used the crystal structure of human MR with DOC [PDB: 2AA7] [19] to analyze the binding of DOC to the MR.

We refined the structure of lamprey CR with 11-deoxycortisol and DOC and human MR with 11-deoxycortisol using Discover 3 in Insight II. For this energy minimization step, Discover 3 was run for 10,000 iterations, using the CVFF force field and a distant dependent dielectric constant of 2 to approximate for water in the protein.

3. Results

In Supplemental Fig. 1, we show the overlap of the C α -backbones of our 3D model of lamprey CR and the crystal structure of human MR. The root mean square deviation [RMSD] of their C α chains is 1.2 Å, which indicates good overall similarity between our 3D model of lamprey CR and human MR.

3.1. Analysis of 11-deoxycortisol binding to the 3D model of lamprey CR

The interactions of key residues in our 3D model of lamprey CR with 11-deoxycortisol are shown in Fig. 2A. Gln-230, Arg-271, Phe-283, Cys-227 and Met-264 stabilize the A ring on 11-deoxycortisol. The C3-ketone on the A ring is 3.2 Å from N ϵ 2 on

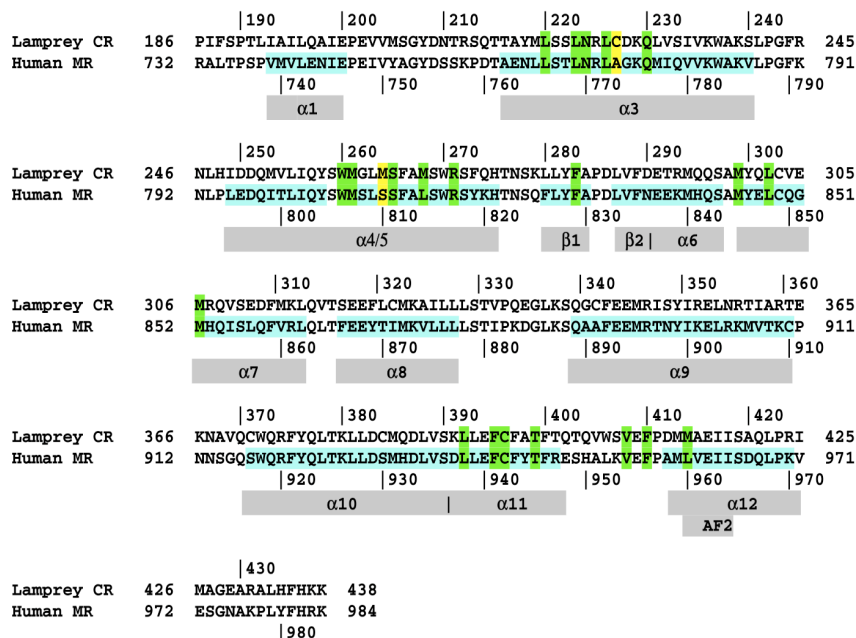


Fig. 1. Alignment of lamprey CR with human MR. α -helices and β -strands from the crystal structure of human MR [PDB: 2A3I] are shown in blue and noted below the alignment. Residues in human MR that contact corticosterone are shown in green [22]. The residues involved in the interaction between helix 3 and helix 5 in human MR in the 3D model of lamprey CR are shown in yellow. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

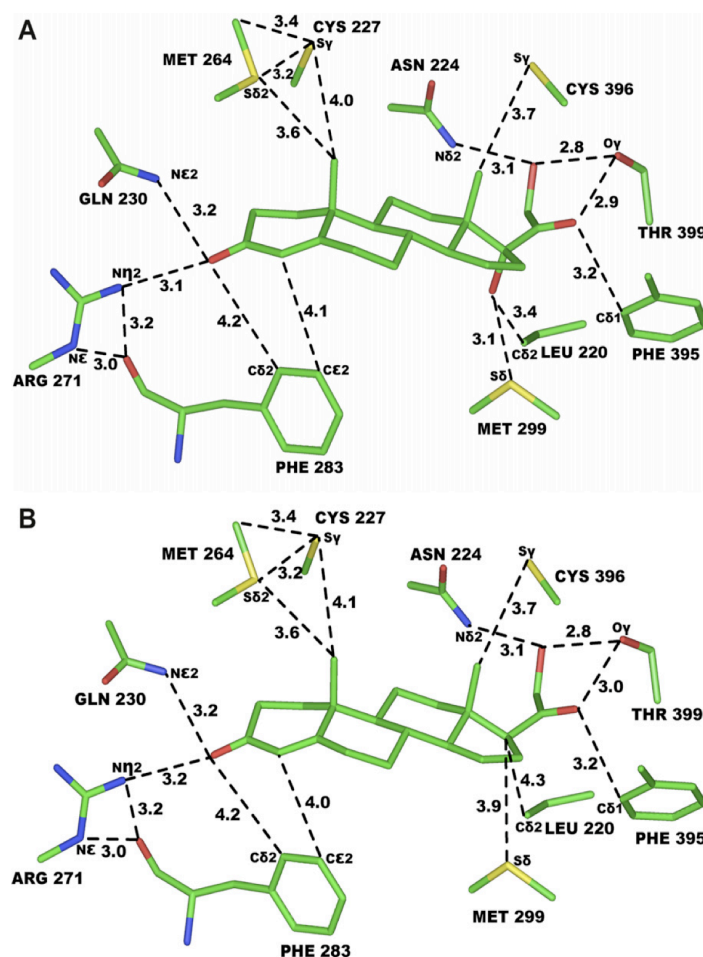


Fig. 2. Interaction of 11-deoxycortisol and DOC with the 3D model lamprey CR. With the exception of the 17 α -hydroxyl on 11-deoxycortisol, both 11-deoxycortisol and DOC have similar stabilizing contacts with lamprey CR. (A) Interaction of 11-deoxycortisol with lamprey CR. Gln-230, Arg-271, Phe-283, Cys-227 and Met-264 in lamprey CR have stabilizing contacts with the A ring of 11-deoxycortisol. Asn-224, Cys-396, Thr-399 and Phe-395 stabilize the D-ring. Leu-220 and Met-299 have van der Waals contacts with the 17 α -hydroxyl on 11-deoxycortisol. Cys-227 and Met-264 have a van der Waals contact. (B) Interaction of DOC with lamprey CR. The stabilizing contacts between DOC and lamprey CR are similar to that for binding of 11-deoxycortisol to lamprey CR. Met-299 has a van der Waals contact with C16 on DOC. Leu-220 is distant from DOC. Cys-227 and Met-264 have a van der Waals contact.

Gln-230 and 3.1 Å from N η 2 on Arg-271. N ϵ on Arg-271 is 3.0 Å from the backbone oxygen on Phe-283. C δ 2 on Phe-283 is 4.2 Å from the C3 ketone. S γ on Cys-227 and S δ 2 on Met-264 are 4 Å and 3.6 Å, respectively from C19. Asn-224, Cys-396, Phe-395 and Thr-399 stabilize the D ring on 11-deoxycortisol. N δ 2 on Asn-224 is 3.1 Å from the C21 hydroxyl; C δ 1 on Phe-395 is 3.2 Å from the C20 ketone; S δ on Cys-396 is 3.7 Å from C18 and O δ on Thr-399 is 2.9 Å from the C20 ketone and 2.8 Å from the C21 hydroxyl on 11-deoxycortisol.

The 17 α -hydroxyl on 11-deoxycortisol has van der Waals contacts with Leu-220 and Met-299. In addition, there is a van der Waals contact between S δ on Cys-227 in helix 3 and S δ on Met-264 on helix 5. As will be discussed later, this helix 3–helix 5 interaction is important in the response to steroids by the MR, GR and PR [24].

3.2. Analysis of DOC binding to the 3D model of lamprey CR

The interactions of key residues in our 3D model of lamprey CR with DOC are shown in Fig. 2B. The distances between these

residues and the A and D rings on DOC are similar to those found for 11-deoxycortisol and lamprey CR. An important exception is that C δ 2 on Leu-220 is 4.3 Å from DOC.

3.3. Analysis of 11-deoxycortisol and DOC binding to human MR

In Fig. 3, we present an analysis of the binding of 11-deoxycortisol and DOC to human MR for comparison with 3D models of lamprey CR (Fig. 2). Our 3D model of human MR with 11-deoxycortisol (Fig. 3A) shows that human MR has similar contacts with 11-deoxycortisol as found our 3D model of lamprey CR (Fig. 2A). For example, Gln-776, Arg-817 are 3.5 Å and 2.7 Å, respectively, from the C3-ketone on 11-deoxycortisol, and Thr-945 is 3 Å from C20 ketone and C21 hydroxyl on 11-deoxycortisol. Of importance is that S δ 2 on Met-845 is 2.6 Å and C δ 2 on Leu-766 is 3.3 Å from the 17 α -hydroxyl on 11-deoxycortisol.

Fig. 3B is based on the solved crystal structure of human MR with DOC [PDB: 2AA7] [19]. The distances between Arg-817, Gln-776 and Phe-829 in the human MR and the C3 ketone in DOC are similar to that for DOC in the 3D model of lamprey CR

Overall, an analysis of the binding of 11-deoxycortisol and DOC to lamprey CR (Fig. 2) and human MR (Fig. 3) reveals that key

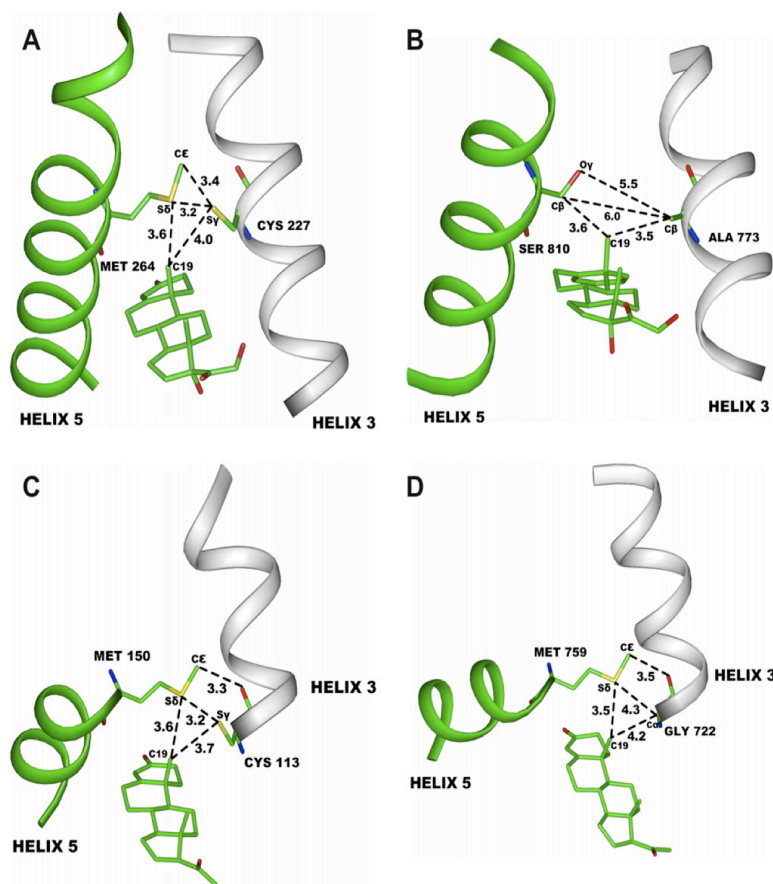


Fig. 4. Analysis of interactions between helix 3 and helix 5 in lamprey CR, lamprey PR, human MR and human PR. 3D models of lamprey CR and lamprey PR identify a contact between helix 3 and helix 5 that is absent in human MR and GR and present in human PR. (A) In the 3D model of lamprey CR with 11-deoxycortisol, Cys-227 on helix 3 and Met-264 on helix 5 are 3.2 Å distant. (B) In the 3D model of human MR with 11-deoxycortisol, Ala-773 on helix 3 and Ser-810 on helix 5 are 5.5 Å distant. (C) In the 3D model of lamprey PR with progesterone, Cys-113 on helix 3 and Met-150 on helix 5 are 3.3 Å distant. (D) In human PR with progesterone, Gly-722 on helix 3 and Met-756 on helix 5 are 3.5 Å distant.

interactions of the A ring of these two steroids with lamprey CR and human MR are conserved, as are many interactions with the D ring.

Of relevance for binding of 11-deoxycortisol to lamprey CR are the contacts of 17 α -hydroxyl on 11-deoxycortisol with S δ on Met-299 and C δ 2 on Leu-220 in lamprey CR (Fig. 2A). The corresponding amino acids in the 3D model of human MR with 11-deoxycortisol are Met-845 and Leu-766, which also have van der Waals contacts with the 17 α -hydroxyl group (Fig. 3A). These stabilizing contacts in human MR are consistent with transcriptional activation of human MR by 1 nM 11-deoxycortisol [20].

Regarding binding of DOC to lamprey CR, Leu-220 is too distant for a van der Waals contact with DOC (Fig. 2B). In the human MR, Leu-766 also is distant from DOC (Fig. 3B) [19]. Nevertheless, DOC has a nM affinity for human MR [25,26]. Thus, it is unlikely that the absence of a stabilizing contact between Leu-220 and DOC explains its low affinity for the CR in lamprey gill cytosol [18]. The lower affinity of DOC for lamprey CR may be due to post-transcriptional modifications of the CR that are specific to lamprey, the absence of inter-domain interactions that are present in human MR [27] or to apparently trivial sequence differences outside of the steroid-binding domain in the CR, as has been found for ligand binding to the estrogen receptor [28].

Interestingly, transcriptional studies in mammalian cells of the ligand-binding domain of lamprey CR fused to a

GAL4-DNA-binding domain indicate that DOC has a K_d of about 4 nM for lamprey CR and 11-deoxycortisol has a K_d of about 80 nM [7]. As noted previously [18], these data showing high affinity of DOC and lower affinity of 11-deoxycortisol for lamprey CR may be due to differences between transcriptional assays in mammalian cells and hormone-binding assays in lamprey gill cytosol or to differences in inter-domain interactions in the lamprey CR and human MR [27] or to post-translational modifications of the receptors or to specific co-regulators in lamprey and human cells [29].

4.2. Evolutionary implications

Various hypotheses have been advanced to explain the origins of the MR and its relationship to the GR and other steroid receptors [7,11,16,17,30,31]. Lamprey and hagfish, two basal vertebrates, contain a CR that is closer to the MR than to the GR [7], which supports the hypothesis that the MR is ancestral to the GR [7,11,17,30,32]. Lamprey also contains a progesterone receptor [PR], leaving unanswered the identity of the common ancestor of the CR and PR and the steroid[s] that regulated the ancestral 3keto-steroid receptor [16,33–34].

Our unexpected finding that the 3D model of lamprey CR predicts the presence of a van der Waals interaction between Cys-227 in helix 3 and Met-264 in helix 5 in lamprey CR provides an important insight into the evolution of steroid specificity for the

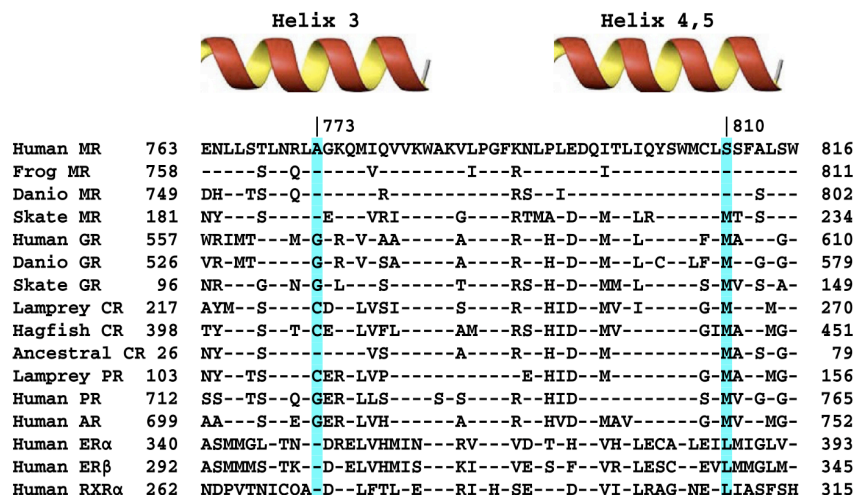


Fig. 5. Sequences in nuclear receptors that correspond to helix 3 and helix 5. Sequences from various steroid receptors that align with helix 3 and helix 5 in the human MR are shown [also see [11, 21]]. Residues involved in the interaction between helix 3 and helix 5 are shown in blue. Residues in other species that are identical to those in human MR are denoted with a -. Residues corresponding to Ala-773 on human MR are cysteine in lamprey CR, hagfish CR and lamprey PR, an alanine in skate MR, frog MR and Danio MR. At this position, skate GR contains a glycine, as do Frog GR, Danio GR, human PR and AR. An alanine is in this position in human ER α , ER β and RXR α , [11,21]. Residues corresponding to human Ser-810 on human MR are methionine in lamprey CR, lamprey PR, hagfish CR, skate MR, skate GR, frog MR, frog GR, human PR and AR. A leucine is in this position in human ER α , ER β and RXR α , [11,21]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

MR and GR. In human MR, the corresponding Ala-773 and Ser-810 lack a van der Waals contact between helix 3 and helix 5 [19,22] (Figs. 4 and 5 and Supplemental Fig. 2). Geller et al. [20] found that replacing Ser-810 with leucine yielded a mutant MR in which there was an interaction between helix 3 and helix 5, and this interaction changed progesterone from an MR antagonist to an agonist. Moreover, 1 nM 19-norprogesterone, pregnenolone and 17 α -OH-progesterone became activators of gene transcription of the Leu-810 mutant MR in Cos-7 cells. Interestingly, cortisone, which does not bind to human MR, is a transcriptional activator of the Leu-810 mutant MR [35]. Further studies with the human GR [21] showed that mutation of Met-604 in helix 5 to leucine provided an interaction with Gly-567 on helix 3, which increased the response to glucocorticoids. Together, these studies established the importance of the helix 3–helix 5 interaction in regulating the specificity and responses to steroids in the human MR and GR.

Re-examination of our 3D model of lamprey PR with progesterone indicates that it predicts the presence of a van der Waals contact between Cys-113 in helix 3 and Met-150 in helix 5 in lamprey PR [16] (Fig. 4C). In human PR, Gly-722 in helix 3 and Met-759 in helix 5 have a van der Waals contact. Interestingly, Zhang et al. [21] found that replacing Gly-722 in helix 3 with alanine yielded a mutant human PR with constitutive activity. This provided additional evidence that the interaction between helix 3 and helix 5 is important in transcriptional activation of steroid receptors.

The evidence from our 3D models of lamprey CR and PR showing a key helix 3–helix 5 contact, and the conservation of the corresponding cysteine and methionine in hagfish CR (Fig. 5) suggests that the helix 3–helix 5 interaction was conserved in the common ancestor of lamprey and hagfish. Moreover, other analyses suggest that this contact is ancient, as it is found in the ER, RXR and other nuclear receptors (Fig. 5) [11,21,24]. However, unequivocal proof of the primordial origin of the helix 3–helix 5 interaction will require crystal structures of lamprey CR with various corticosteroids.

Interestingly, skate MR appears to be an evolutionary intermediate in the loss of the helix 3–helix 5 interaction in vertebrate MRs. Skate MR has an alanine at the position corresponding to

lamprey Cys-227, while skate MR contains a methionine corresponding to lamprey Met-264 in helix 5. Based on studies showing that the human Ser810Met mutant MR responds to 19-nor-progesterone [20], skate MR would be expected to retain an interaction between helix 3 and helix 5. It appears that this interaction was lost later, when methionine was replaced by serine in helix 5 in teleosts and land vertebrates (Fig. 5) [20,30].

Based on the changes in the response of human MR and human GR to steroids due to mutations in helix 3 and helix 5 [20,21], it appears that the loss of this interaction was important in the evolution of corticosteroid specificity in vertebrate MR and GR, although mutations at other positions in the MR and GR also appear to be important [7,17,36].

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.steroids.2011.07.015.

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Chapter 3. Evolutionary analysis of the segment from helix 3 through helix 5 in vertebrate progesterone receptors



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Evolutionary analysis of the segment from helix 3 through helix 5 in vertebrate progesterone receptors

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ABSTRACT

The interaction between helix 3 and helix 5 in the human mineralocorticoid receptor [MR], progesterone receptor [PR] and glucocorticoid receptor [GR] influences their response to steroids. For the human PR, mutations at Gly-722 on helix 3 and Met-759 on helix 5 alter responses to progesterone. We analyzed the evolution of these two sites and the rest of a 59 residue segment containing helices 3, 4 and 5 in vertebrate PRs and found that a glycine corresponding to Gly-722 on helix 3 in human PR first appears in platypus, a monotreme. In lamprey, skates, fish, amphibians and birds, cysteine is found at this position in helix 3. This suggests that the cysteine to glycine replacement in helix 3 in the PR was important in the evolution of mammals. Interestingly, our analysis of the rest of the 59 residue segment finds 100% sequence conservation in almost all mammal PRs, substantial conservation in reptile and amphibian PRs and divergence of land vertebrate PR sequences from the fish PR sequences. The differences between fish and land vertebrate PRs may be important in the evolution of different biological progestins in fish and mammalian PR, as well as differences in susceptibility to environmental chemicals that disrupt PR-mediated physiology.

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1. Introduction

The adrenal steroids, aldosterone and cortisol, and sex steroids, estradiol, progesterone and testosterone, regulate a wide range of physiological processes including reproduction, development and homeostasis. The physiological actions of these vertebrate steroids are mediated by nuclear receptors, a large and diverse group of transcription factors that arose in multi-cellular animals [1–5].

Lamprey, a basal vertebrate [6–8], contains an estrogen receptor [ER], progesterone receptor [PR] and a corticoid receptor [CR] [9,10]. Our interest in the evolution of adrenal and sex steroid receptors [11–13] stimulated us to construct 3D models of lamprey ER [14], PR [15] and CR [16]. An unexpected outcome from an analysis of our 3D model of lamprey CR was the discovery of a van der Waals contact between Cys-227 on helix 3 and Met-264 on helix 5 [16]. This contact was unexpected because in the human MR, complexed with corticosteroids, the distance between corresponding sites in helix 3 [Ala-773] and helix 5 [Ser-810] is a little over 5 Å, which is too far for a van der Waals contact [17–19]. Moreover, Geller et al. [20] demonstrated that the distance between Ala-773 and Ser-810 in human MR is important in its response to steroids.

Mutations of either Ala-773 or Ser-810 that decrease the distance between these sites on helix 3 and helix 5 alter the response of human MR to progesterone, cortisone, other steroids and spironolactone [20–22]. Thus, for the Leu-810 mutant MR, progesterone and spironolactone become agonists instead of antagonists [20,22], and cortisone, which does not activate wild-type MR, becomes an agonist [21]. Mutations at the corresponding sites, Gly-722 [helix 3] and Met-759 [helix 5] in human PR and Gly-567 [helix 3] and Met-604 [helix 5] in the human glucocorticoid receptor [GR] also alter their response to progesterone and corticosteroids, respectively [23,24]. Interestingly, in the human PR, Gly-722 is required for binding of RU486 [25], a progesterone antagonist that is used as a contraceptive [26]. Replacement of Gly-722 with either cysteine [25] or alanine [23] leads to loss of binding of RU486.

In lamprey PR, the corresponding residues in helix 3 and helix 5, are Cys-113 and Met-150, respectively [15]. Examination of our 3D model of lamprey PR revealed the presence of a van der Waals contact between Cys-113 on helix 3 and Met-150 on helix 5, which indicates that this interaction between helix 3 and helix 5 in the PR and CR is ancient.

These data and the intriguing observation by Hu and Funder [27] that the Cys/Met pair in helix 3 and helix 5, respectively, is conserved in eel PR motivated us to investigate the evolution of this interaction between helix 3 and helix 5 on vertebrate PRs. Our goal was to determine when glycine first evolved as a partner for

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the conserved methionine in helix 5 in the PR. BLAST analyses of PR sequences in GenBank reveal that the helix 3 cysteine is conserved in the PR in all fish, skates, amphibians and reptiles. It is in the PR in platypus, a monotreme, which evolved at the base of the mammalian line [Fig. 1], that the cysteine to glycine replacement first appears, as well as three other replacements in the segment containing helix 3 through helix 5. We propose that replacement of the previously conserved cysteine with a glycine in helix 3 in a platypus PR was important in mammalian evolution. In mammalian PRs, the functions of this glycine in helix 3 and of three other amino acids that first appear in mammalian PRs in the segment containing helix 3 through helix 5 are not yet known. They may be important in steroid specificity for the PR, as different progestins are active in fish and mammals [28,29] [Fig. 2], or in the binding of different co-regulators to PRs in various vertebrates [30,31] or both.

2. Experimental

2.1. Analysis of PR sequences

A 59 residue segment from the human PR corresponding to helix 3 through helix 5 [15,16,27,32–34] was used as a probe by BLAST to search GenBank and Ensembl for the corresponding segment in

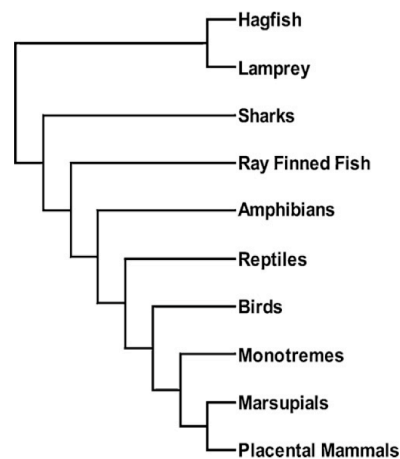


Fig. 1. Vertebrate phylogeny. Platypus is a monotreme, which evolved at the base of the mammalian line. In platypus PR, four amino acids replacements first appear in a segment containing helix 3 through helix 5.

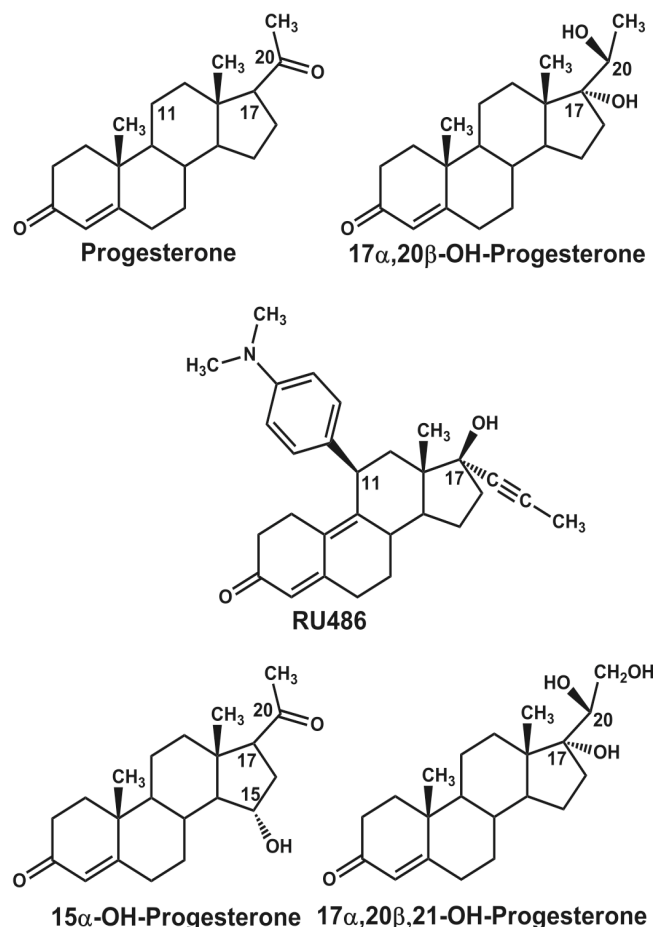


Fig. 2. Structures of progesterone and fish progestins. Progesterone is the active progestin in mammals. 17 α ,20 β -Hydroxy-progesterone and 17 α ,20 β ,21-hydroxy-progesterone are active in fish [29,46], but not in humans [49,50]. 15 α -Hydroxy-progesterone may be the active progestin in lampreys [15,47,48]. RU486 is a progesterone antagonist that is used as a contraceptive [26,42].

other animal PRs. The accessions for each gene are presented in Table 1 in the Supplementary data.

The crystal structure of human PR with progesterone [PDB: 1A28] [32] was used to investigate the effects on the interaction between progesterone and the human PR containing the four amino acid replacements that first appear in platypus PR. For this purpose, the Biopolymer software was used to construct human PR with four replacements: Cys-722, Cys-728, Ala-762 and Met-763. This 3D model was refined with Discover 3 using the CVFF force field and a distant dependent dielectric constant of 2 for 50 iterations.

3. Results

3.1. Sequence analysis of helix 3 through helix 5 in the land vertebrate PRs

The conservation of amino acids in the segment containing helix 3 through helix 5 in human PR, lamprey PR, skate PR and selected PRs from amphibians, reptiles and mammals is shown in Fig. 3. Also shown are sequences from human and skate MR, GR and AR and lamprey CR. The positions corresponding to Gly-722 and Met-759 in human PR are shown in blue.

Analysis of the sequences in Fig. 3 reveals that Met-759 is conserved in all vertebrate PRs, while a glycine corresponding to Gly-722 first appears in platypus PR. Opossum, wallaby [35] and horse PRs contain an alanine, and Golden hamster [*Mesocricetus auratus*] [25] and Chinese hamster [*Cricetulus griseus*] PRs contain a cysteine and glycine, respectively, at this position. All other available mammalian sequences in GenBank contain a glycine corresponding to Gly-722 in human PR.

Three other amino acids, corresponding to Ser-728, Gly-762 and Leu-763 in human PR, first appear in platypus PR. In chicken PR, the corresponding amino acids are Cys-581, Ala-615 and Met-616. Other bird and amphibian PRs also contain a cysteine, alanine and methionine that correspond to the chicken residues [Fig. 4]. Residues corresponding to Ala-615 and Met-616 are conserved in skate PR and lamprey PR [Fig. 3].

There is 100% conservation of helix 3 to helix 5 in most mammal PRs and about 90% conservation between mammal and bird PRs, indicating that this segment is under strong constraints in these vertebrates. Interestingly, macaque PR and the baboon PR have a leucine corresponding to Ser-735 in human PR [Fig. 3].

We also note that human and skate AR and GR contain a methionine and glycine, corresponding to Met-759 and Gly-722 in human PR, indicating that a glycine at this site in helix 3 evolved at least twice in adrenal and sex steroid receptors.

3.2. Sequence analysis of helix 3 through helix 5 in fish PRs

Fig. 5 shows the alignment of the segment containing helix 3 through helix 5 in fish PRs with selected land vertebrate PRs, skate PR and lamprey PR. All of the fish PRs contain the helix 3 cysteine that corresponds to Gly-722 in human PR. Conservation of this cysteine in lamprey, skate and fish PRs suggests an important role in progesterone action. Interestingly, residues corresponding to Ala-615 and Met-616 in human PR, which are conserved in lamprey PR and skate PR, are not conserved in teleost PRs [Fig. 5].

3.3. 3D model of human PR containing four replacements in helix 3 through helix 5

To begin to investigate the role in progesterone binding of the four amino acids that first appear in platypus PR, we constructed a 3D model of the progesterone in human PR containing Cys-722, Cys-728, Ala-762 and Met-763. Fig. 6 shows the overlap at these four sites and some other residues in the steroid binding domain

of the “ancestor-like” 3D model and the crystal structure of wild-type human PR complexed with progesterone [15,32–34]. There is excellent overlap of all amino acids.

Fig. 7 shows a detailed analysis of the interaction between the progesterone and the side chains in wild-type PR [Fig. 7A] and the “ancestor-like” PR [Fig. 7B]. Comparison of Fig. 7A and B reveals that the “ancestor-like” PR and the crystal structure of the PR have similar contacts between Gln-725, Arg-766, Phe-778 and Gln-725 and the ketone on the progesterone A ring; between Asn-719 and the progesterone C21 methyl group; between Thr-894 and the progesterone C20 ketone and between Met-909 and the progesterone C18 methyl group.

There also is excellent conservation of the positions of the four replacements in helix 3 through helix 5. In wild-type PR [Figs. 6 and 7A], Gly-762 and Leu-763 have similar interactions as found for Ala-762 and Met-763 in the “ancestor-like” PR [Figs. 6 and 7B]. Neither Ser-728 [Fig. 7A] nor Cys-728 [Fig. 7B] are close to either progesterone or amino acids in the steroid binding pocket.

In Fig. 8 we show a close-up analysis of the region containing Gly722 [Fig. 8A] and Cys-722 [Fig. 8B]. Interestingly, in the wild-type PR [Fig. 8A], the contacts between Gly-722 with Leu-726 and Met-759 are similar to that between the backbone of Cys-722 and Leu-726 and Met-759 in the “ancestor-like” PR [Fig. 8B]. However, due to presence of the side chain of Cys-722 in the “ancestor-like” PR [Figs. 7B and 8B], there are several contacts that are missing in Gly-722, which lacks a side chain. Thus, Sy of Cys-722 contacts Sy on Met-759, Sy on Met-909, Cε3 and Cζ3 on Trp-755 and the C19-methyl group on progesterone; and Cβ on Cys-722 contacts the backbone oxygen on Asn-719 and the C19 methyl group on progesterone [Fig. 8B].

4. Discussion

The PR mediates the actions of progesterone, which regulates many aspects of female reproductive physiology, including fertilization, preparation of the endometrium for implantation and parturition [36–38]. Crystal structures of human PR with progesterone [32] and synthetic progestins [33,34,39], as well as RU486 [40], an important anti-progestin [26,41,42] have provided important insights into the interaction of the PR with different steroids. Although most of the focus on progesterone has been directed towards its role in females, progesterone also has important physiological actions in males [43,44]. These important physiological actions of progesterone in male and female vertebrates motivated us to examine the evolution of a key structural region in the ligand-binding domain.

4.1. A role for the cysteine to glycine replacement in helix 3 in the evolution of mammal PRs

Analysis of vertebrate PR sequences in GenBank revealed that Cys-113 in helix 3 in lamprey PR is conserved in eel [27] and other fish PRs, as well as skate, amphibian, reptile and bird PRs [Figs. 3–5]. Glycine first appears at this site in the PR in platypus, a monotreme, which evolved at the base of the mammalian line [Fig. 1], suggesting that this glycine was important in the evolution of mammal PRs. An intriguing finding is that the Golden hamster PR has a cysteine, while the Chinese hamster PR has a glycine at this site. Met-150 in helix 5 in lamprey PR is conserved in all available PR sequences. The occurrence of a transition between glycine and cysteine during the evolution of placental mammals and the presence of alanine in marsupial PRs raises the possibility that the ancestral monotreme had either a cysteine or an alanine at the site corresponding to Gly-722 in human PR. Sequencing of the echidna [Spiny Anteater]

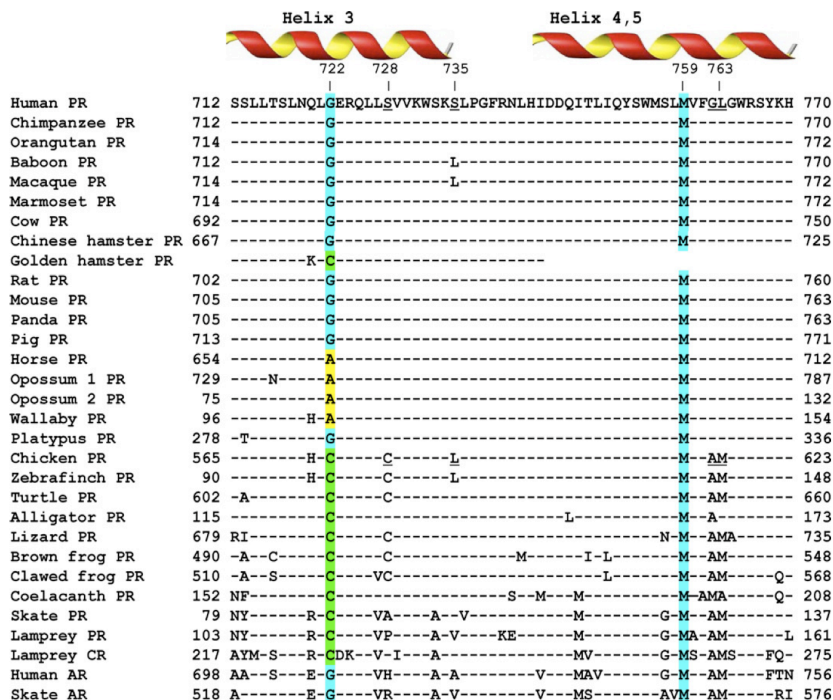


Fig. 3. Alignment of helix 3, 4 and 5 in lamprey, skate and land vertebrate PRs. Alignment of sequences from lamprey PR, skate PR and various land vertebrate PRs and selected MRs, GRs and androgen receptors [AR] [also see [24,27]]. Gly-722 and Met-759 in human PR correspond to Ala-773 and Ser-810 in human MR. The sequences of this segment in several primates: Northern white cheeked gibbon, white handed gibbon, gorilla, African green monkey, black and white colobus monkey, dusky leaf eating monkey, tufted capuchin monkey, gray saki monkey and black spider monkey are 100% identical to that of human PR [Supplementary Table 1]. Opossum 1 = gray short-tailed opossum; opossum 2 = gray brush-tailed opossum. Partial sequence of Golden hamster PR is reported in [25,35].

PR would provide additional evidence for or against the emergence of this glycine in helix 3 in monotremes and not in placental mammals.

Compared to wild-type PR, human PR with a Cys-722 mutation has a 40,000-fold lower affinity for RU486 [25]. Moreover, RU486 does not inhibit binding of progesterone to Golden hamster PR [45], which contain a cysteine, corresponding to the Gly-722 in human PR.

Alanine is the other amino acid found at the position corresponding to Gly-722 in human PR. Opossum, wallaby [35] and horse PRs contain an alanine at this site [Fig. 3]. Human PR with an Ala-722 mutation has a similar response to progesterone as wild-type PR and a low affinity for RU486 [23].

4.2. Conservation of helix 3 through helix 5 in land vertebrate PRs

Almost all of the amino acids in helix 3 through helix 5 are 100% conserved in mammalian PRs [Fig. 3]. Comparative analyses of this 59 residue segment in land vertebrate PRs uncovered additional insights into the evolution of the PR in amphibians, reptiles and mammals. As shown in Figs. 3 and 4, Cys-581, Ala-615 and Met-616 in chicken PR, which are conserved in reptile and amphibian PRs, are replaced by serine, glycine and leucine, respectively, in mammal PRs. The Ala-Met pair in chicken PR is found in lamprey PR and CR, skate PR and AR, and probably was present in their common ancestor. Thus, during the emergence of mammals, four replacements corresponding in human PR to Cys722Gly, Cys728Ser, Ala764Gly

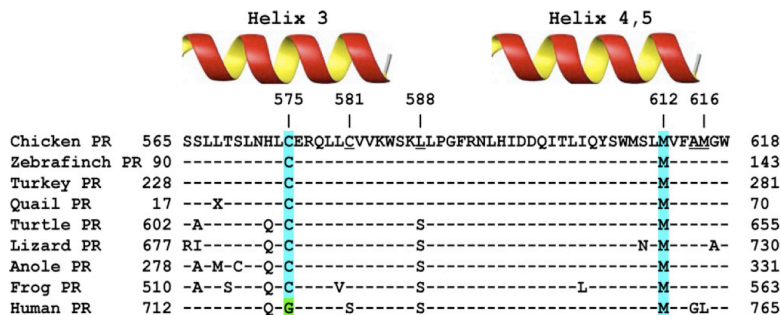


Fig. 4. Alignment of helix 3 through helix 5 in bird, lizard and turtle PRs. Bird, lizard, turtle and amphibian PRs contain the conserved cysteine/methionine pair and other conserved residues that differ from platypus and other mammals. The cysteine/methionine pair is found in skate and lamprey PRs.

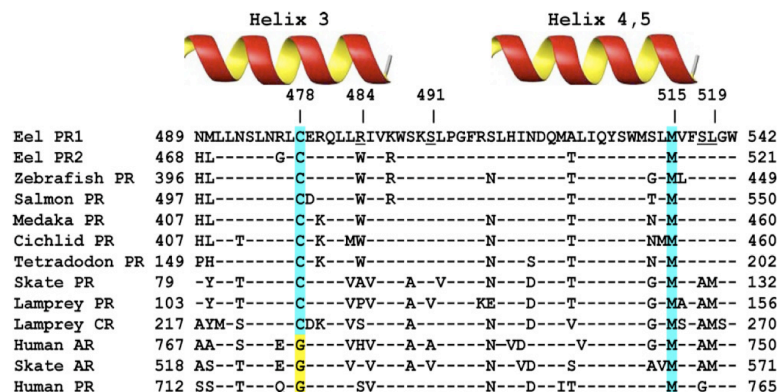


Fig. 5. Alignment of helix 3 through helix 5 in fish PRs. Alignment of sequences from fish PRs and lamprey PR, skate PR, frog, chicken and human PRs. Cys-478 and Met-515 in eel PR2 correspond to Ala-722 and Met-759 in human PR and Ala-773 and Ser-810 in human MR.

and Met765Leu occurred in the 59 residue segment containing helix 3 through helix 5 [Figs. 3 and 4].

4.3. Conservation of helix 3 through helix 5 in fish PRs

There is a much smaller dataset of fish PRs in GenBank for an evolutionary analysis. Nevertheless, BLAST analysis finds that the helix 3 cysteine is conserved in all fish PRs [Fig. 5]. In fish PRs, serine and leucine, replace Ala-129 and Met-130, respectively, in skate PR, which are conserved in amphibian, reptile and bird PRs, and which are glycine and leucine, respectively, in mammals [Figs. 3 and 4]. Thus, these serine and leucine replacements are unique to fish.

It is interesting to note that various hydroxylated progestins are active in fish [28,29,46]. In males, $17\alpha,20\beta$ -dihydroxy-progesterone is important in spermiation [28]. Novel hydroxylated progestins are present in lamprey [15,47,48] [Fig. 2]. Unlike fish PRs, human PR and chicken PR have low affinity for 17α -hydroxy-progesterone [49–51]. The divergence of the segment containing helix 3 through helix 5 in fish and land vertebrates may be relevant for differences in the responses of fish and mammal PRs to hydroxylated progestins [28,29,46].

4.4. Functional implications

Comparison of the 3D structure of the wild-type PR with the 3D model of the “ancestor-like” PR indicates that the four amino acid replacements retain the contacts found between wild-type PR and progesterone. In particular, there is a van der Waals contact between Cys-722 in helix 3 and Met-759 in helix 5 [Figs. 7 and 8]. Similarly, the Gly762Ala and Leu763Met replacements have similar contacts with progesterone or the PR as found in wild-type PR. Conservation of these interactions is consistent with the report by Benhamou et al. [25] that wild-type chicken PR and chicken PR mutated to contain Gly-575, Ser-581, Gly-615 and Leu-616 had similar binding and transcriptional responses to progesterone agonists as found for human PR.

RU486 is not a natural ligand for either chicken PR or human PR. Nevertheless, the loss of binding of RU486 to chicken PR due to Cys-755 in chicken PR indicates that ligand selectivity of the PR is influenced by substituents at C11. Also, RU486 does not inhibit progesterone binding to Golden hamster PR [45], which is consistent with it containing a cysteine corresponding to Gly-722 in human PR. Moreover, RU486 does not inhibit progesterone binding wallaby PR [35], which has an alanine corresponding to

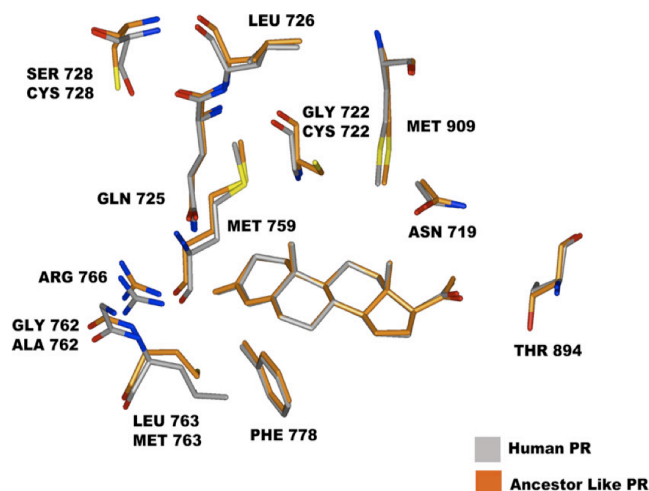


Fig. 6. Superposition of wild-type human PR and human PR containing Cys-722, Cys-728, Ala-762 and Met-763 in helix 3 through helix 5. There is excellent overlap of key amino acids that stabilize the A and D rings on progesterone as well as the backbone of Cys-722 and Gly-722.

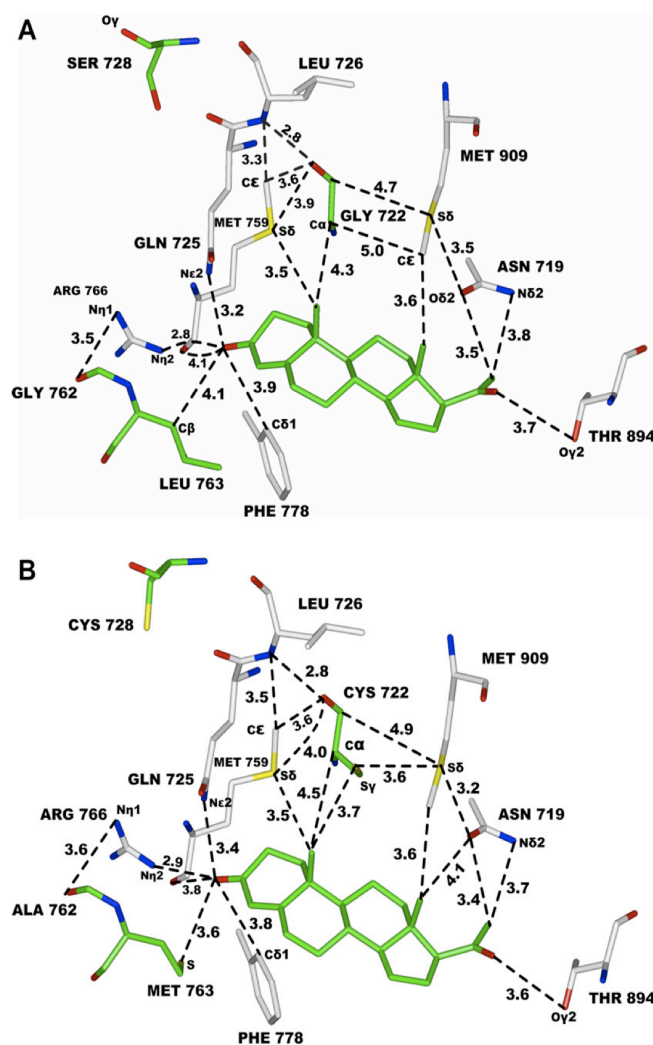


Fig. 7. Progesterone in wild-type human PR and human PR containing Cys-722, Cys-728, Ala-762 and Met-763 in helix 3 through helix 5. (A) Contacts between progesterone and wild-type PR [32]. Contacts between progesterone and wild-type PR have been described previously [32–34]. Note that C α on Gly-722 is 5 Å from C ϵ in Met-909. (B) Contacts between progesterone and human PR containing Cys-722, Cys-728, Ala-762 and Met-763. The distances between the A and D rings on progesterone and the “ancestor-like” PR are similar to that in wild-type PR. Note that S γ on Cys-722 is 3.4 Å from C ϵ in Met-909.

Gly-722 in human PR. Another marsupial, the opossum, also contains an alanine corresponding to Gly-722 in human PR. Human PR with a Gly722Ala mutation does not bind RU486 [23]. Because all of these PRs bind progesterone with high affinity, the functional implications of the presence of Gly-722 on human PR are not yet known.

A clue may come from studies with analogs of RU486 containing aliphatic and aromatic substituents at C11, which indicate that it is the 11 β aromatic substitution that causes the loss of binding of RU486 to chicken PR and to human PR with Cys-722 [25,35]. A model of the crystal structure of human PR with RU486 indicates that it would have steric clashes with Cys-722 [40]. Moreover, as shown in Fig. 8B, the side chain of Cys-722 has numerous contacts with Met-909, Met-759 and Trp-755, which are absent in human PR due to the lack of a side chain in Gly-722. Cys-722 stabilization of this region of the steroid binding domain in chicken PR would be disrupted by steroids with an 11 β aromatic substituent. This cysteine in the PR in fish, amphibians, reptiles and birds functions may

function to exclude an endogenous or an exogenous compound that is not relevant in mammals.

A role for Gly-722 in the binding of co-regulators to human PR also is possible. Helix 3 and helix 5 are part of the groove that together with the activation function 2 [AF2] site on helix 12 form a binding site for co-regulators on the PR [32,52–54] and other nuclear receptors [17,55–59]. Our 3D models [Figs. 7 and 8] predict that Cys-722 stabilizes the region containing helix 3 and helix 5, while the crystal structure of human PR indicates the Gly-722 provides more conformational flexibility for this region. These differences would be expected to have consequences for the interaction of chicken PR and human PR with AF2 and co-regulators. That is, one or more co-regulators may have different effects on transcriptional activation by progesterone of human PR and chicken PR.

The additional contacts between Cys-722 and Met-909 are relevant to this possibility because Met-909 is part of the AF2 site, and Petit-Topin et al. [33] found that Met-909 was important in

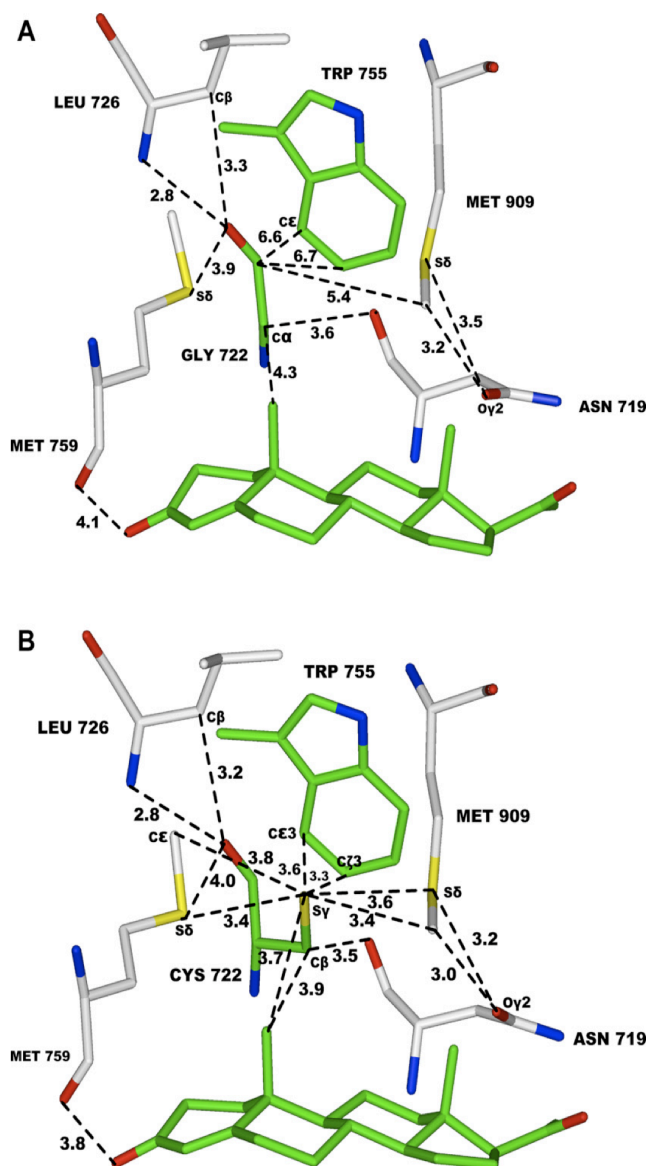


Fig. 8. Closeup of Gly-722 in wild-type human PR and Cys-722 in the “ancestor-like” PR. (A) Contacts between Gly-722 and other residues in wild-type human PR. The backbone carbon on Gly-722 is 5.4 Å from Cε on Met-909 and 6.6 Å from Cε3 on Trp-755. Gly-722 contacts Met-759 and Leu-726. (B) Contacts between Cys-722 and other residues in the “ancestor-like” PR. Sγ on Cys-722 has numerous stabilizing contacts with nearby residues. Sγ on Cys-722 is 3.4 Å from Cε in Met-909, 3.6 Å from Cε3 on Trp-755 and 3.4 Å from Sδ on Met-759. Sγ on Cys-722 also is 3.7 Å from C19 on progesterone.

transcriptional activation of human PR to progesterone and levonorgestrol. They reported that although the Met909Ala mutant was able to bind 1 nM progesterone and levonorgestrol indicating that Met-909 was not essential for steroid binding. However, even at 100 nM, neither steroid was able to activate transcription of the Met909Ala mutant. Moreover, levonorgestrol at 1 μ M concentration was not able to recruit the Transcriptional Intermediary Factor 1 α [TIF1 α] coactivator to the PR. This suggests that the absence of an interaction between Gly-722 and Met-909 in the human PR or the stabilization of Met-909 by Cys-722 in the ancestor-like PR [Fig. 8B] may influence the recruitment of co-regulators. Effects on co-regulator binding and steroid binding are not mutually exclusive, as seen in studies on the influence of ligands on binding of co-regulators to nuclear receptors. That is, binding of different

steroids to the PR and other steroid receptors can influence the interaction with co-regulators and the ultimate biological response [30,31,57,59–61].

4.5. Endocrine disruption

The evidence that changes in the contacts between helix 3 and helix 5 influence the binding of steroids to the MR, PR and GR [20,24] indicates that differences in the sequences in the segment containing helix 3 through helix 5 in fish, amphibian, reptile and mammal PRs are likely to lead to differences in the physiological effects of phytochemicals and synthetic chemicals [2,62–66] in these species. That is, a chemical that disrupts progesterone action in fish may not have the same effect in humans and vice versa. Indeed, a relevant

example is the absence of RU486 binding to the chicken PR and wallaby PR due to a single amino acid difference with human PR.

4.6. Next steps

At this time, the selective advantage for the four replacements in the PR that arose in segment containing helix 3 through helix 5 in platypus PR is not known. We hope that this report will stimulate studies to resolve this conundrum because the strong sequence conservation at these four sites in the ancestors of platypus PR, the subsequent conservation of the four replacements in mammal PRs and Dobzhansky's insight [67] "Nothing in biology makes sense except in the light of evolution" argues for a functional role in the evolution of mammals.

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.jsbmb.2012.04.007>.

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Chapter 4. Study of Coactivator-ligand binding domain interaction in ER α and ER β

Introduction. Estrogens receptors regulate many important physiological functions that are of interest to many scientists from different fields. ER α regulates the differentiation and maintenance of neural, skeletal, cardiovascular, and reproductive tissues (Shiau et al. 1998). ER β also have been known to regulate many physiological functions including, but not limited to, synthesis of neurons and regulation of colorectal epithelium cells (Edvarsson et al. 2011 and Shiau et al. 1998). The compounds that modulate ER α and ER β transcriptional activities are of high importance and they are currently being used to treat osteoporosis, cardiovascular disease, breast cancer, colon cancer, and prostate cancer (Edvarsson et al. 2011 and Shiau et al. 1998).

The relationship between the coactivator proteins and ligand binding domain of the ER α and ER β was analyzed in this chapter. Certain residues in the ligand binding domain have been known to be required for transactivation but not necessarily for ligand binding (Weatherman et al. 1999). This region, called AF-2, is well conserved throughout the members of nuclear receptor superfamily (Wurtz et al. 1996). The key residues of AF-2 play important role in transactivation and certain mutations in AF-2 can decrease the binding affinity of coactivator. The AF-2 region is primarily in helix 3-5 and helix 11-12 and loop before helix 12 can also influence the helix 12 conformation.

Previous studies have shown that the nuclear receptor interaction domains, also called NR boxes, of the coactivator interact with a hydrophobic cleft of the nuclear receptor (Weatherman et al. 1999). NR boxes contain a conserved sequence of LXXLL where L represents the Leucine and X represents other amino acids and residues preceding or following the sequence can also modulate the affinity of the LBD-coactivator interaction (Weatherman et al. 1999).

A study done by Shiau et al. on the structural analysis of ER α complexed with diethylstilbestrol (DES), an agonist of ER α , describe the deep hydrophobic groove and shallow groove formed by residues from helices 3,4,5 and 12 and the turn between helices 3 and 4 (Shiau et al. 1998). In addition, the LBD further stabilizes the main chain conformation of the NR box by forming “charge clamp” at the both ends of the peptide helix (Shiau et al. 1998). His study have stimulated us to perform more detailed structure analysis the LBD-coactivator interaction in ER α to take a better look at the both grooves and study the corresponding key residues in ER β to see whether the interaction is conserved across the ER α and ER β . Furthermore, we analyzed the sequence of fragment of helix 3, 5, and 12 from different vertebrates to analyze the evolution.

Structure analysis of coactivator binding to ER α and ER β . We analyzed the crystal structure provided by Shiau et al. and performed detailed structure analysis of binding of the NR box II peptide to the hydrophobic groove of ER α (Shiau et al. 1998). As discussed by Shiau et al, the NR box II peptide-

binding site was the groove composed of residues Ile-358, Ala-361, and Lys-362 from helix 3; Gln-375, Val-376, Leu-379 from helix 5; and Leu-539, Glu-542, and Met-543 from helix 12 (Shiau et al. 1998). Also, the LBD interaction with the hydrophobic face of the NR box II peptide a helix formed by the side chains of LXXLL motif leucines (Leu-690, Leu-693, and Leu-694) (Shiau et al. 1998) (Figure 4-2 through 4-5).

The alignment between ER α and ER β showed conservation of key residues from nuclear receptor that bind to NR box peptide between the ER α and ER β (Figure 4-1). However, not all of the amino acids from helix 3, helix 5, and helix 12 were identical and relevance of different amino acids needs to be analyzed further.

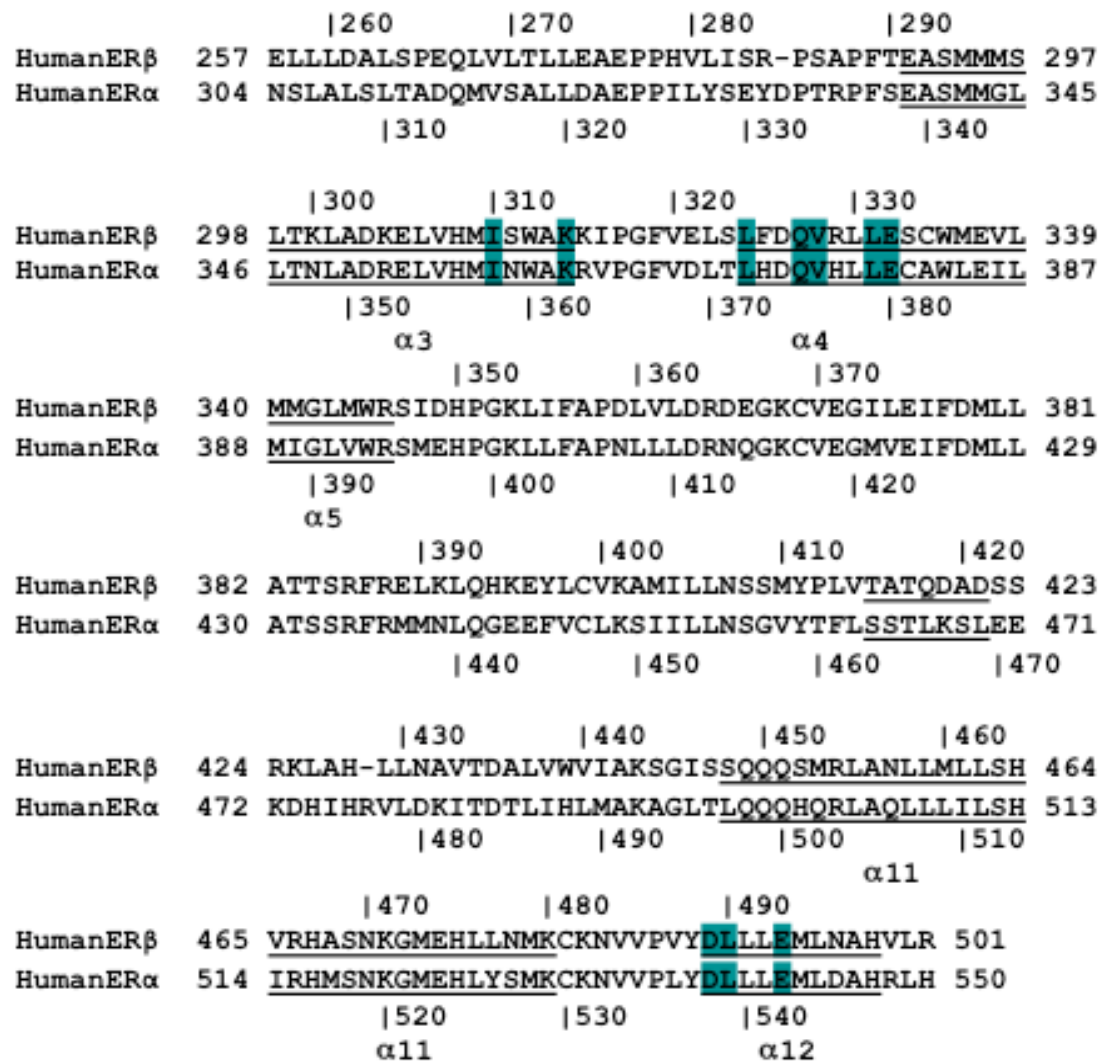


Figure 4-1. Alignment of Human ERα and Human ERβ. The sequence alignment of ERα and ERβ is shown. The helices are underlined and only the relevant helices in peptide binding site of nuclear receptor is shown. The highlighted residues represent the key residues that bind to the coactivator peptide helix (Shiau et al. 1998).

The interaction of first leucine from NR box peptide with LBD of nuclear receptor is conserved between ER α and ER β . As Shiau et al. discussed (Shiau et al. 1998), the side chain of Leu-690 forms close interaction within the groove by forming a van der Waals contacts with the side chains of Ile-358, Val-356, Leu379, Glu-380, and Met-543 (Figure 4-2). Likewise, the corresponding key residues from crystal structure of ER β crystallized by Mockinghoff et al. was analyzed (Mockinghoff et al. 2010). The side chain of Leu-690 also forms close interaction within the groove by forming a van der Waals contacts with the side chains of Ile-310, Leu-331, and Val-328 (Figure 4-3).

Human ER α with Corregulator (DES)

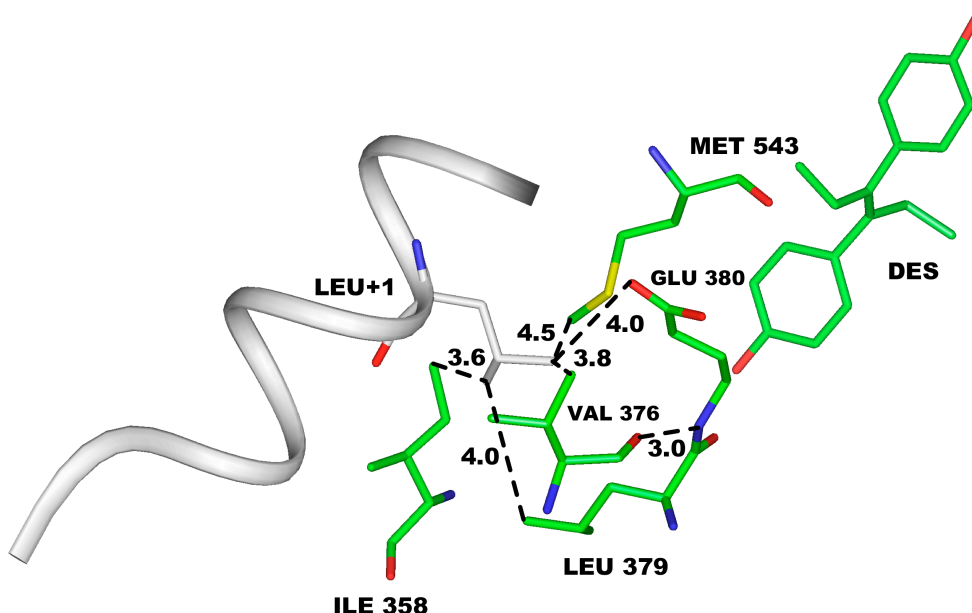
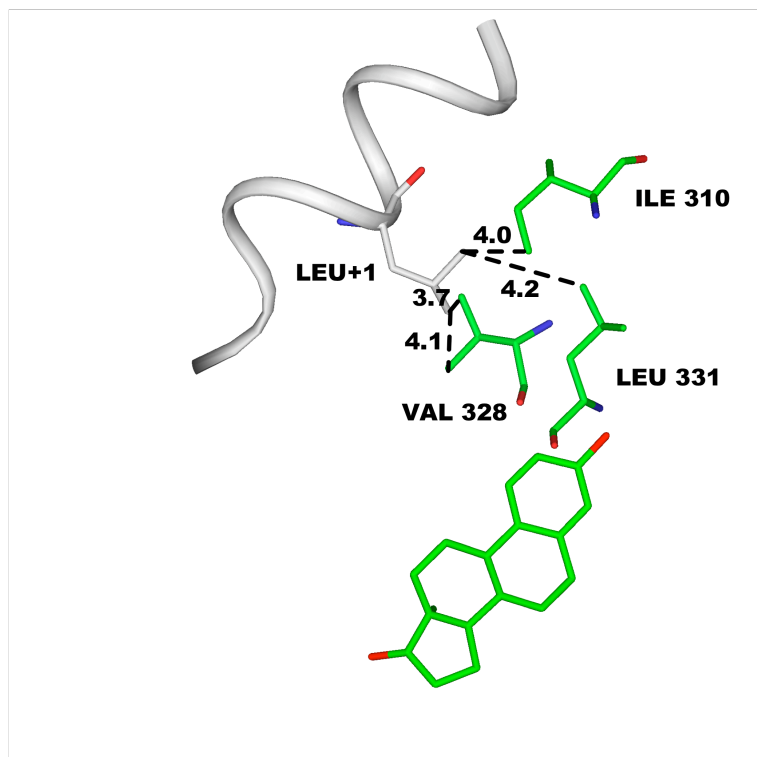


Figure 4-2. The Leu+1 from NR Box II Peptide and LBD interaction in ER α . A detailed structure view of the coactivator peptide bound to the ER α LBD. The region of LBD that does not contact the coactivator peptide is not shown. The coactivator peptide is shown as white ribbon and Leu-690 is labeled Leu+1 as it is common nomenclature used for its first position in LXXLL. The side chains of residues of LBD is colored by atom type (Carbon as green, sulfur atom as yellow, oxygen atom as red, and nitrogen atoms as blue).

Human ER β with Corregulator



SRC1 Box II

Figure 4-3. The Leu+1 from SRC1 BoxII Peptide and LBD interaction in ER β . A detailed structure view of the coactivator peptide bound to the ER β LBD. The region of LBD that does not contact the coactivator peptide is not shown. The coactivator peptide is shown as white ribbon and Leu-690 is labeled Leu+1 as it is common nomenclature used for its first position in LXXLL. The side chains of residues of LBD is colored by atom type (As specified in Figure 4-2).

The conservation of Leu+5 interaction within the groove and difference in interaction of Leu+4 and Ile-1 between the ER α and ER β . As Shiau discussed (Shiau et al. 1998), the side chain of Leu+5 (Leu-694) has van der Waals contacts within the groove with the side chains of Ile-358, Lys-362, Leu-372, Gln-375, Val-376, and Leu-379 (Figure 4-3). However, the side chains of Leu+4 (Leu-693) and Ile-1 (Ile-689) rests against the groove. Ile-689 lies within the shallow depression formed by the side chains of Asp-538, Leu-539, and Glu-542 and the side chain of Leu-693 makes nonpolar contacts with the side chains of Ile-358 and Leu-539 (Figure 4-3).

The structure analysis of corresponding key residues in ER β showed interesting result. The interaction of Leu+5 (Leu-694) also has van der Waals contacts within the groove with the side chains of Ile-310, Lys-314, Leu-324, Gln-327, Val-328, and Leu-331. However, much larger distance was observed between the side chains of Ile-1 (Ile-689) and the side chains of Asp-489, Leu-490, and Glu-493. This suggests that contact between the Ile-1 and shallow depression was lost in ER β .

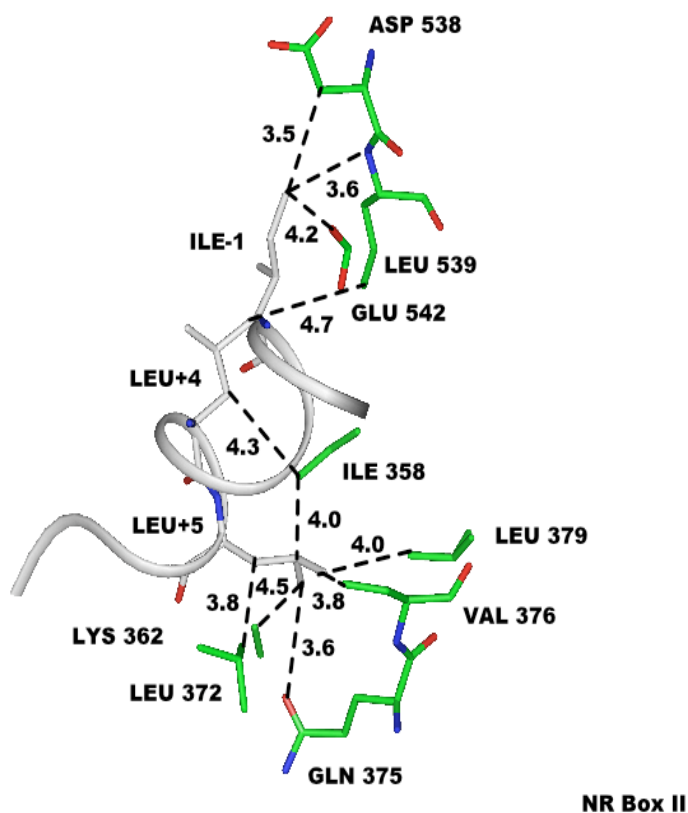


Figure 4-4. The Leu+4, Leu+5, and Ile-1 from NR Box II interaction with LBD in ER α . A detailed structure view of the coactivator peptide bound to the ER α LBD. The region of LBD that does not contact the coactivator peptide is not shown. The coactivator peptide is shown as white ribbon and the label of Leu-693 as Leu+4, Leu-694 as Leu+5, and Ile-689 as Ile-1 in consensus with common nomenclature used for their positions in LXXLL. The side chains of residues of LBD is colored by atom type (As specified in Figure 4-2).

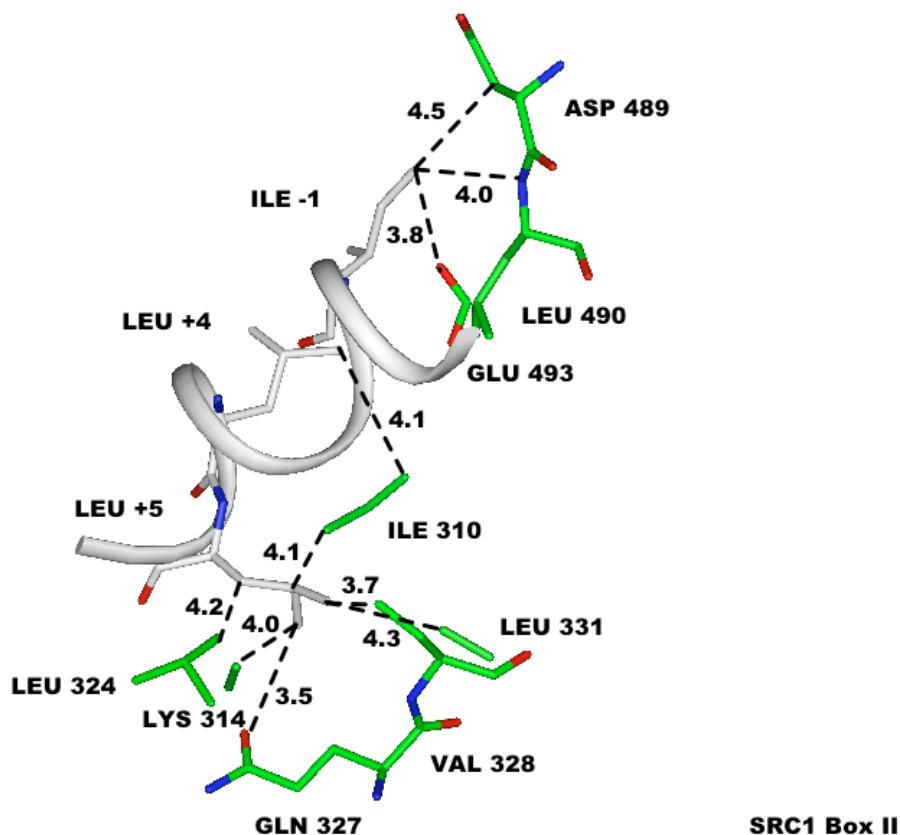


Figure 4-5. The Leu+4, Leu+5, and Ile-1 from SRC1 Box II interaction with LBD in ER β . A detailed structure view of the coactivator peptide bound to the ER β LBD. The region of LBD that does not contact the coactivator peptide is not shown. The coactivator peptide is shown as white ribbon and the label of Leu-693 as Leu+4, Leu-694 as Leu+5, and Ile-689 as Ile-1 in consensus with common nomenclature used for their positions in LXXLL. The side chains of residues of LBD is colored by atom type (As specified in Figure 4-2).

The Charge Clamp at each end of the coactivator peptide was conserved in the ER α and ER β . In addition to the interaction of the coactivator peptide and hydrophobic face of the LBD, the LBD stabilizes the main chain conformation of the NR box peptide by forming the charge clamp at both ends of the peptide helix. As discussed by Shiau et al, the coactivator (NR Box II Peptide) with ER α LBD has a charge clamp at the ends of the coactivator. The Glu-542 positioned at end of the LBD has columbic interactions with the amides of the residues of N-terminal and Lys-362 positioned at the other end of the LBD also has columbic interactions with the carbonyls of the residues of the C-terminal turn of the peptide helix (Figure 4-6).

The corresponding key residues from crystal structure of ER β also have a formation of charge clamp at the both ends of coactivator peptide. The Glu-493 positioned at end of the LBD has columbic interactions with the amides of the residues of N-terminal and Lys-314 positioned at the other end of the LBD also has columbic interactions with the carbonyls of the residues of the C-terminal turn of the peptide helix (Figure 4-7).

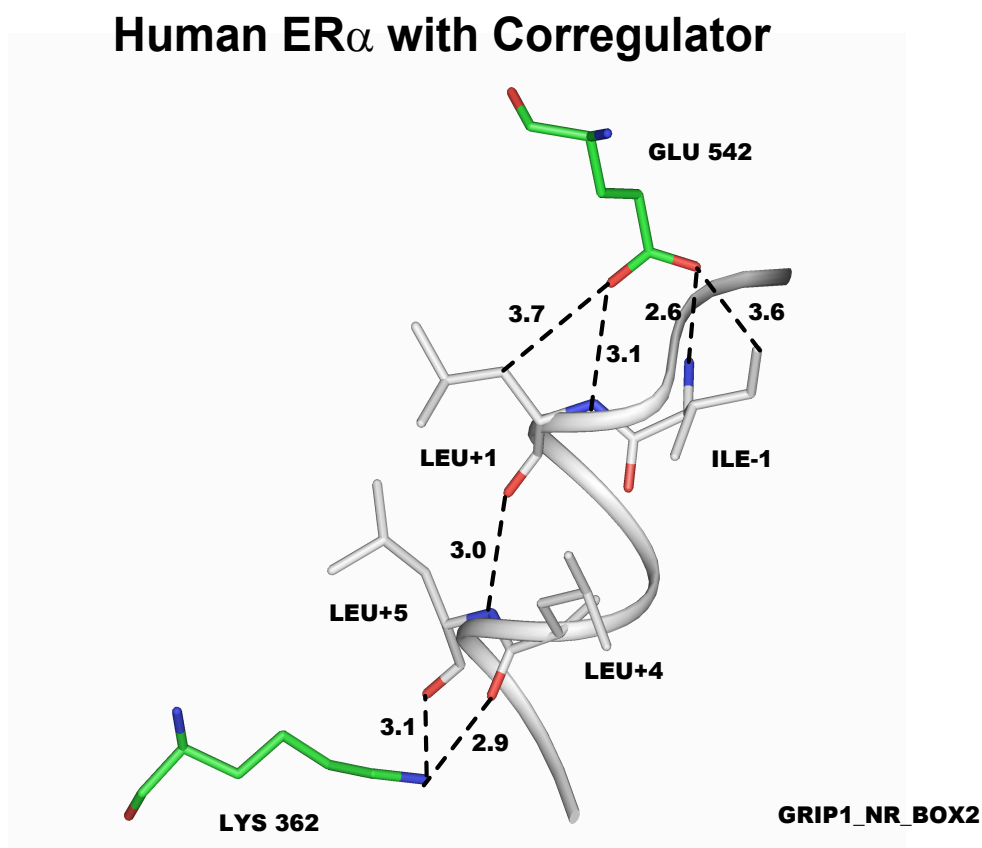


Figure 4-6. The presence of charge clamp at both ends of NR Box II peptide in ER α . The interaction of residues from both ends of LBD with coactivator peptide is shown. The coactivator peptide is shown as white ribbon. Ile-689, Leu-690, Leu-693, and Leu-694 are labeled Ile-1, Leu+1, Leu+4, and Leu+5, respectively, in consensus with common nomenclature for their positions in LXXLL. The side chains of residues of LBD is colored by atom type (Carbon and sulfur atom as yellow, oxygen atom as red, and nitrogen atoms as blue).

Human ER β with Corregulator

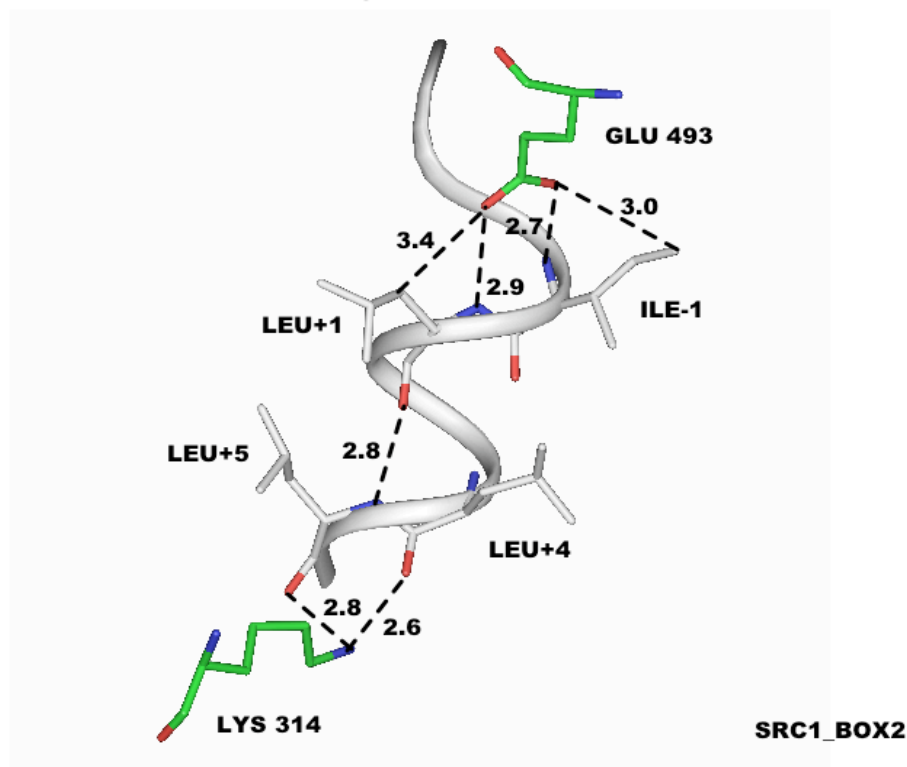


Figure 4-7. The presence of charge clamp at both ends of SRC1 Box II peptide in ER β . The interaction of end residues from both ends of LBD with coactivator peptide is shown. The coactivator peptide is shown as white ribbon. Ile-689, Leu-690, Leu-693, and Leu-694 are labeled Ile-1, Leu+1, Leu+4, and Leu+5, respectively, in consensus with common nomenclature for their positions in LXXLL. The side chains of residues of LBD is colored by atom type (As specified in Figure 4-8).

Sequence analysis of fragment from helix 3 through helix 5 and helix 11 through helix 12 in vertebrate ER α and ER β . Concurrent with structure analysis, the sequences of helix 3 through helix 5 and helix 11 through helix 12 in vertebrates were collected from the evolutionary study. The fragment from helix 3 through helix 5 of both ER α and ER β was used as probe for a BLAST search for the corresponding sequences of different vertebrates. The conservation of the residues was highest in mammal then decreased as we went down the evolutionary tree to marsupials, birds, amphibians, and fish for ER α and ER β (Figure 4-9, Figure 4-10).

Likewise, the fragment from helix 11 through helix 12 of both ER α and ER β was used as probe for BLAST search for the corresponding sequences of different vertebrates. The sequences within the ER α seemed much more conserved through birds and amphibians and ER β showed relative high conservation of sequence through birds and amphibians. (Figure 4-11, Figure-4-12).

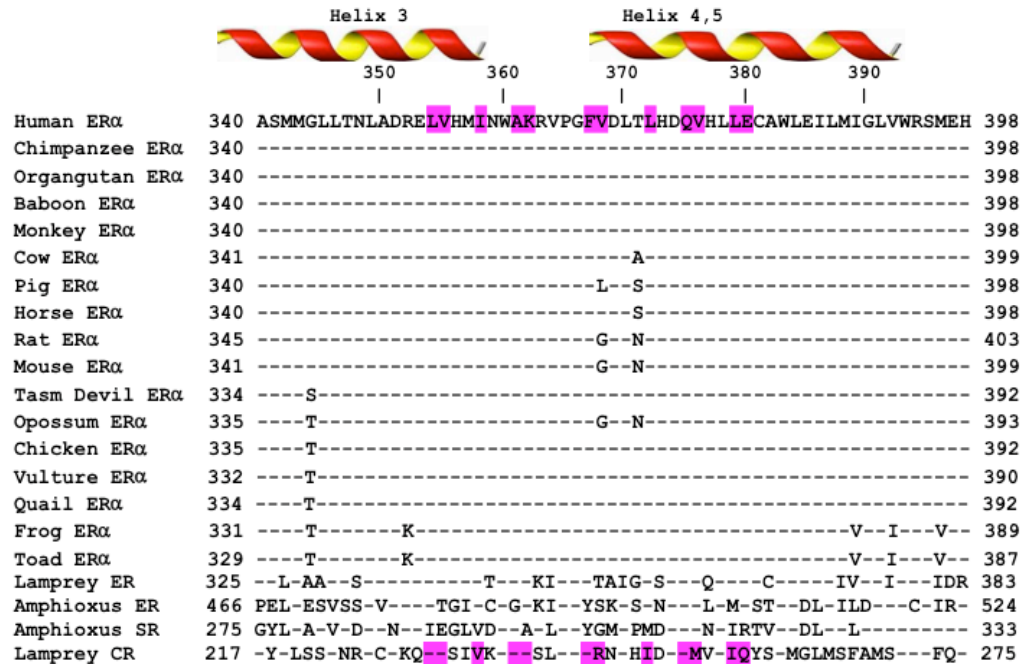


Figure 4-8. The sequence analysis of Helix-3 through Helix-5 in ERα vertebrates. The important vertebrates from categories of mammals, marsupials, birds, amphibians, and fish are shown. The dash represents the same amino acid in comparison to the sequence of Human ERα. The key residues of coactivator binding site is highlighted.

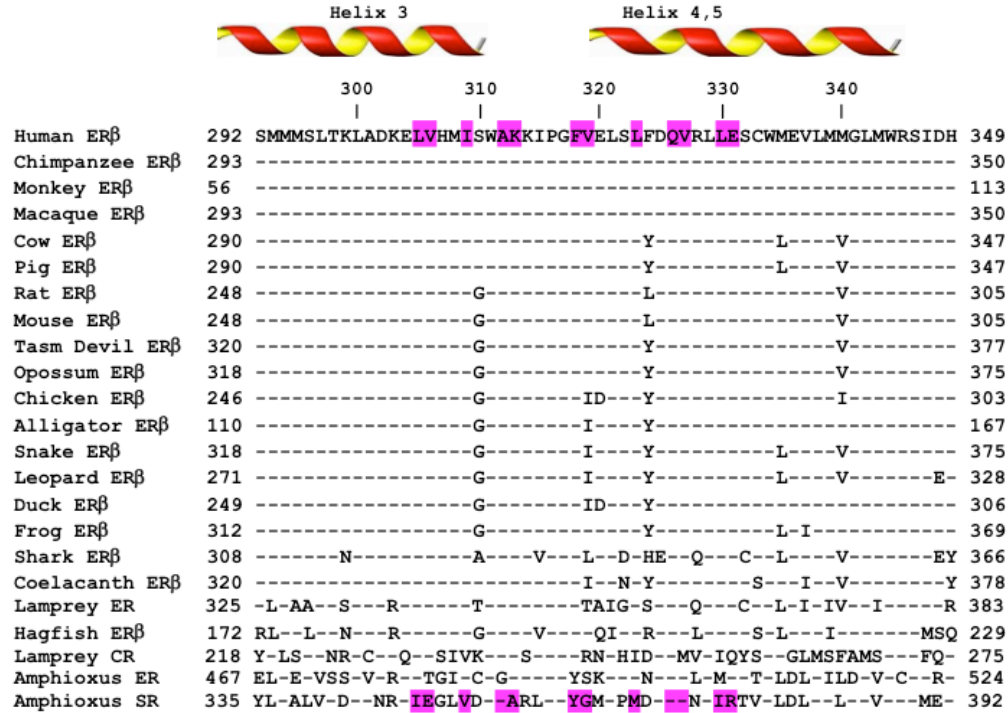


Figure 4-9. The sequence analysis of Helix-3 through Helix-5 in ERβ vertebrates. The important vertebrates from categories of mammals, marsupials, birds, amphibians, and fish are shown. The dash represents the same amino acid in comparison to the sequence of Human ERβ. The key residues of coactivator binding site is highlighted.

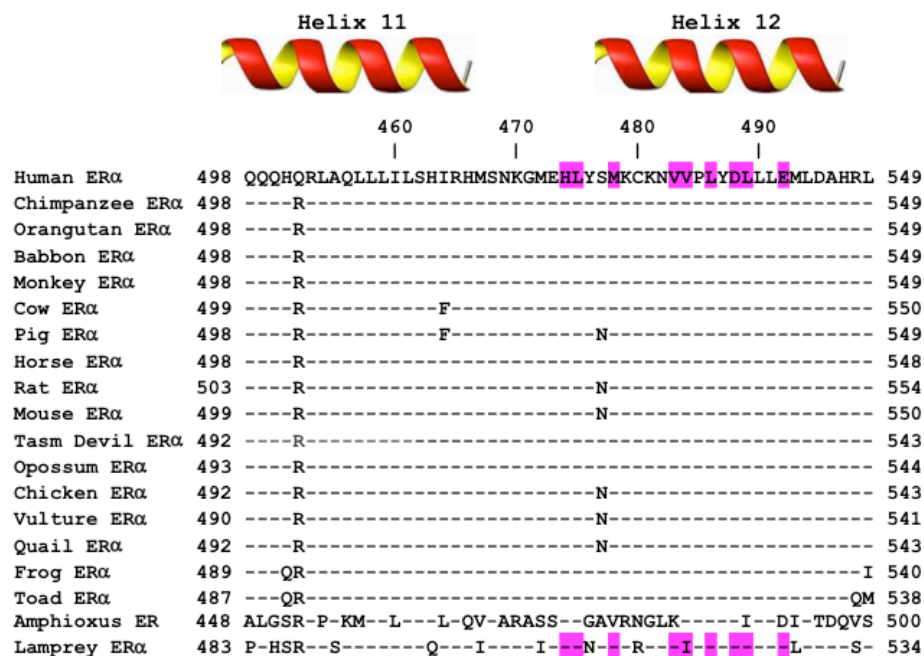


Figure 4-10. The sequence analysis of Helix-11 through Helix-12 in ERα vertebrates. The important vertebrates from categories of mammals, marsupials, birds, amphibians, and fish are shown. The dash represents the same amino acid in comparison to the sequence of Human ERα. The key residues of coactivator binding site is highlighted.

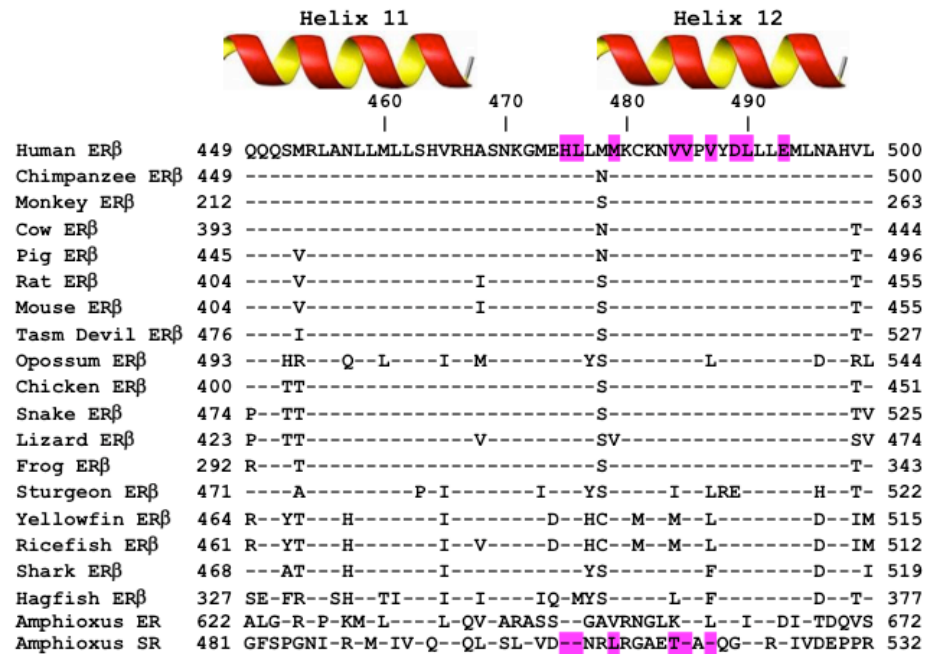


Figure 4-11. The sequence analysis of Helix-11 through Helix-12 in ERβ vertebrates. The important vertebrates from categories of mammals, marsupials, birds, amphibians, and fish are shown. The dash represents the same amino acid in comparison to the sequence of Human ERβ. The key residues of coactivator binding site is highlighted.

Detailed sequence analysis of helix-3 through helix-5 in birds, amphibians, and fish. The general trend of increase of divergence as we move down the evolutionary tree from mammals, marsupials, birds, amphibians, and fish stimulated us to perform more detailed sequence analysis of birds, amphibians, and fish of Helix-3 through Helix 5 and Helix-11 through Helix-12 in both ER α and ER β .

Sequence analysis of Helix-3 through Helix-5 in ER α showed high conservation of sequence upon till fish. Within the birds and amphibians, there is an interesting replacement from Gly-345 to Thr. Within the fish, many more distinct replacements appeared such as Arg-353 replaced by Lys, Asp-360 replaced by Ala, Arg-364 replaced by Lys, Thr-372 replaced by Ser, His-378 replaced by Glu, Ala-383 replaced by Ser, Ile-386 replaced by Val, and Val-393 replaced by Ile (Figure 4-13).

Sequence analysis of Helix-3 through Helix-5 in ER β showed more residue replacements within the birds and amphibians compared to ER α . They include Ser-310 replaced by Gly, Val-319 replaced by Ile, Phe-324 replaced by Tyr, and Met-340 replaced by Ile. Within the fish, more divergence of sequence was observed (Figure 4-14).

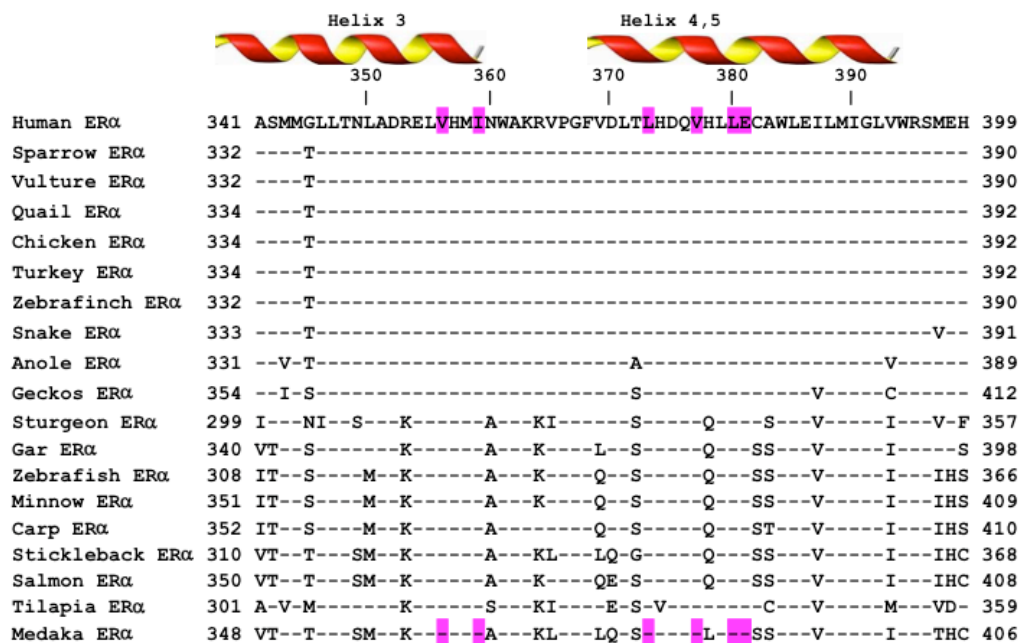


Figure 4-12. The detailed sequence analysis of Helix-3 through Helix-5 in ERα birds, amphibians, and fish. The important vertebrates from categories of birds, amphibians, and fish are shown. The dash represents the same amino acid in comparison to the sequence of Human ERα. The key residues of coactivator binding site are highlighted.

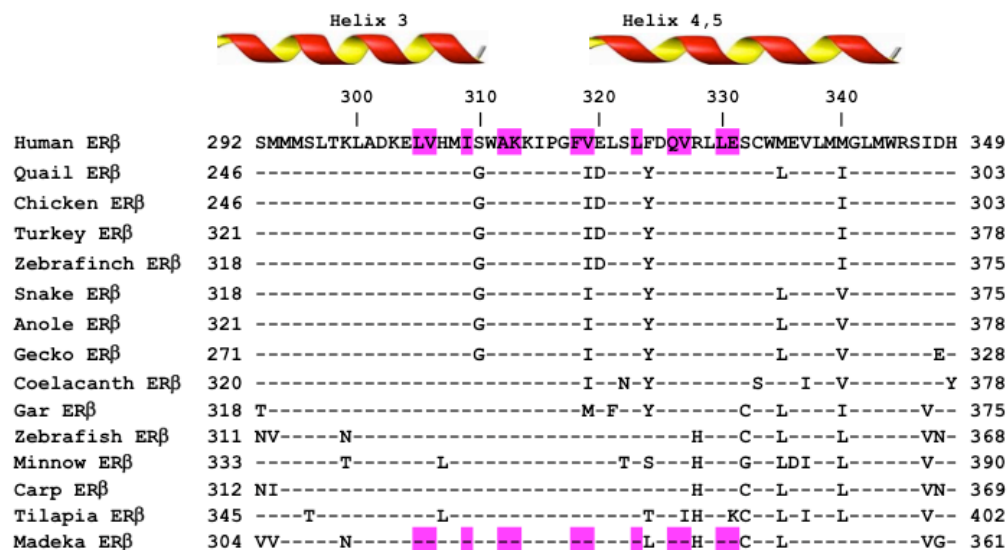


Figure 4-13. The detailed sequence analysis of Helix-3 through Helix-5 in ERβ birds, amphibians, and fish. The important vertebrates from categories of birds, amphibians, and fish are shown. The dash represents the same amino acid in comparison to the sequence of Human ERβ. The key residues of coactivator binding site are highlighted.

Detailed sequence analysis of helix-11 through helix-12 in birds, amphibians, and fish. Likewise, more detailed analysis of sequence fragment from helix-11 through helix-12 in birds, amphibians, and fish was performed in both ER α and ER β . In ER α , the sequences showed better conservation within the birds and amphibians with two distinct replacements of Gln-502 to Arg and Ser-478 to Asn. Within the fish, more divergence was found (Figure 4-15).

In ER β , few more replacements were shown within the birds and amphibians. Ser-452 and Met-453 were both replaced by Tyr, Met-478 replaced by Ser, and Val-499 replaced by Tyr. Within the fish, much more divergence was found (Figure 4-16).

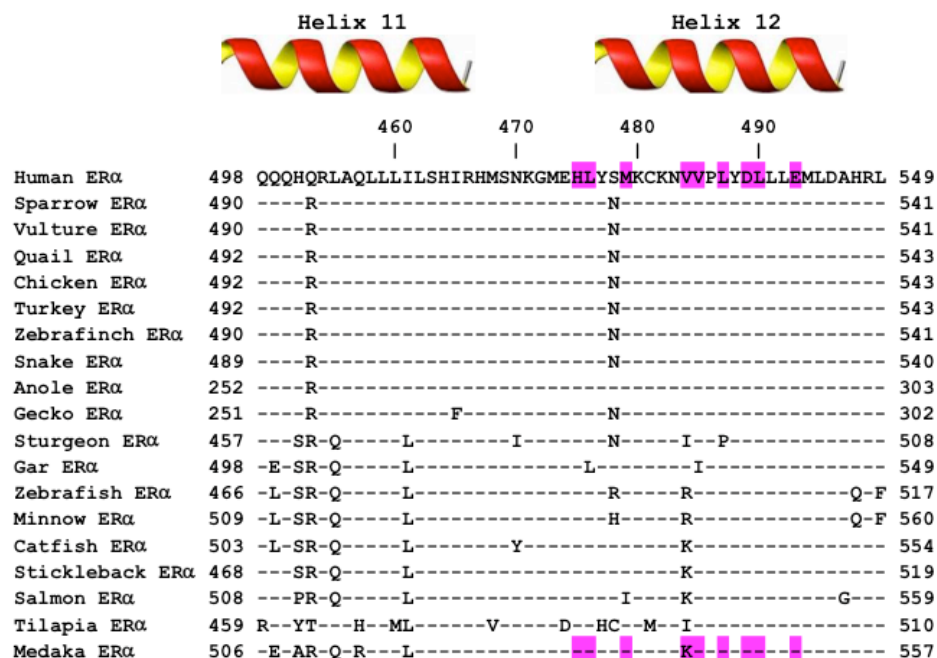


Figure 4-14. The detailed sequence analysis of Helix-11 through Helix-12 in ERα birds, amphibians, and fish. The important vertebrates from categories of birds, amphibians, and fish are shown. The dash represents the same amino acid in comparison to the sequence of Human ERα. The key residues of coactivator binding site are highlighted.

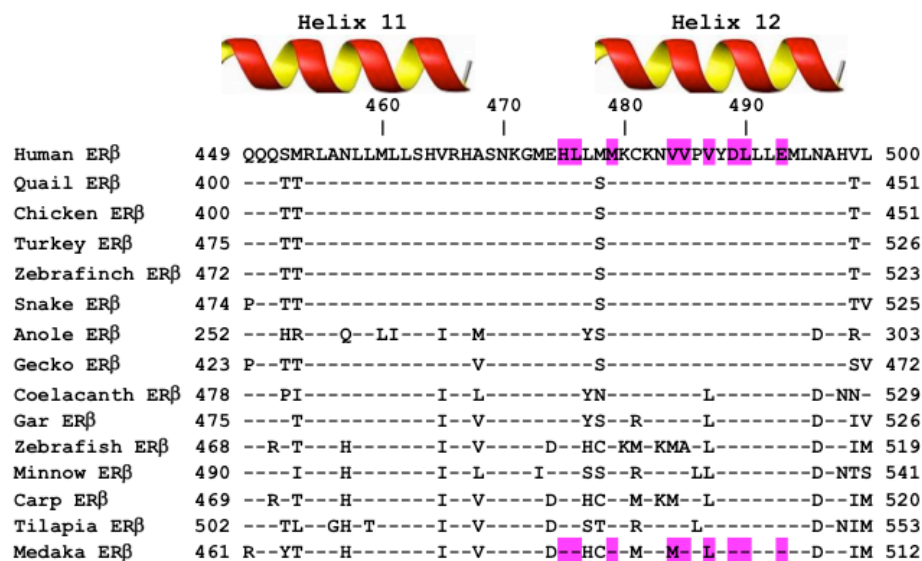


Figure 4-15. The detailed sequence analysis of Helix-11 through Helix-12 in ERβ birds, amphibians, and fish. The important vertebrates from categories of birds, amphibians, and fish are shown. The dash represents the same amino acid in comparison to the sequence of Human ERβ. The key residues of coactivator binding site are highlighted.

Chapter 5. Conclusions and future directions

Structure analysis of the various receptors. The structure analysis of various steroid receptors provided us with explanation behind the previous findings. The structural analysis of the Lamprey CR and Human MR complexed with 11-deoxycortisol and deoxycorticosterone allowed more detailed explanation of the findings of Close et al. that only 11-deoxycortisol has high affinity binding to lamprey gill cytosol (Close et al. 2010). Especially, the discovery of the van der Waals contact between Cys-277 in helix 3 and Met-264 in helix 5 in lamprey CR was quite interesting because such contact is lost in MR and GR. Furthermore, it is known that the contact between the helix 3 and helix 5 can alter the response of MR to progesterone from antagonist to agonist and such change can lead to condition known as pre-eclampsia (Chapter 2).

The structure analysis of Human PR complexed with progesterone and RU-486 provided insight into binding of RU-486 to the PR. Also, the key residues from helix-3 and helix-5 identified through structure analysis allowed us to perform sequence analysis of PR in vertebrates and to find the interesting replacement of cysteine to glycine replacement in helix 3 that first appears in mammals (Chapter 3).

The structure analysis of ER α and ER β complexed with coactivator peptide NR Box II and SRC1 Box II, respectively, allowed more detailed explanation of study of Shiau et al. and discovery of a different shallow depression formed by helix 12 in ER β (Shiau et al. 1998). From the structure

analysis of crystal structure provided by Shiau et al, the binding of Leu+1 and Leu+5 within the hydrophobic groove was shown in detail (Figure 4-2, Figure 4-4). The binding of Leu+1 and Leu+5 was conserved between the ER α and ER β but difference between the ER α and ER β was observed in binding of Ile-1 to LBD of nuclear receptor. The Ile-1 showed higher binding to shallow depression in ER α compared to Ile-1 binding to shallow depression in ER β (Figure 4-4 and Figure 4-5). The reason and relevance of the difference needs to be further analyzed.

Sequence analysis provides insights into evolution of steroid receptors. Sequence analysis of fragment from helix 3 through helix 5 in vertebrate progesterone receptors allowed the discovery of the role of cysteine to glycine replacement in helix 3 in the evolution of mammal PR. Analysis of vertebrate PR showed a conserved Cys-113 in eel, fish, amphibian, reptile and bird PRs. However, the cyteine to glycine replacement first appears in the PR in platypus, which is located at the base of the mammalian line. This suggests that the appearance of glycine is important in the evolution of mammal PRs (Chapter 3).

Sequence analysis of frangment from helix 3 through helix 5 in vertebrate estrogen receptor alpha and beta showed high conservation of sequence within the mammals and marsupials and increased divergence from birds, amphibians, to fish. Through the sequence analysis, some distinct residue replacements

were observed within the birds, amphibians, and fish and consequences of such residue replacements needs to be further analyzed.

Mutagenesis studies. As mentioned above, mutations in key residues can alter the binding and response of steroid receptor to its ligand. The helix 3 through helix 5 and helix 11 through helix 12 sequence analysis of estrogen receptors has shown some distinct residue replacements within the birds, amphibians, and fish. Such residue replacement should be further analyzed through mutagenesis study. Especially, the residue replacements that took place near the key residues of coactivator binding site should be analyzed in detail. The residue of human ER α and human ER β can be replaced with residues that starts to appear in birds, amphibians, and fish and consequent structure analysis can provide new insight into the evolution of estrogen steroid receptor.

Chapter 6. Experimental

6.1 Analysis of Lamprey CR and Human MR complexed with 11-

deoxycortisol and deoxycorticosterone. The 3D structure of human MR co-crystalized with corticosterone [PDB: 2A3I] was used as template for constructing the 3D model of lamprey CR [Genbank: AAK20930] (Li et al. 2005). The sequences of the steroid-binding domain of lamprey CR and human MR was performed using BLAST and two sequences showed 67% match and 76% match with no gaps after including conservative replacements. The strong similarity between lamprey CR and its template gives us confidence in the accuracy of our lamprey 3D model. The 3D model of lamprey CR was constructed using the Homology option in Insight II.

After the apo- 3D model of lamprey CR was obtained, the corticosterone from hman MR was inserted into lamprey CR by overlapping lamprey CR with human MR and extracting corticosterone from human MR then inserting corticosterone into lamprey CR using the biopolymer option in Insight II. The corticosterone in lamprey CR and human MR was converted to 11-deoxycortisol by adding 17 α -hydroxyl and removing the 11 β -hydroxyl using biopolymer option in Insight II. In the same manner, corticosterone was converted to DOC by removing 11 β -hydroxyl. The crystal structure of human MR with DOC [PDB:2AA7] was used to analyze the binding of DOC to the MR (Bledsoe et al. 2005).

The structure of lamprey CR with 11-deoxycortisol and DOC and human MR with 11-deoxycortisol was refined using Discover 3 in Insight II. For the energy minimization step, Discover 3 was run for 10,000 iterations, using the CVFF force field and a distant dependent dielectric constant of 2 to approximate for water in the protein.

6.2 Analysis of Progesterone receptor. In order to perform analysis of PR sequences, a 59 residue segment from the human PR corresponding to helix 3 through helix 5 (references) was used as a probe by BLAST to search GenBank and Ensembl for the corresponding segment in progesterone receptor of other animals.

The crystal structure of human PR with progesterone [PDB:1A28] was used to analyze the effects on the interaction between progesterone and the human PR containing four amino acid replacements that first appear in platypus PR (Williams and Sigler 1998). The 3D model of human PR with four amino acid replacement was constructed using Biopolymer option in Insight II by placing four replacements followed by refinement using Discover 3 with CVFF force field and distant dependent dielectric constant of 2 for 50 iterations.

6.3 Analysis of co-regulator in Estrogen Receptors. In order to perform analysis of ER sequences, a 53 residue segment from the human ER α and ER β corresponding to helix 3 through helix 5 was used as a probe by BLAST to search GenBank and Ensembl for corresponding segment in Estrogen Receptors of other animals.

The crystal structure of human ER α co-crystallized with DES [PDB: 2ERD] was used to analyze the effects on the interaction between coactivator peptide and the human ER α (Shiau et al. 1998). For ER β , the crystal structure [PDB: 3OLS] reported by Mocklinghoff et al. was used to analyze the effects on the interaction between coactivator peptide and the human ER β (Mocklinghoff et al. 2010).

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