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Molecular determinants of ERα and ERβ involved in selectivity of 16α-iodo-17β estradiol

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Abstract

The two known estrogen receptors, ERα and ERβ, are hormone inducible transcription factors that have distinct roles in regulating cell proliferation and differentiation. The natural ligand, 17β-estradiol (E2), binds with high affinity to both ERα and ERβ. However, a close analogue, 16α-iodo-17β-estradiol (16αIE2) showed about 10-fold selectivity for ERα over ERβ. From X-ray studies, it has been shown that the ligand-binding domains (LBD) of the two receptors are strikingly similar, and that only two changes fall within the binding cavity (ERα Leu384 to ERβ Met336, and ERα Met421 to ERβ Ile373). To understand the molecular basis for the ERα selectivity of 16αIE2, mutants and chimeras of ERα and ERβ were generated, and ligand-binding and transactivation functions were studied. The ERα Leu384 Met mutant behaved like ERα WT in the presence of 16αIE2; whereas the profile of the ERα Met421 Ile mutant was similar to that of ERβ WT. The ERβ mutant Ile373 Met behaved like ERα with 16αIE2. The results clearly demonstrate the role of ERα Met421 in the ERα selectivity of 16αIE2.

Keywords: Estrogen receptors; ERα; ERβ; Estradiol; 16α-Iodo–17β estradiol; Molecular modeling

1. Introduction

Estrogens regulate many important physiological processes, and their targets include reproductive tissues, the cardiovascular system, bone, and the brain [1–3]. Two estrogen receptor (ER) subtypes, ERα, and ERβ, encoded by two different genes, mediate the actions of estrogens [4–7]. The two ER subtypes are similar in their structural organization. They contain an amino terminal domain that contributes to transactivation function; a central domain responsible for the specific DNA binding, dimerization and nuclear localization; and a C-terminal domain involved in ligand-binding and ligand-dependent transactivation function. The two estrogen receptors share about 95% homology in their DNA binding domains, but their ligand-binding domains (LBD) share only 56% of amino acid sequence identity [5–9].

The two ERs bind to the natural ligand 17β-estradiol (E2) with high and nearly equal affinity. Extensive binding studies utilizing E2, its analogues and synthetic ligands with ER and various mutants of ER ligand-binding domain (LBD) have provided a detailed description of the ER pharmacophore [10,11]. Molecular modeling studies have provided valuable information of the residues involved in the interaction of E2 with the ERα receptor [10–12]. The crystal structures of the LBD of ERα and ERβ with well-known agonists and antagonists have been resolved, and the structures have provided a molecular basis for the distinctive pharmacophore of ER and its binding properties and also have provided the structural evidence for the mechanism of antagonism [13–15].

A number of natural and synthetic ER ligands display marked differences in the binding affinity and activation of the estrogen receptor. Several ER sub-type selective ligands have been identified. Some of the compounds, both steroidal and non-steroidal, are more potent in activating ERα than ERβ [16–19]. A novel class of ERα selective ligands that are full antagonists of ERβ has been identified [20]. Ligands that are ERβ selective are also known. Several phytoestrogens preferentially bind and activate ERβ [21,22]. A non-steroidal estrogen that is considerably more potent on ERβ than on ERα has also been discovered [20].

A close analogue of E2, 16α-iodo-17β-estradiol (16αIE2) has shown 10-fold selectivity toward ERα [21,23,24]. In

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order to understand the molecular basis for this selectivity and to identify the determinants involved in the ER subtype-selectivity, functional studies were performed with mutants of ERα and ERβ and their chimeras. The results of the ligand-binding and transactivation studies show that residue Met421 in ERα plays a significant role in the ERα selectivity of 16αIE2.

2. Materials and methods

2.1. Plasmids and mutagenesis

ERα WT cDNA was cloned into *Bam*HI–*Xho*I sites of pcDNA 3(Invitrogen). The ERβ coding sequence was cloned as a *Bam*HI–*Xba*I fragment into a pcDNA 3-expression vector. To generate ER mutants, site-directed mutagenesis was performed utilizing primers containing the desired mutation followed by amplification of the ERα mutant segment using PCR and the subsequent replacement of the amplified ERα segment into the ERα WT plasmid.

2.1.1. Mutation of ERα Leu384 Met

The following primers were used. 5′ primer CACCTTCTAGAATGTGCCTGAATGGAGATCCTG 3′ primer GAATAGGGCCCGCTAGCTGCATGCTCGAGTTATCAG. ERα WT plasmid was used as a template in the PCR reaction, and the following conditions were used: 94 °C—3 min, 5 cycles of 94 °C—30 s, 50 °C—30 s, 72 °C—90 s, and 20 cycles of 94 °C—30 s, 55 °C—30 s, and 72 °C—90 s, and finally, 72 °C—10 min. The PCR product was purified on a QIAquick spin column (Qiagen), digested with *Xba*I and *Apa*I, and the gel purified fragment was cloned into the corresponding sites in ERα WT cDNA plasmid.

2.1.2. Mutation of ERα Met421 Ile

5′ primer CAAAGGGATCCACCATGACCATGACCCTCCACACC and 3′ primer with Met to Ile codon mutation CATGTCGAAGATCTCCACGATGCCCTCTACACA were used for PCR amplification utilizing the conditions described above. The purified PCR product was digested with *Bgl*II and *Xba*I and the gel-purified fragment was cloned into the corresponding sites in the ERα WT plasmid.

2.1.3. Mutations of ERα Leu384 Met and Met421 Ile

The mutant plasmids were generated in a similar fashion as the Met421 Ile mutant, except, Leu384 Met ERα mutant plasmid DNA was used as a template for PCR amplification.

2.1.4. Mutation of ERβ Ile373 Met

The mutant plasmid was generated by a similar approach as described above. The following primers were used: 5′ primer TCCAGATCTTGTTCTGGACAGGGATGAGGGGAAATGCGTAGAAGGAATGCTGG and 3′ primer GGATCTAGAGTCGACGCGTCACTGAGACTGAGGGTTCTGG. PCR amplification was performed with the above conditions using ERβ WT as the template DNA. The purified PCR fragment was digested with *Bgl*II and *Xba*I and along with the *Hin*dIII–*Bgl*II fragment of the 5′ end of ERβ was cloned into *Hin*dIII–*Xba*I fragment of pcDNA3 expression vector.

2.1.5. Isolation of ERα and ERβ chimera plasmids

The ERβ sequence contained a unique *Eco*RV site in the DNA binding domain. A derivative of ERα without any changes in amino acids was generated by changing GGCTACCAT (Gly, Tyr, and His) to GGATATCAT(Gly, Tyr, and His) resulting in the generation of a unique *Eco*RV site in the DNA binding domain corresponding to ERβ (Fig. 1A). The ER specific primer GTGTGCAATGACTATGCTTCAGGATATCATTATG with desired changes, along with the ERα 3′ primer with an *Xho*I site, was used to amplify the ERα sequence, and the purified, amplified fragment was digested with BSrD1 and *Xho*I and was cloned into the pcDNA 3 vector along with the *Bam*HI and BsrD1 fragment from the ERα plasmid. The chimera of ERα and ERβ was generated by switching the *Bam*HI–*Eco*RV fragments of the two receptors.

The sequences of all of the above constructs were confirmed by sequencing to assure accuracy.

2.2. Bacterial expression of ligand binding domain of ERα WT, ERα mutants(Leu384 Met, Met421 Ile), and ERβ WT

The LBD of ERα was amplified using PCR primer 5′ primer AGCCATATGATGAAAGGTGGGATACGAAAAG and 3′ primer ATAGGATCCTTATTACTTGTCATCGTCGTCTTTGTAGTCGACTGTGGCAGGGAAACCCTCTGCCT and using the conditions mentioned above. The purified PCR fragment was digested with *Nde*I and *Bam*HI and was cloned into the corresponding sites of pET 15b vector. The ERα LBD mutant plasmids were generated by utilizing the corresponding ERα mutant plasmid as the template for PCR amplification. The plasmids were transformed into BL21 (DE3) and protein expression was induced by 1 mM IPTG at 25 °C for 2–3 h. The cells were harvested and resuspended in 50 mM Tris pH 7.4 and 150 mM NaCl. The cell suspension was lysed using the French Press. The lysate was clarified by centrifugation and was stored at −70 °C and was used for ligand-binding studies.

2.2.1. Ligand binding assay

Ligand binding reactions were performed in Wallace high binding cross talk free 96-well plates containing 2 nM [^{3}H] 17β-estradiol (NEN, Boston, MA), unlabelled compounds and 1 μg crude lysate in Dulbecco's phosphate buffered saline supplemented with 1 mM EDTA. After incubation at room temperature for 5–18 h, unbound material was removed by rinsing, and bound DPMs were determined by liquid scintillation counting [25].

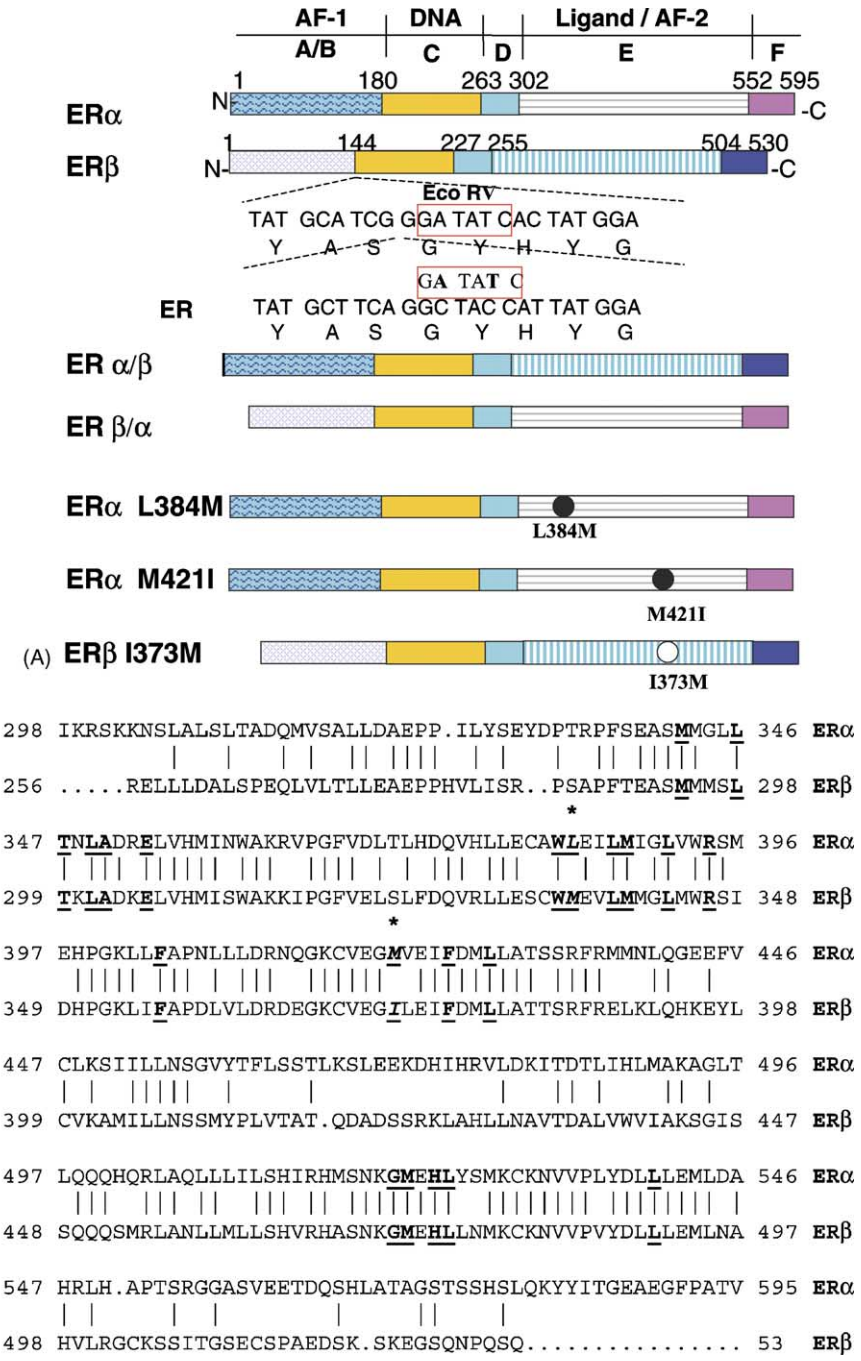

Fig. 1. (A) Schematic representation of ERα, ERβ, ERα–ERβ chimeras, and ERα and ERβ mutants. The linear sequence represents part of the DNA binding domain and the strategy to generate ERα–ERβ chimeras. (B) Sequence alignment of the LBD of human ERα and ERβ proteins. The vertical line (I) indicates the identical amino acids. The dots in the ERβ and ERα sequence annotate the presence of the gaps. The bold, underlined residues represent the amino acids that line the hormone binding cavity and/or interact with the bound ligand. The residues marked in italics and (*) represent the amino acid changes in ERα and ERβ LBD. The figure was produced using the Seq. Web program.

2.3. Cell culture and transient transfection

The HepG2 cell line was routinely cultured in MEM (Invitrogen) supplemented with 10% fetal bovine serum (Hyclone). The HepG2 cells (3×10^5/well) were plated in a 24-well tissue culture dish in phenol red-free MEM containing 10% charcoal treated fetal bovine serum. The cells in each well were transfected with 50 ng of receptor plasmid, 0.5 μg of 2X ERE-tk-luciferase reporter plasmid, 0.25 μg of pRSV β-galactosidase plasmid as internal control and 0.2 μg of calf thymus DNA in a volume of 50 μl using the calcium phosphate precipitation method. After incubation of the cells with the DNA for 4 h, the cells were glycerol shocked with 20% glycerol in 1 × Tris saline pH 7.9 for 1 min and washed

with media containing 2% charcoal/dextran treated serum. The cells were then treated with ligand in maintenance media. After 20 h of incubation, the cells were lysed, and the cell lysates were assayed for luciferase and β-galactosidase activity. The ER activity was determined by normalizing the luciferase activity with β-galactosidase activity as described previously [7].

2.3.1. Homology model of ERβ LBD

The homology model of ERβ LBD was built using the LOOK version 3.5 software package.[1] The X-ray structure of ERα [13] was selected as a template to predict the 3-dimensional structure of LBD of ERβ. The alignment of the ERβ LBD sequence (residues 258–500) with the ERα LBD sequence (residues 305–549) was done using the automated alignment feature within the program, and the final model was generated using the segment matching modeling method [26].

2.3.2. Computational methodology

Published coordinates for ERα complexed with E2 were obtained from the protein data bank (pdb) [13, 1ERE (pdb code)]. Coordinates for the ERβ–E2 complex were obtained from an in-house ERβ–E2 complex structure [27]. All docking calculations were performed using the QXP software package [28]. After adding explicit hydrogens using the hadd/hopt utilities, the X-ray ligand was minimized in the active site. The resulting structure was used as an input to a series of constrained simulated annealing dynamics calculations, wherein all atoms were allowed to move in a 0.1 Å radius flat potential after which a 20 kJ/mol/A^2 quadratic constant penalty was applied. Successive runs where the atoms were constrained in increments of 0.1 Å radius were performed until the energy of the complex started to increase. The resulting structure was then kept rigid, and the complex was subjected to 1000 steps of Monte Carlo search (MCDOCK) to explore all of the poses of the ligand [28]. Visualization of X-ray structures and docking results was performed using the InsightII software package.[2]

3. Results

3.1. LBD of ERα and its homology to ERβ

The sequence identity of the ERα and ERβ LBD is 56%, indicating that the secondary structure elements would probably be highly conserved. The homology model of the ERβ LBD was built based on the LOOK package, and the results showed that the binding site of ERβ reveals only two amino acid differences within a shell of 6 Å around the ligand pocket. The two amino acid differences are ERα Met421/ERβ Ile373 on the alpha face of the steroid ring and ERα Leu384/ERβ Met336 on the beta face. Subsequent X-ray structures of the ERβ/Genestein complex (15), as well as in-house ERβ/E2 structures (solved at a resolution of 2.4 Å, (27)), confirm that these two amino acids are indeed different in the binding pocket.

3.2. Ligand induced responsiveness of WT, mutant receptor ERα and ERβ

The transactivation function of the WT and mutant ERα and ERβ receptors with E2 and 16αIE2 were studied in HepG2 cells utilizing a 2X ERE-tk-luciferase reporter, and the results are given in Fig. 2A–H. ERα WT showed about an 80-fold increase in luciferase activity in the presence of 100 nmol/l E2, and the pattern of activation with 16αIE2 was very similar to that with E2 (Fig. 2A). ERβ WT was about five-fold less active in its ability to trans-activate the ERE-tk-luciferase reporter in the presence of E2. The steroidal 16αIE2 was less active with ERβ-WT compared to E2 and showed about two- and eight-fold less activity at 10 and 100 nmol/l, respectively. There was a right shift in the transactivation profile compared to E2 transactivation (Fig. 2B). To determine the extent to which the selectivity of 16αIE2 depends on the LBD versus other receptor domains, E2 and 16αIE2 were tested against two ER chimeras in which only the LBDs are switched within the context of the remainder of the receptor. The ERβ/ERα chimera gave a similar transactivation profile with E2 and 16αIE2, and the pattern was very similar to that of ERα WT (Fig. 2C). With ERα/ERβ, 16αIE2 was about four-fold less active than E2, and the pattern was similar to that of ERβ WT (Fig. 2D). The data suggest that the LBD of ERα is the major factor in determining the selectivity of 16αIE2 for the ERα subtype. Several reports, including the X-ray crystallographic studies, have identified the amino acid residues that are in close contact with the ligand. Of these, all but two are identical in ERα and ERβ, and the two residues in ERα, Leu384, and Met421, are replaced by Met336 and Ile373, respectively in ERβ. To determine the role of these amino acids in the selectivity of ERα for 16αIE2, ERα mutants with Leu384 and Met421 replaced by the corresponding ERβ residues (Met and Ile, respectively) were generated. The ERα mutant Leu384 Met was about 60% as active as WT ERα. Both E2 and 16αIE2 were equally active and gave a transactivation profile similar to the ERα WT receptor (Fig. 2E). The mutation of ERα at 421 from Met to Ile did not affect the transactivation function with E2. However, 16αIE2 was less active compared to E2, and the pattern of the transactivation profile was very similar to that of ERβ WT (Fig. 2F). The results suggest that methionine at 421 is critical for the efficient transactivation function of ERα WT, and its conservative change to isoleucine residue affects the binding of 16αIE2 (see below) and its transactivation function. The ERα mutant with two mutations (ERα Leu384 Met, Met421 Ile) behaved in a similar fashion to the ERα single mutant, ERα Met421 Ile (Fig. 2G). If the role of ERα

[1] LOOK version 3.5 Molecular application group, Palo Alto, CA, USA.
[2] Insight II Software Package, Accelrys, San Diego, CA, USA.

Met421 is critical for the efficient binding of 16αIE2 and transactivation function, then a change of Ile at 373 to Met in the ERβ LBD should change the transactivation profile of 16αIE2 from ERβ to ERα. A mutation of Ile at 373 to Met was generated and tested for its transactivation function (Fig. 2H). The mutation had no effect on the transactivation function with E2 compared to ERβ WT. However, 16αIE2 was equally as active as E2, and the transactivation profile

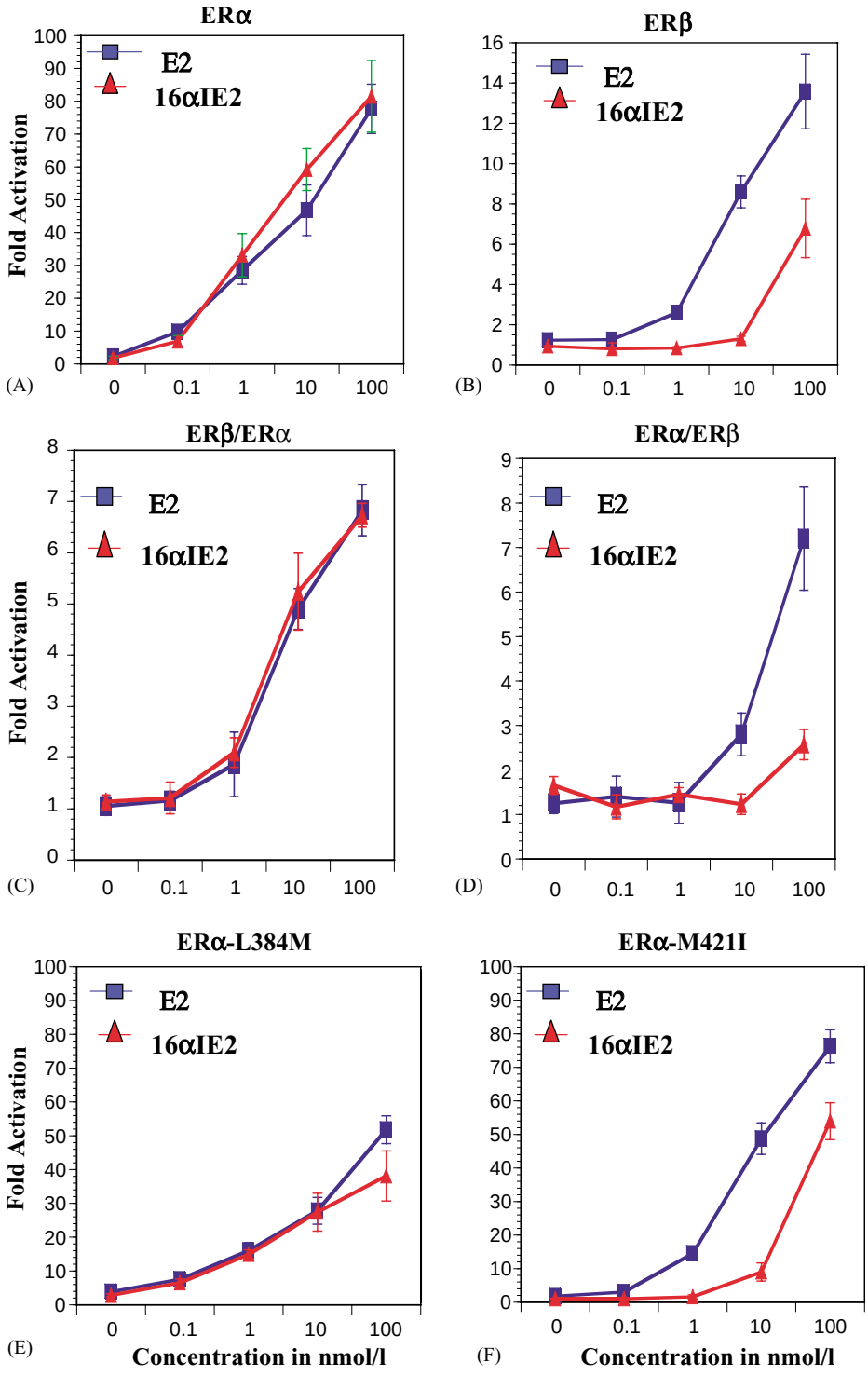

Fig. 2. Transactivation profile of ERα, ERβ, their chimeras, and mutant ERα and ERβ receptors with E2 and 16αIE2. HepG2 cells were transfected with the expression vector for ERα and ERβ WT (A & B), their chimeras (C & D), and mutant plasmids (E–H) along with the 2X ERE-tk-luciferase and pRSV-β-galactosidase reporter plasmids. Cells were treated with the indicated concentrations of E2 and 16αIE2 for 24 h. Luciferase activity was normalized with β-galactosidase activity.

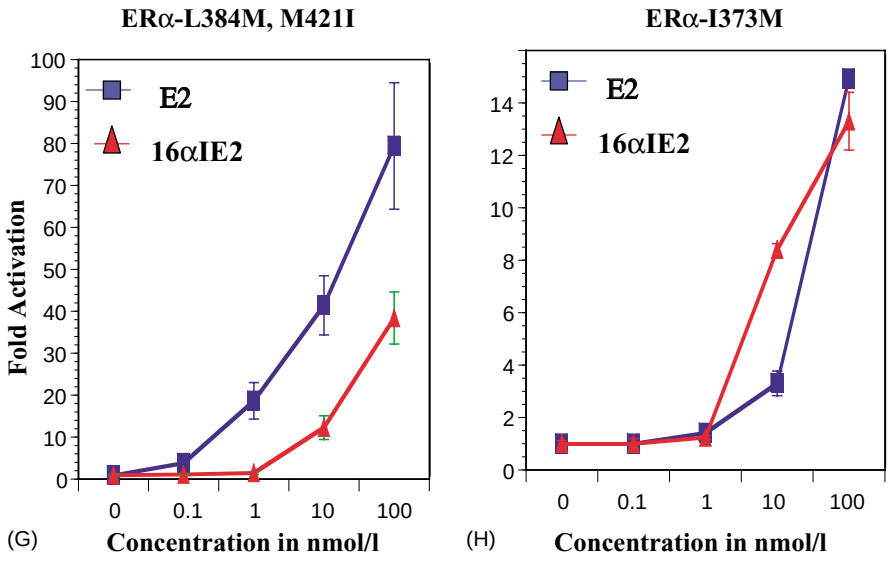

Fig. 2. (*Continued*).

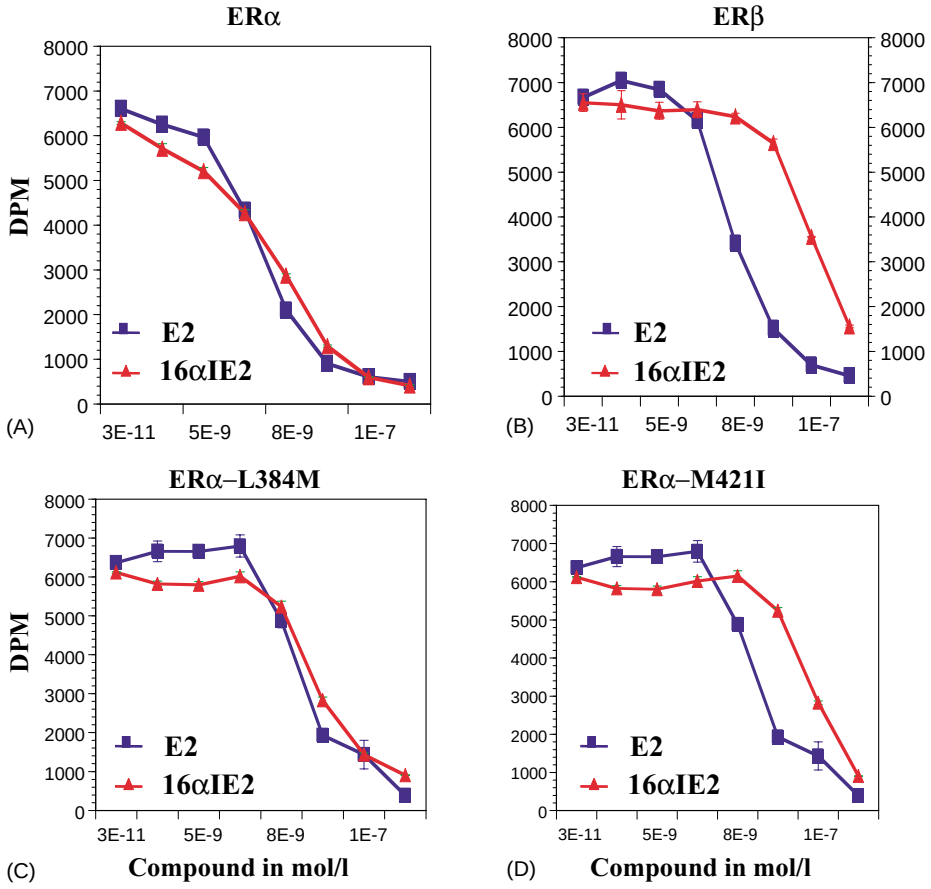

Fig. 3. Competitive binding of E2 and 16αIE2 to LBD of ERα, ERβ, and mutant ERα receptors.

was very similar to ERα WT. The loss of ERα selectivity for 16αIE2 with the mutation of Met421 Ile indicates the importance of Met421 for ERα selectivity toward 16αIE2. Moreover, the gain of selectivity for ERβ by changing Ile373 Met confirms the importance of the methionine residue in the LBD for the sub-type selective interaction of 16αIE2.

3.3. Ligand binding

The binding of ligands, E2 and 16αIE2, was studied using bacterially expressed LBDs of ERα, as well as mutants of ERα and ERβ receptors, and the results are shown in Fig. 3. Radioinert E2 inhibited the binding of [^{3}H] 17β estradiol to the ligand binding domains of ERα and ERβ with the same potency, as expected given the nearly identical binding affinity to both receptors [22]. The ligand, 16αIE2, showed binding very similar to E2 to the ERα LBD (Fig. 3A), whereas it was 15 times less potent on ERβ (Fig. 3B). While ERα mutant Leu384 Met showed overall reduced binding (Fig. 3C), the binding affinities for E2 and 16αIE2 were similar, suggesting that the mutation Leu384 Met does not discriminate between E2 and 16αIE2. However, the ERα mutant Met421 Ile showed reduced binding of 16αIE2 compared to E2, and the pattern of the binding of the two ligands was similar to that of ERβ. The above binding studies indicate that the methionine residue at 421 of the LBD of ERα plays an important role in the interaction with 16αIE2 and its selectivity toward ERα. Mutation of this Met to Ile as seen in ERβ, results in reduced binding to 16αIE2 (Fig. 3D).

4. Discussion

Several approaches, including molecular modeling, affinity labeling and site directed mutagenesis, have been taken to formulate the general model for the interaction that occurs between the receptor and its ligands upon the formation of the receptor–ligand complex. Utilizing the above approaches and close structural analogs of E2, the orientation and residue contacts of E2 with ER have been predicted [10–12]. The crystal structures for the LBD of ERα bound to E2, raloxifene, 4OH-tamoxifen, and diethylstilbestrol (DES), as well as a peptide derived from the coactivator GRIP1, have also been elucidated [13–15]. These crystal structures have provided the details of the pharmacophore for the ER and its binding properties. The estrogen-binding cavity is completely partitioned with an external environment and is buried deeply within the hydrophobic core of the LBD and surrounded by parts of helixes H3, H6, H8, H11, and H12. Hormone recognition is achieved through a combination of specific hydrogen bonds and hydrophobic interactions between the core residues and the non-polar E2 skeleton. Upon ligand binding, the LBD undergoes conformational changes whereby the C-terminal H12 is realigned over the ligand-binding pocket against H3, H5/6 and H11. The activity of the ERα activation function domain 2 (AF2) is dependent upon the integrity of a hydrophobic interaction surface generated by conserved amino acid changes in H3 H5/6 and H12, which is critical for the interaction of members of the p160 coactivator family [13,14].

The structure of the ERβ LBD in the presence of phytoestrogen genistein and raloxifene has also been determined [15]. The overall structure of the ERβ LBD is very similar to that of ERα. Twenty-two residues, predominantly hydrophobic in character, line the cavity and interact with the bound ligand. The contact residues are identical to those of ERα with two changes: amino acids Leu384 and Met421 are replaced by Met336 and Ile373. The binding cavity in the ERβ-genistein complex is slightly smaller than that of ERα–E2, and this reduction is primarily due to the replacement of leucine at position 336 in ERα with a bulkier methionine in ERβ. The methionine 336 residue in the ERβ LBD may result in the tight packing of ligand in ERβ [15]. As part of our effort to understand the molecular basis for the non-selective nature of E2 and the ERα selectivity of 16αIE2, we have analyzed the mutation results using molecular modeling.

4.1. Estradiol is a non-selective ligand

Insights into the non-selective nature of the E2 ligand can be rationalized from examining the X-ray structures of ERα/E2 [13] and ERβ/E2 [27]. The overall topology of our in-house ERβ/E2 X-ray structure is similar to that of the published ERα/E2 structure [13] with H12 packed against H3, H5/H6 and H11 in a conformation that has been observed in agonist-bound structures. The overall RMS for all of the backbone atoms of these two structures is 0.89 Å. In both of these structures, E2 adopts a similar orientation in the ligand-binding pocket with the phenolic A ring making critical hydrogen bonds with Arg and Glu residues at one end of the cavity, and the 17β–OH group hydrogen bonding to His at the other end. The rigid framework of E2 does not allow any of its atoms to approach any of the residue substitutions within either of the ER subtypes. Fig. 4A and B shows the X-ray structures of ERα/E2 and ERβ/E2 along with critical residues in the binding pocket. The alpha face of both of these complexes shows that the closest distance between the ligand (16C of the D ring) and the substituted residues is very similar (4.2 and 4.3 Å). This is not the case for the beta face of the region where distances of 4.4 Å in ERα and 3.5 Å in ERβ are observed from the closest atom, which in this case is the 18 methyl group to the substituted residues. Although the distance of 3.5 Å is shorter in the case of ERβ, we believe it does not contribute to the energy of the complex. Our QM calculations [27] performed on model systems suggest that both of these distances fall in the region of a relatively flat potential with no change in relative energy. Thus, we conclude that these distances do not influence the selectivity of E2 toward the ER subtypes and support the results of our mutation studies.

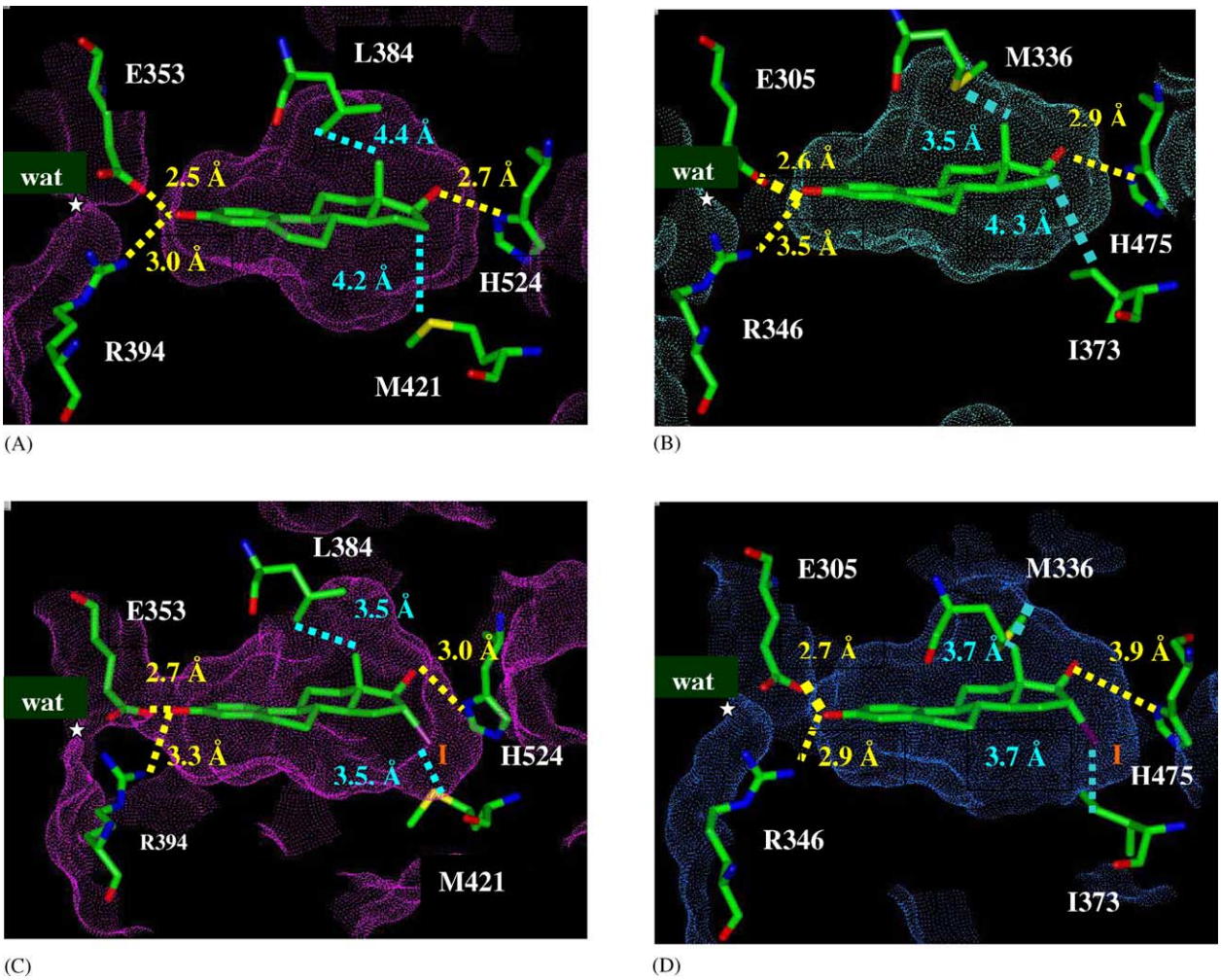

Fig. 4. Model of E2 and 16αIE2 in the ERα and ERβ ligand-binding pocket. (A and B): X-Ray structure of (A) ERα/E2(Green) and (B)ERβ/E2 with critical residues and a Connolly surface of the binding pocket. Hydrogen bonds are shown with yellow dotted lines, while the distance of closest approach to the two substituted residues are shown in cyan dotted lines. The numbering of residues in 4A is from ERα, 4B is from ERβ; (C and D) docked structure of 16αIE2 (green) in ERα and ERβ with critical residues in the binding pocket. Distances from the iodo (I) group to the nearest atom of (C) the ERα Met421 and (D) ERβ Ile373 residue in the pocket are shown in cyan. All of the figures were generated using the Insight II software package .

4.2. 16αIE2 is an ERα selective ligand

Based on binding studies, several reports have shown that 16αIE2 is an ERα selective ligand, with selectivity ranging from 10- to 30-fold in favor of ERα over ERβ [23]. The results of the present study clearly demonstrate that both E2 and 16αIE2 showed a similar binding and transactivation profile with ERα. However, with ERβ, 16αIE2 bound with less affinity as shown earlier [23], and also produced less transactivation function compared to E2, thus, demonstrating its selectivity toward ERα in both binding and transactivation function. In the present study, we show that the change of ERα Met421 to isoleucine changes the behavior of 16αIE2 from ERα to the ERβ type. The corresponding change of ERβ Ile373 to Met makes it behave like ERα with the16αIE2 ligand. The result clearly demonstrates that Met421 is an important determinant in the ERα selectivity of 16αIE2.

In an effort to understand the origin of the ERα selectivity of 16αIE2 (10-fold in activation and 20- to 30-fold in binding), docking studies were performed [28] within the binding site of ERα and ERβ. The results of the docking studies (Fig. 4C and D) show 16αIE2 adopts similar orientations in both ERα and ERβ sites with the 16α-iodo group in the vicinity of the ERα Met421/ERβ Ile373 residue. While both docked orientations have the hydroxyl group of the A ring interacting with the Arg and Glu residues, the 17β–OH distance in 16αIE2/ERβ complex is much further i.e. 3.9 Å versus 3.0 Å from the His residue. This may be a result of the iodine atom (C–I bond length = 2.13 Å) experiencing an unfavorable steric interaction with the ERβ Ile373 residue, which would position the 17β–OH group further away from His475. This interaction is roughly estimated to contribute approximately a 7- to 20-fold loss in binding when one compares E2 versus 17-deoxy-E2 [27,29]. Thus, one might expect a similar or a smaller loss

in potency for the 16αIE2 ligand with ERβ on account of this longer distance. In addition, a distance of 3.5 Å from iodine to sulfur of Met of ERα421 in the docked orientation of 16αIE2 represents a favorable interaction in the ERα site according to quantum mechanical calculations [27]. Thus we believe that an unfavorable steric interaction between the iodo group and ERβ Ile373, as well as an attractive interaction between the iodo group and ERα Met421 both contribute to the selectivity of 16αIE2.

Several subtype-selective ligands of ERα and ERβ have been described, and the selectivity mainly revolves around very few residues of the LBD. Diarylpropionitrile (DPN) is an ERβ-selective ligand, and a single ERα point mutation, Leu384 Met, is largely sufficient to switch the DPN response of this ER to that of the ERβ type; however, more extensive studies with mutants have shown the residues in helix three are also important in achieving the full ER β selectivity of DPN [20]. A recent study with the enantiomers of indanesterol (IA) have shown that although the binding of IA–R and IA–S was not affected by ERα Leu384 Met mutation, IA–R showed higher potency in activating Leu384 Met mutated ERα and WT ERβ, demonstrating that a single residue in the LBD determines the stereoselectivity of ERα and ERβ for indenosterol ligands [30]. The success with the synthesis of many ER sub-type specific ligands due to the specific interaction with a limited number of LBD amino acids indicates that the approach can be used to design selective ligands for ERα and ERβ receptors.

Comparison of structure-function relationships specific for the very similar yet distinct ERα and ERβ subtypes of the ER offers a unique opportunity for dissecting the role of ligand-binding and ultimately mediating biological function. Detailed studies involving the combination of structural and functional analysis of wild-type, as well as mutated ERα and ERβ bound to subtype-specific ligands, coupled with molecular modeling and crystallographic studies will help to develop ER subtype-specific ligands. These studies will provide the basis for the structure-based design of improved agonists and antagonist as tools to probe the function of estrogen receptors and also for the treatment of estrogen-related diseases.

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