Molecular and Cellular Endocrinology 246 (2006) 83–90

Understanding the human estrogen receptor-alpha using targeted mutagenesis

Debra F. Skafar a,b,∗, Shohei Koide c

a Department of Physiology, Wayne State University School of Medicine, 540 E. Canfield, Detroit, MI 48201, United States
b The Barbara Ann Karmanos Cancer Institute, Detroit, MI 48201, United States
c Department of Biochemistry and Molecular Biology, University of Chicago, Chicago, IL 60637, United States

Abstract

The estrogen receptor-alpha (ERα) is a wonderfully complex protein important in normal biology, breast cancer, and as a target for anti-cancer agents. We are using the available structures of the hERα as well as secondary structure predictions to guide site-directed mutagenesis in order to test the importance of specific interactions and regions in the ligand-regulated activity of the protein.

In one area of interest, we are investigating the role of the F domain in the ligand-stimulated activity of the hERα. Results from our laboratory and others suggest that the F domain modulates the activity of the hERα. In order to better understand the role of the F domain in the hERα, we have constructed mutants within this region. Mutations within a predicted alpha-helical region alter the response of the ER to estradiol (E2), eliminate or impair the agonist activity of 4-hydroxytamoxifen (4-OHT), and alter the ability of E2 to overcome 4-OHT’s antagonist activity. Deleting the F domain increases the affinity of the receptor for E2; by contrast, mutating a residue in the middle of the predicted helix to a proline does not alter the affinity for E2, but does change the binding mechanism from a positive cooperative to a noncooperative interaction. These and other results show the F domain exhibits substantial functional complexity, and support the idea that this domain modulates the activity of the hERα.

In a second area of interest, we are investigating the role of hydrophobic and hydrogen-bonding interactions at the start of helix 12 in the activity of the hERα. Leucine-536 (L536) has been proposed to participate in hydrophobic interactions that form part of a capping motif stabilizing the start of helix 12. When mutated, the resulting receptors exhibit a reduced response, or even an inverted response, to E2 and 4-OHT on both ERE-driven and AP-1-driven promoters. Interestingly, these mutated receptors also exhibit altered interactions with probes that recognize the agonist-bound and 4-OHT-bound conformations of the ERα. Thus, L536 couples the binding of ligand with the conformation of the receptor.

Overall, these results show that combining structure-based hypotheses with functional tests of the ER’s activity can identify regions and interactions that are important in the ligand-stimulated activity of the protein.

© 2005 Elsevier Ireland Ltd. All rights reserved.

Keywords: Estrogen receptor-alpha, Mutagenesis, 4-Hydroxytamoxifen

1. Introduction

The estrogen receptor-alpha (ERα) is a wonderfully complex protein important in normal biology, breast cancer, and as a target for anti-cancer agents. We want to understand the function of two regions of the alpha form of the estrogen receptor. The first part of this paper considers work on the extreme C-terminal region of the receptor, the F domain. The second part considers work on the region at the start of helix 12 in the ligand-binding domain (LBD) of the estrogen receptor. Although the estrogen receptor can modify the activity of cells through a number of mechanisms, we will focus on its classical, ERE-driven, mechanism of action (Nilsson and Gustafsson, 2000).

1.1. The F domain in the human estrogen receptor-alpha

Among the members of the nuclear receptor superfamily, the F domain is one of the most variable regions (Evans, 1988). Although many LBD crystal structures have been determined, little is known about the conformation of the F domain. The F domain is generally considered devoid of well-defined structure. Why is the F domain important? First, consider the F domain in another member of the nuclear hormone receptor superfamily, the orphan receptor hepatic nuclear factor-4-alpha (HNF4-α). HNF4α possesses one of the longest F domain in the nuclear
The secondary structure, \( h = \) helix, \( e = \) extended, \( c = \) coil, was predicted using GOR IV (Garnier et al., 1996). Mutated residues are in bold.

<table>
<thead>
<tr>
<th>551</th>
<th>552</th>
<th>553</th>
<th>554</th>
<th>555</th>
</tr>
</thead>
<tbody>
<tr>
<td>G551L/G557L</td>
<td>APSGQGAV</td>
<td>EEIDGSLAT</td>
<td>AGSTSHS5LQ</td>
<td>KYITGEAEQ</td>
</tr>
<tr>
<td>G556E/G558A</td>
<td>APSGQGAV</td>
<td>EEIDGSLAT</td>
<td>AGSTSHS5LQ</td>
<td>KYITGEAEQ</td>
</tr>
<tr>
<td>S559A/E562A</td>
<td>APSGQGAV</td>
<td>EEIDGSLAT</td>
<td>AGSTSHS5LQ</td>
<td>KYITGEAEQ</td>
</tr>
<tr>
<td>Q565F</td>
<td>APSGQGAV</td>
<td>EEIDGSLAT</td>
<td>AGSTSHS5LQ</td>
<td>KYITGEAEQ</td>
</tr>
<tr>
<td>S554E/C557G</td>
<td>APSGQGAV</td>
<td>EEIDGSLAT</td>
<td>AGSTSHS5LQ</td>
<td>KYITGEAEQ</td>
</tr>
</tbody>
</table>

The secondary structure, \( h = \) helix, \( e = \) extended, \( c = \) coil, was predicted using GOR IV (Garnier et al., 1996). Mutated residues are in bold.
in the ER: the results of these functional studies do not depend on whether the predicted structure corresponds with the actual structure. We focused on the region of the ERs F domain near and within the predicted alpha helix. When testing a prediction for a helix, the classic experiment is to disrupt it by inserting a proline residue into the middle of the predicted helical region, as we did by mutating glutamine-565 to proline (Q565P). Next, since hydrogen bond formation between the side chains of residues at the start of an alpha helix and the peptide backbone can stabilize the helix (Aurora and Rose, 1998), we wanted to disrupt this potential interaction, and so mutated serine-559 (S559) and glutamic acid-562 (E562) to alanines (S559A/E562A mutant). The two vicinal glycines, G556 and G557, are also of interest. Since glycine has the smallest side chain, comprising only a hydrogen atom, we mutated these to bulky, hydrophobic, leucines in the G556L/G557L mutant. Finally, mutating serine-554 to a stop codon (S554stop) truncated the receptor and deleted the entire F domain.

We tested the activity of the wild-type or mutated estrogen receptors using a transient transfection assay in HeLa cells. We use this system because although there is no standard promoter and cell model for evaluating the effects of mutations of the estrogen receptor, when reports in the literature describe a mutated estrogen receptor, its activity on an ERE-driven reporter in HeLa cells is generally included.

We first examined the effect of mutating the F domain on the ability of the estrogen receptor to stimulate transcription of an ERE-driven promoter in response to estradiol (fold-stimulation) (Fig. 3). As you would expect, the addition of estradiol increased the activity of the wild-type receptor. Next, note that each mutant was stimulated by estradiol, showing that mutation or deletion of the F domain did not eliminate the ability of the receptor to respond to estradiol (Schwartz et al., 2002). Of these mutated proteins, the S559A/E562A mutant exhibited the greatest effect—it’s activity at the highest ligand concentrations was substantially greater than that of the wild-type protein (Schwartz et al., 2002). In addition, substituting a proline in the middle of the predicted helical region may have attenuated the response to estradiol (Schwartz et al., 2002). Thus, mutations in the F domain altered the response of the human estrogen receptor to estradiol.

We next examined the effects of mutations within the F domain on the weak agonist activity of 4-hydroxytamoxifen, using the same ERE-driven promoter (Fig. 4). Tamoxifen is not solely an antagonist—it is a selective estrogen receptor modulator (SERM), and can exert weak agonist activity in some circumstances. Tamoxifen approximately doubled the activity of the wild-type receptor on an ERE-driven reporter (Schwartz et al., 2002). Also, as originally shown by Montano et al., deleting the F domain (S554stop) eliminated the ability of tamoxifen to stimulate the activity of the receptor. Mutating the vicinal glycines to leucines (G556L/G557L) did not eliminate the agonist activity of tamoxifen (Schwartz et al., 2002). However, the mutations directed to the predicted helical region of the protein did alter the agonist activity of tamoxifen. The Q565P mutant lost the ability to respond to tamoxifen as an agonist, while the S559A/E562A mutant responded to 4-OHT as an agonist only at the very highest concentration of ligand used, if at all (Schwartz et al., 2002). Thus, not only deletion of the entire F domain, but mutation of the predicted helical region, greatly impaired or eliminated the agonist activity of tamoxifen.

We next examined the effects of mutations in the F domain on tamoxifen’s antagonist activity. In this series of experiments, we maintained the concentration of 4-hydroxytamoxifen constant at 100 nM, and added increasing concentrations of estradiol. By comparing the activity of the receptor in the presence of...
both tamoxifen and estradiol with its activity in the presence of estradiol alone, we calculated the concentration of estradiol that would be needed to overcome tamoxifen’s inhibition of the activity of the receptor (Table 2) (Schwartz et al., 2002). For the wild-type protein, 160 nM estradiol was needed to overcome 50% of the inhibitory effect of 100 nM 4-OHT (Schwartz et al., 2002). The G556L/G557L and the S559A/E562A mutations had little or no effect on the concentration of estradiol needed to overcome tamoxifen inhibition (Schwartz et al., 2002). However, when the F domain was deleted, only 8 nM estradiol was required to overcome inhibition by tamoxifen—1/20 of the amount of estradiol needed by the wild-type protein to overcome tamoxifen’s inhibitory activity (Schwartz et al., 2002). By contrast, we calculated it would take substantially more estradiol—320 μM to overcome the inhibition by tamoxifen in the Q565P mutant (Schwartz et al., 2002). (Note that this value is obtained by extrapolation, and would be unlikely to be achieved in aqueous solution.) Thus, mutations in the F domain increased (S554stop) or decreased (Q565P) the ability of estradiol to overcome inhibition by tamoxifen.

Key aspects of the ability of the estrogen receptor to respond to ligands are the affinity of binding, as well as the mechanism of interaction between the ligand and the receptor. A non-cooperative interaction is characterized by a Hill coefficient of 1, whereas a positive cooperative interaction is characterized by a Hill coefficient greater than 1 (Hill, 1910). We measured the binding of [3H]-estradiol to baculovirus-expressed wt ER, the Q565P mutant, and the S554stop mutant in an equilibrium binding assay (Fig. 5, Table 3). As we and others have reported, the wild-type receptor bound estradiol with high affinity, having a Kd of 0.05 nM (Schwartz et al., 2002). Substitution of a proline at position 565 in the middle of the predicted helical region did not affect the affinity for estradiol—the Kd of the Q565P mutant was ∼0.2 nM—but reduced the Hill coefficient to a value near 1, which indicates that the binding mechanism had been converted from a positive cooperative to a non-cooperative interaction (Schwartz et al., 2002). These results show that mutations in the F domain altered the affinity of the receptor for estradiol, as well as the site–site interactions of the receptor.

Taken together, our results show that mutations in the F domain altered the responses of the ER to estradiol and 4-hydroxytamoxifen, as well as the affinity for estradiol and the site–site interactions of the receptor. This raises an important question: how might this be? In other words, how could a region of only 42 amino acids modulate so many different activities of the receptor?

To address this question, consider a model of the estrogen receptor alpha F domain based on secondary structure predictions (Fig. 2) (Schwartz et al., 2002). In this model, there is a predicted alpha-helical region, a predicted extended region, and two additional predicted extended residues almost at the extreme C-terminus of the protein. Of note, the larger predicted extended region contains residues—isooleucine and threonine—that are characteristic of surface beta-strands (Palliser et al., 2000; Palliser and Parry, 2001). Most importantly, this model shows that although the F domain contains only about 42 residues, it is potentially 100 Å or more of peptide.

Table 2

<table>
<thead>
<tr>
<th>Receptor</th>
<th>E2 concentration (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt hERα</td>
<td>0.16</td>
</tr>
<tr>
<td>Q565P</td>
<td>0.16</td>
</tr>
<tr>
<td>S559A/E562A</td>
<td>0.16</td>
</tr>
<tr>
<td>G556L/G557L</td>
<td>0.16</td>
</tr>
<tr>
<td>S554stop</td>
<td>0.008</td>
</tr>
</tbody>
</table>

Table 3

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Hill coefficient, nH</th>
<th>Affinity, Kd (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt hERα</td>
<td>1.58 ± 0.18</td>
<td>0.64 ± 0.41</td>
</tr>
<tr>
<td>S554stop</td>
<td>1.65 ± 0.16</td>
<td>0.05 ± 0.007</td>
</tr>
<tr>
<td>Q565P</td>
<td>0.94 ± 0.1</td>
<td>0.23 ± 0.01</td>
</tr>
</tbody>
</table>

Estradiol-binding was measured using baculovirus-expressed S554stop, Q565P, and wt ER receptors (Schwartz et al., 2002). The values of the affinity (Kd) and Hill coefficient (nH) were calculated by fitting the untransformed binding data to the Hill equation by nonlinear regression using GraphPad Prism. These values are the mean ± S.E.M. of two to three independent experiments (Schwartz et al., 2002).

Fig. 5. Binding of the wt and mutant hERα to [3H]estradiol. The binding of [3H]estradiol to wt hERα (filled squares), the S554stop mutant (open circles), or the Q565P mutant (filled triangles) was measured in [3H]estradiol containing baculovirus-expressed receptor (Schwartz et al., 2002). (Left) Non-transformed saturation binding data, the lines shown are the best fit by nonlinear regression to the Hill equation using GraphPad Prism. (Right) Scatchard plot of the same data (Scatchard, 1949). Similar data are in Schwartz et al. (2002).
Now consider the size of the ligand-binding domain of the estrogen receptor-alpha. The LBD monomer is approximately 50 Å × 40 Å × 30 Å, and has different surfaces involved in dimerization and coactivator binding, as well as surfaces that are potentially involved in ligand association and dissociation.
different parts of the F domain could interfere with or help to form different surfaces on the ligand-binding domain. In this way, the F domain could modulate multiple activities of the receptor.

1.2. What interactions determine the conformation of the human estrogen receptor-alpha?

One of the fundamental properties of the estrogen receptor is to change shape, or conformation, in response to the binding of ligand. Most notably, the position of helix 12 changes, depending on the ligand that is bound—it covers the ligand-binding pocket in the presence of a strong agonist, DES, and it blocks the coactivator binding site in the presence of a SERM, 4-hydroxytamoxifen (Brzozowski et al., 1997; Shiu et al., 1998).

We want to understand what determines the conformation of the estrogen receptor. Consider the region at the start of helix 12: the residue in blue, leucine-536 (L536), occupies different positions, and makes totally different contacts in the two different structures of the receptor (Fig. 7). In the agonist-bound receptor, it appears to be involved in a hydrophobic interaction with leucine-541 (L541) in magenta, in the tamoxifen-bound receptor, the interaction between the two residues is completely disrupted (Shiu et al., 1998). We therefore wanted to understand the role of this residue in the conformation changes of the protein.

We mutated L536 to a number of different residues: to alanine (A), which has a small side chain, to glutamic acid (E), which has a negatively charged side chain, to glycine (G), which has only a hydrogen atom for a side chain, to isoleucine (I), which is similar to leucine, to lysine (K), which is large and positively charged, and to asparagine (N), which is small and polar. We evaluated the activity of the mutants in response to estradiol on an ERE-driven luciferase reporter (Fig. 8) (Zhao et al., 2003).

As expected, estradiol stimulated the activity of the wild-type receptor. All of the mutated receptors except the isoleucine substitution exhibited increased basal activity of the receptor (Zhao et al., 2003). The glycine and asparagine mutants exhibited the highest basal activity (Zhao et al., 2003). However, of the mutated receptors, only the substitution with isoleucine retained the ability to be stimulated by estradiol (Zhao et al., 2003). Thus, mutations of L536 eliminate the ability of estradiol to stimulate the activity of the receptor on an ERE-driven promoter.

We next investigated the effect of these mutations on the weak agonist activity of 4-hydroxytamoxifen (Fig. 9). Tamoxifen stimulates the activity of the wild-type receptor; the antagonist ICI-182,780 has no effect by itself (Zhao et al., 2003). Again, the mutated receptors exhibited an increased basal activity (Zhao et al., 2003). Most interestingly, tamoxifen either had no effect on the activity of a mutated receptor, or decreased its basal activity—that is, tamoxifen exhibited an “inverse agonist” effect.

Fig. 10. Interactions of hERalpha mutants with hER agonist complex-specific probes as measured using a yeast two-hybrid system. β-Galactosidase activity (Miller Units, Y-axis) was measured in the presence of 1 μM of the indicated ligand, from left to right: E2 (E), ICI-182,780 (I), 4-hydroxytamoxifen (T), raloxifene (R), vehicle control (V) or progesterone (P). Column A, results obtained using the receptor-interacting domain of SRC-1 (aa190–400); column B, results obtained using monobody “E3#6”. The data are from triplicate measurements. Reactivity with the wt (top panels) and L536E mutant (lower panels) is shown. Different panels represent experiments using different yeast cells and thus the absolute activity cannot be normalized (or quantitatively compared) across panels (Zhao et al., 2003). Republished with permission of the American Society for Biochemistry and Molecular Biology, from Zhao et al. (2003); permission conveyed through Copyright Clearance Center.
The antagonist ICI-182,780 decreased the activity of all of the mutated receptors (Zhao et al., 2003). Thus, mutations at L536 also eliminate the ability of 4-hydroxytamoxifen to act as an agonist on an ERE-driven promoter.

In order to understand how these mutations affected the ability of the estrogen receptor to interact with coactivator proteins, we used a two-hybrid system. In this system, interaction between the two proteins of interest drives expression of a reporter gene. We use a two-hybrid system in yeast to investigate the interaction between estrogen receptor and the coactivator protein SRC-1 (Koide et al., 2002). In addition, probes based on a fibronectin backbone, called "monobodies", are used that specifically recognize the agonist-bound conformation of the receptor; these are designated E3#6 and E2#23 (Koide et al., 2002). For the sake of simplicity, focus on the activity of the wild-type protein and one mutant, the L536E mutant (Fig. 10).

Each probe, SRC-1, E3#6 and E2#23, reacts with the wild-type receptor in the presence of estradiol, but not in the absence of ligand, or in the presence of ICI-182,780, 4-hydroxytamoxifen, raloxifene, or progesterone (Koide et al., 2002; Zhao et al., 2003). When leucine-536 is mutated, two of the probes, SRC-1 and E3#6, show increased reactivity with the receptor in the absence of ligand that is blocked by 4-hydroxytamoxifen (Zhao et al., 2003). Similar results are observed with the other mutants, with the exception of the isoleucine substitution (Zhao et al., 2003). Thus, mutations at leucine-536 increase the basal reactivity with two probes that specifically recognize the agonist-bound conformation of the receptor.

Monobody probes that recognize the tamoxifen-bound conformation of the receptor have also been developed (Koide et al., 2002). Again, focus on the wild-type receptor and the glutamic acid mutant (Fig. 11). Each probe – OHT#1 and OHT#33 – reacts with the wild-type receptor in the presence of tamoxifen, but shows little reactivity in the absence of ligand or in the presence of estradiol, ICI-182,780, raloxifene, and progesterone (Zhao et al., 2003). Mutation of leucine-536 to glutamic acid substantially increases the reactivity of the receptor with OHT#1.

![Figure 11](image-url)
in the presence of raloxifene, but not in the absence of ligand. The mutated receptor also interacts with OHT#1 in the presence of tamoxifen. All mutants, except the isoelucine substitution, show increased reactivity with this probe in the presence of raloxifene (Zhao et al., 2003). This means the raloxifene-bound, mutated receptor, resembles the tamoxifen-bound, wide-type receptor, in the part of the protein that is recognized by this probe.

When reactivity with the other probe, OHT#33 is examined, the glutamic acid mutant shows increased reactivity in the presence of ICI-182,780, but not in the absence of ligand (Fig. 11) (Zhao et al., 2003). The mutated receptor also interacts with OHT#33 in the presence of tamoxifen (Zhao et al., 2003). All mutants, with the exception of the isoelucine substitution, show increased reactivity with this probe in the presence of ICI-182,780. This means the ICI-182,780-bound, mutated receptor, resembles the tamoxifen-bound, wide-type receptor, in the part of the protein that is recognized by this probe (Zhao et al., 2003).

Thus, mutations of leucine-536 increase the interaction of the raloxifene-bound and ICI-182,780-bound receptor with probes that recognize the tamoxifen-bound conformation of the protein.

What do all these results mean? They show that leucine-536 is critical for coupling the binding of agonist and antagonist ligands to the conformation of the receptor. In other words, this residue is a tie rod (Zhao et al., 2003). (A tie rod is part of a car’s steering linkage, which connects the steering wheel with the driving wheels.) These results also show that leucine-536 essentially “reads” the side chain of tamoxifen, raloxifene, and ICI-182,780, and so distinguishes the conformations of these SERM-bound or antiestrogen-bound ligand-binding domains.

Acknowledgements

We thank the members of our laboratories who have contributed so much over the years. We also thank the agencies who have provided support for our research over the years, including the National Institutes of Health, the National Science Foundation, and the Department of Defense Breast Cancer Research Program, and internal funds from the Karmanos Cancer Institute, the Environmental Health Sciences Center, and the Wayne State University School of Medicine.

References