Mutations Targeted to a Predicted Helix in the Extreme Carboxyl-terminal Region of the Human Estrogen Receptor-α Alter Its Response to Estradiol and 4-Hydroxytamoxifen

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The human estrogen receptor-α, a member of the nuclear receptor superfamily, is a ligand-regulated transcriptional modulator. Because comparatively little is known about the extreme carboxyl-terminal region of the estrogen receptor (F domain), we used secondary structure prediction to design mutations that delete the F domain (S554stop), disrupt a possible turn (G556L/G557L), and alter a predicted helix (S559A/E562A, Q565P), and we evaluated the effects of these mutations on hormone binding and transcription activation in response to estradiol and the mixed agonist/antagonist 4-hydroxytamoxifen. Mutations that deleted the F domain (S554stop) or targeted the predicted helix (S559A/E562A, Q565P) greatly reduced or eliminated the agonist activity of 4-hydroxytamoxifen. Deleting the F domain increased the affinity of the receptor for estradiol and decreased the antagonist activity of 4-hydroxytamoxifen. The Q565P mutant exhibited a non-cooperative hormone-binding mechanism, as well as an impaired response to estradiol and increased antagonist activity of 4-hydroxytamoxifen. Our results show that mutations in the F domain alter not only the response to estradiol, the affinity for hormone, and the interaction between receptor subunits but can uncouple the agonist and antagonist activities of 4-hydroxytamoxifen. These results suggest that the F domain modulates the activity of the estrogen receptor-α by multiple mechanisms.

The members of the nuclear receptor superfamily act as transcriptional regulatory factors and exhibit a multidomain structure characterized as domains A-E/F (1, 2). The F domain, which is present only in certain members of this large superfamily, is located at the extreme COOH terminus of the receptor distal to the larger ligand binding domain (LBD)† or domain E (Fig. 1). Among the nuclear receptors for which this region is present, substantial variability exists in both the length of the F domain, from 19 to more than 80 amino acids long, and its sequence. For example, the F domains in the α- and β-forms of the human estrogen receptor (hER) exhibit no significant sequence homology and are suggested to be in part responsible for the differences in the biological activity of these forms (3). Although structural information is available for the DNA-binding domains and the ligand-binding domains of the hERα, no structural information is yet available for the F domain.

Residues in this domain are not required for ligand binding or transcriptional activation and have no independent activity attributed to them (4). Indeed, in some cases, deletion of the F domain is reported to enhance receptor activity (3–5). Not only is the AF ERα mutant an effective transactivator in response to E2, it is also similar to the wt protein in its ability to induce distortions in DNA and directed bend angles (6), as well as its half-life (7). However, Wrenn and Katzenellenbogen (8) have shown that although the S554 frame-shift ER, which contains 35 codons not present in the F domain of the wt ERα, is a potent mediator of E2-stimulated transactivation in yeast, its activity in Chinese hamster ovary cells is markedly impaired despite the demonstration of an almost normal E2 binding affinity in both cell types. Ince et al. (9) showed that when the same mutant is coexpressed with the wt receptor, transactivation is suppressed. Thus, although deleting the F domain has little effect on the response of the hERα to E2, certain mutations of the F domain can impair responses to E2.

In contrast to the minimal effect of deleting the F domain of the hERα on its response to E2, deleting this domain eliminates the ability of tamoxifen to act as an agonist (10). Furthermore, by using a yeast fusion protein assay, Nichols et al. (11) have shown that the F domain is involved in the antagonist activity of tamoxifen as well. Thus, the presence of the F domain is a key determinant of the ability of the hERα to respond to tamoxifen.

Because the carboxyl terminus of the E2-bound hERα LBD is located at the dimerization interface between ER monomers (12, 13), it is likely that residues in the F domain influence dimerization (5). In addition, the ER is an allosteric protein that binds E2 with high positive cooperativity, which indicates that information is transferred efficiently between subunits of the homodimer (14). Because of the position of the F domain relative to the dimerization interface, it is likely to be involved in subunit-subunit interactions and the transfer of ligand-binding information between subunits. Finally, because helix 12 is reoriented in the 4-hydroxytamoxifen- (4-OHT-) and raloxifene-bound ER LBDs, it is likely that the F domain will also become reoriented and through this reorientation play a role in the response of the receptor to these ligands (12, 13).
The poor conservation in sequence and length combined with the relative lack of structural and functional information make the F domain one of the least well understood segments in the multidomain structure of nuclear receptors. Our aim is to understand better the contribution made by residues in the F domain of hERα to the changes in conformation and transcriptional activation induced by the binding of different ligands.

We have examined the F domain of hERα by performing secondary structure prediction analyses and by constructing mutants designed to perturb selected predicted elements of structure. We have tested the effects of these mutations on hormone binding and on the response to E2, 4-OHT, and the combination of E2 and 4-OHT in transient transfection assays. We have also used energy minimization to construct a model for the F domain. Our results show that specific mutations in the F domain alter the response to E2, the agonist and antagonist activities of 4-OHT, the affinity of the ER for E2, and the subunit-subunit interactions of the ER. They also show that the effects of mutations on these activities are separable.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Reagents—**All cell culture reagents were purchased from Invitrogen with the exception of [3H]cloromphenicol (PerkinElmer Life Sciences), acetyl coenzyme A lithium salt (Amersham Biosciences), 17β-estradiol (E2), and 4-hydroxytamoxifen (4-OHT) which were obtained from Sigma. HeLa (human cervical carcinoma) cells, obtained from the ATCC (Manassas, VA), were routinely maintained in a 5% CO2 incubator using DMEM/F-12 media (Dulbecco's modified Eagle's F-12) supplemented with 10% fetal bovine serum (FBS) (Invitrogen) plus 0.5% gentamicin antibiotic (Sigma), and were subcultured weekly. SF9 insect cells, Bac-N-Blue transfection kit, and the Hill coefficient were obtained by non-linear regression analysis of data generated. The F domain alter the response to E2, the agonist and antagonist activities of 4-OHT, the affinity of the ER for E2, and the subunit-subunit interactions of the ER. They also show that the effects of mutations on these activities are separable.

**Expression of the ER in SF9 Cells—**The cDNA encoding the wt and mutant ERs was excised from their respective pSG5 vectors by digestion with EcoRI and MspI and ligated into the pBlueBac2B vector using standard molecular biological techniques to generate transfer plasmids for generation of recombinant baculovirus. The correct insertions were confirmed by DNA sequencing. The transfer plasmids containing the wild-type and mutant ERs were cotransfected with the linear viral DNA AcMNPV by cationic liposome-mediated transfection using procedures suggested by the manufacturer (Invitrogen). Identification of recombinant virus and generation of a high titer virus stock were carried out using procedures suggested by the manufacturer (Invitrogen). The recombinant wt and mutant ER proteins were expressed by infecting SF9 cells (106 cells/ml) with the corresponding high titer virus stocks for 48 h. After expression, the cells were washed with phosphate-buffered saline and pelleted in 50-ml aliquots. The pellets were stored at -80°C.

**Preparation of the wt and Mutant ERs from SF9 Cells—**An SF9 cell pellet was ultracentrifuged to remove supernatant. The cell pellet was resuspended in 1 ml of an expression plasmid for wt or mutant ER was lysed with a Wheaton Potter-Elvehjem tissue grinder in buffer consisting of 40 mM Tris, 5 mM dithiothreitol, 0.1 mM EDTA, 0.3 M NaCl, 15% glycerol, pH 7.5 (TEDG + salt buffer), containing 20 mg/ml ovalbumin and final concentrations of 0.4 mM 4-(2-aminoethyl)benzenesulfonfluryl fluoride, 47 μg/ml leupeptin, 1 μg/ml pepstatin, and 2 μM E64. The cell extracts were clarified by centrifugation at 220,000 × g for 30 min at 4°C (19). The recombinant wt and mutant ER proteins were assayed for CAT activity as described previously (17). Cell extracts were prepared as described above. Aliquots (200 μl) of the cell extracts containing wild-type or mutant ERs were incubated with increasing concentrations of [3H]estradiol from 0.5 to 40 nM for 2–4 h at 25°C to reach equilibrium. The non-specific binding was determined in a parallel set of incubations containing a 200-fold molar excess of unlabeled estradiol. Free and bound steroid were separated by dextran/charcoal assay. Preliminary experiments were conducted to determine the time necessary for each mutant to achieve equilibrium. The affinity of the receptor for estradiol and the Hill coefficient were obtained by non-linear regression analysis using the programs Lotus 1–2–3 and GraphPad Prism to fit the binding data to the Hill equation (20). The data were also graphed according to the method of Scatchard (21). Experiments in which receptor inactivation was greater than 10% were discarded; for the wt, S554stop, and the subunit-subunit interactions of the ER. They also show that the effects of mutations on these activities are separable.

**Molecular Modeling—**Molecular modeling was carried out using Biopolymer and Discover within InsightII 98 (Molecular Simulations, Inc.) using a Silicon Graphics O2 work station and the consistent valence force field. The F domain, residues 551–595 of the hERα (624 atoms), was constructed based on the secondary structure predictions shown in Fig. 1. (GOR IV); hydrogens were added (pH 7.0), and the

**Plasmids (DNA Constructs)—**The pERE-BLCAT reporter (4) contains an estrogen-response element (ERE) derived from the vitellogenin A2 promoter upstream of sequences from the herpes simplex viral thymidine kinase gene linked to the coding region of the bacterial gene for chloramphenicol resistance. The transcription vector containing the wild-type (wt) hERα was the generous gift of Drs. Pierre Chambon and Hinrich Groneneymer (15). Mutants of hERα were constructed as described below.

**Site-directed Mutagenesis of the hERα—**Oligonucleotide-directed mutagenesis was performed following procedures suggested by the manufacturer (Promega). A 786-bp fragment containing the cDNA correspondence to the amino terminus of the ER, including the juncture between domains E and F, was subcloned from HEG0-pSG5 into the mutagenesis vector pALTER1 (Promega) by digesting with EcoRI and HindIII endonucleases. Three oligonucleotide primers in which one or two amino acids were altered as designed were obtained from Operon (Alameda, CA). The mutations and the corresponding oligonucleotides are as follows: 1) 5′GG555, 5′-CAAGTGGCTGTCCTGCCCCTTC-3′; 2) 5′-TTGTCGCTGGCTCGCCCCCTCC-3′; 3) 5′-CTCCTACGAGATGCAATACCGCTAGTGGGCCC-3′; and 3) G556L/G557L, 5′-CTCCTACGAGATGCAATACCGCTAGTGGGCCC-3′. The F domain deletion mutant (S554stop) was constructed by altering residue Ser-554 of the F domain to a stop codon, using the oligonucleotide 5′-GGATCCCCTACGTCGATTTCTGGG-3′. The oligonucleotide primers were annealed into the hERα-pALTER1 single-stranded DNA along with two antibiotic resistance-altering primers (the tetracycline knockout primer and the ampicillin repair primer, provided by Promega) in the present of T4 DNA ligase.

**Transient Cotransfections and CAT Assays—**For transient transfection experiments, HeLa cells were plated at 2 × 104 cells/60-mm dish in DMEM/F-12 growth media supplemented with 5% dextran-coated charcoal-stripped FBS and were allowed to recover for 24 h. Transfection experiments were carried out using the Superfect reagent (Qiagen, Valencia, CA), according to the manufacturer’s instructions. Each 60-mm plate received 2.0 μg of the CAT reporter plasmid (pERE-BLCAT) along with 0.5 μg of an expression plasmid for wt or mutant hERα. The vitellogenin ERE-containing reporter was chosen for the cotransfection to high level of expression and for the fact that it confers to transiently transfected HeLa cells. The transfected cells were incubated overnight and then refed with fresh media (DMEM/F-12 plus 5% dextran-coated charcoal-stripped FBS) that had been supplemented with either E2 or 4-OHT at the indicated concentrations or with ethanol vehicle (0.01%). In experiments designed to test the ability of 4-OHT to antagonize E2-induced stimulation, cotreatment assays were performed using the indicated concentration of E2, in the presence of a single concentration of 4-OHT, 10−7 M. The transfected cells were exposed to hormone- or vehicle-supplemented media for 24 h before harvesting. In each experiment, the abilities of both the mutant and the wt ER to activate the reporter plasmid were measured in parallel, in the same assay, using duplicate cell culture dishes for each experimental condition. The samples were normalized for protein concentration (16) and assayed for CAT activity as described previously (17, 18).

Data quantitation was performed using AMBISS Imaging Acquisition Software (San Diego, CA). The percentage conversion of chloramphenicol to its acetylated products was determined for each sample and is expressed as the fold activity over control or as the percent of the wild-type ER activity induced by corresponding ligand concentrations. Data are the means of 2–7 independent experiments performed in duplicate.
Fig. 1. The domains of the human ERs, the sequence and 
predicted secondary structure of the F domain, and the mutants 
used in the present study. The domains of the full-length hERα 
(residues 1–595) are shown. The darkly shaded box indicates the 
F domain, residues 553–595. The amino acid sequence of the carboxy-
terminal region of the protein (residues 551–595), including the F 
domain, of hERα is shown below. Residues predicted to be an 
α-helix are in bold; residues predicted to be β-strand are underlined. 
Fig. 2. Energy-minimized model of residues 551–595 of the 
hERα. Minimization was carried out using Biopolymer and Discover as 
described under "Experimental Procedures." The backbone ribbon 
and side chains are shown in gray. The residues mutated in the current 
study are in black. The side chain of Arg-555 has been omitted for 
clarity. The dashed lines indicate the approximate positions of the 
predicted helix and a region of β-strand. Two additional residues near 
the carboxyl terminus of the protein are predicted to be β-strand. 
The remainder of the domain, including the region between the predicted 
helix and β-strand and the region following the β-strand, is predicted 
to be random coil.

RESULTS

Design of Mutations in the F Domain of hERα—In the ab-
ence of structural information, we used secondary structure 
prediction (GOR IV, 22) to target areas of the F domain for 
mutagenesis (Fig. 1). The major predicted elements of structure 
include an α-helix (residues 559–570, Fig. 1, shown in 
boldface) and a β-strand (residues 580–585, Fig. 1, underlined); 
in addition, the two glycines at 556 and 557, just up-
stream of the predicted helix, may be a region of high flexibility 
or a turn, and two residues in the extreme carboxyl-terminal 
region are also predicted to be β-strand (Fig. 1). The remainder 
of the domain is predicted to be random coil. Similar predictions 
were obtained using the methods of Chou-Fasman, 
Delange-Roux, and Levitt (Refs. 23–25 and results not shown).

Molecular modeling based on the secondary structure pre-
dictions of residues 551–595 of the hERα was also carried out. 
In the minimized model, the amino-terminal region contains a 
helical region, whereas the residues carboxyl-terminal to this 
form an extended region —60–70 Å long (Fig. 2). The Gly-556 
and Gly-557 occupy a turn between the end of the LBD and the 
predicted helix (Fig. 2). To test whether the initial assignment 
of secondary structure to residues could influence the mini-
mized model, a region that is not predicted to be α-helical was 
modeled beginning with a helical secondary structure and sub-
jected to energy minimization. This peptide did not remain 
helical but unfolded during the early rounds of minimization to 
a more extended structure (not shown). These results provide 
additional support for the existence of an α-helix in the amino-
terminal region in the F domain, as well as a more extended 
region that is potentially able to interact with other regions of 
the receptor and/or other proteins.

For the experimental studies, we targeted residues within 
and near the predicted α-helix for mutation, as well as con-
structing a receptor lacking the entire F domain (Fig. 1). The 
S554stop mutant was designed to eliminate the entire F 
domain. The G556L/G557L mutant was designed to disrupt the 
potential turn or to reduce the local flexibility of the region 
located on the amino-terminal side of the predicted helix. The 
S559A/E562A mutant was designed to alter the hydrogen 
bonding at the beginning of the putative α-helix, whereas the 
Q565P mutant was intended to disrupt the predicted α-helix.

Basal Activity of F Domain Mutants of hERα—We found that 
mutations to the F domain of ERα have widely differing effects 
on the levels of basal reporter activity in transiently trans-
fected HeLa cells. The basal activity of each of the ERα mu-
tants is expressed as a percentage of the basal CAT activity 
(arbitrarily set at 100%) induced by wt ER transfectants in 
ethanol-treated control cells, measured in parallel assays. The 
Q565P and ΔF ER mutants exhibited basal activity approxi-
ately 2–3-fold greater that of the wt ER (Table I). Basal 
activity of the S559A/E562A mutant was similar to that of the 
wt ER. By contrast, the G556L/G557L mutant ER retains a 
reduced level of basal activity, ~53% of the wt ER (Table I). 
Western immunoblotting showed that all mutants were ex-
pressed at levels similar to that of the wt protein (not shown).

Estradiol-stimulated Transcription by the F Domain 
Mutants—We measured the abilities of wt and mutant hERα 
to activate transcription from the ERE-driven reporter in the 
presence of increasing concentrations of E2, expressed relative 
to the level of activity in the absence of E2. In these experi-
ments, E2 increased reporter gene activity levels mediated by 
the wt ER in a dose-dependent manner; the maximum increase 
was 9.7-fold in response to 10⁻⁷ m. At 1 μM E2, a decrease in 
the level of transactivation was observed, so that the overall dose-
response curve was bell-shaped. All of the F domain mutants of 
ERα were able to increase transcription in response to stimu-
lation with E2; the extent of induction, however, varied among 
the ER transfectants (Fig. 3A).

The S554stop or ΔF hERα truncation mutant, in which the 
entire F domain is missing, exhibited a robust response to 
stimulation with E2. The E2 dose-response curve produced by 
the ΔF ERα transfectant is qualitatively and quantitatively 
similar to that of the wt receptor, with the exception that it 
retained activity at the highest E2 concentration used (1 μM), 
whereas the wt receptor exhibited decreased activity at this 
concentration (Fig. 3A). These results show that although de-
leting the F domain produces a receptor mutant that exhibits 
increased basal activity, the ΔF ERα retains a wt-like sensitivi-
ty to stimulation by E2. Our results also show that the pres-
ence of the F domain is dispensable for E2-stimulated gene 
expression by hERα. However, experiments that measure the 
effects of single and double point mutations of residues in the F
domain of ERα on E2-stimulated expression from the same CAT reporter provide additional insight into the role of this segment.

Transcription activation by the S559A/E562A mutant exhibited an interesting dose response. At the middle concentrations of E2 (1.0–10 nM E2), the activity of the mutant was similar to that of the wt protein; however, at the highest concentrations (0.1 and 1 μM), the mutant exhibited nearly three times the activity of the wt protein (Fig. 3A). Thus, this double mutation led to magnified E2-stimulated transactivation at the higher ligand concentrations.

The double point mutant G556L/G557L hERα was also tested for its responsiveness to stimulation by E2. Estradiol (1 nM) was a potent agonist of the G556L/G557L mutant hERα-driven transactivation response, stimulating a 16-fold increase in the level of reporter activity, roughly twice the activity of the wt protein at this same concentration (Fig. 3A). Transcriptional activation mediated by the G556L/G557L hERα at other E2 concentrations was similar to that of the wt protein. It is worth noting that the G556L/G557L hERα is the only F domain mutant that, like the wt hERα, exhibits a bell-shaped dose-response curve. It exhibited maximal activity at low/intermediate concentrations of E2 and decreasing activity at higher E2 concentrations.

An entirely different profile is observed for the Q565P mutant. Transient cotransfection assays used to evaluate the impact of this single amino acid change on transcriptional activity revealed a reduction (relative to that of wt hERα) in the response of the mutant to stimulation at each concentration of E2 used up to 10−6 M (Fig. 3A). Moreover, the maximum response of the Q565P ERα mutant to E2 is ~45% lower than that of the wt ERα at the same concentration (10−7 M E2) (Fig. 3A). The decreased response to E2 and the increased constitutive activity exhibited by this mutant underscore the importance of Gln-565 in mediating optimal E2-regulated responsiveness of the ERα.

F Domain Mutations Diminish the Capacity of hERα to Mediate Partial Agonism by 4-OHT—To examine the influence of F domain residues on the agonist activities of the selective estrogen receptor modulator 4-OHT, we tested the dose-dependent effects of 4-OHT on the activity of the same ERE-CAT reporter induced by wt and F domain mutants of ERα in transient cotransfection assays. As has been described previously (10) using other reporters incorporating a vitellogenin-derived promoter, our results show 4-OHT to be a weak agonist of the wt ERα-driven transactivation response. CAT expression levels in wt ERα-transfected cells increased by 1.7–1.9-fold in response to administration of 10−10 to 10−7 M 4-OHT and by 2.7-fold in response to 1 μM 4-OHT (Fig. 3B).

We, like others, found that deleting the F domain eliminated the ability of 4-OHT to stimulate transcription from a vitellogenin-derived promoter (Ref. 10; Fig. 3B). The S559A/E562A mutant has a substantially reduced response to 4-OHT; it is only able to stimulate transcription at the μM concentration of 4-OHT (Fig. 3B). The Q565P mutant did not respond to 4-OHT as an agonist at any of the concentrations tested. Moreover, at the highest concentration used, 10−6 M 4-OHT, repression of the basal transcriptional activity by the Q565P mutant was observed (Fig. 3B). Thus, mutations to residues within the putative α-helix of the F domain produce hERα mutants that have either a substantially reduced or complete loss of their ability to mediate 4-OHT agonism.

By contrast, in the experiments using the G556L/G557L mutant, the weak agonistic activity associated with 4-OHT is maintained over much of the concentration curve. Reporter activity levels initiated by the G556L/G557L mutant ERαs in response to 10−10 to 10−7 M 4-OHT are similar to those of the wt. However, at 1 μM 4-OHT, the agonistic activity is lost (Fig. 3B). Thus, unlike the substantial loss of 4-OHT agonism observed in the deletion mutant or the mutants having changes to residues in the predicted α-helix, this mutant, which targeted residues outside of the predicted α-helix, had little effect on the ability of 4-OHT to exert mild agonism.

In summary, these results show that the integrity of the F domain of hERα, and of the predicted α-helix within that domain, is required for 4-OHT to act as a weak agonist at an ERE-driven reporter. Moreover, a mutation that increased the response to E2, S559A/E562A, also substantially decreased the ability of 4-OHT to function as a partial agonist of ERα-driven transcriptional activation. This shows that the effects of these mutations on E2-stimulated and 4-OHT-stimulated transcription activation are separable.

4-OHT-mediated Repression of the E2-stimulated Transactivation Response Is Differentially Affected by Mutations in the F Domain of hERα—To understand better the role played by F domain residues in mediating the anti-estrogenic effects of 4-OHT, we performed coadministration assays with 4-OHT and E2. For these experiments, HeLa cells were transiently cotransfected with the pERE/BL-CAT reporter and expression vectors for the wt hERα or the F domain mutants as before, incubated either with ethanol vehicle alone (controls) or with increasing concentrations of E2 (10−10 to 10−6 M) in the presence of a single concentration of 4-OHT (10−7 M) for 24 h, and assayed for ERE-CAT reporter activity. By using this approach, we were able to measure the ability of E2 to overcome the inhibition by 4-OHT and assess the contribution of specific residues in the F domain of hERα to this process. The 10−7 M concentration of 4-OHT was chosen for its ability to repress wt transcriptional activation stimulated by administration of up to 100 nM E2 (data not shown). In addition, the S559A/E562A and Q565P mutants were transcriptionally inactive in response to 10−7 M 4-OHT alone; the G556L/G557L mutant and the wt exhibited some activity (Fig. 3B). Note that no concentration of 4-OHT alone lacked agonist activity for all proteins tested (Fig. 3B).

For the wt protein and all mutants, increasing E2 concentrations in the presence of 4-OHT led to at least some degree of increased transcription (Fig. 3C). There are notable differences, however, in the responses of the individual proteins. Coadministration of 4-OHT and E2 to the S559A/E562A mutants led to a dose-dependent increase in reporter activity levels, greatly exceeding those produced by the wt hERα at the highest ligand concentrations (Fig. 3C). A more complex response is exhibited by the G556L/G557L mutant ERαs. At E2 concentrations from 10−10 to 10−7 M, transcription increases in a manner similar to that of the wt protein (Fig. 3C). However, at 10−6 M E2, transcription was substantially reduced compared with the wt protein (Fig. 3C). Finally, although transcription by the Q565P ERα mutant was somewhat stimulated by increasing concentrations of E2, it never reached the level produced by the wt protein (Fig. 3C).
We also compared the activity of each protein in the presence of 4-OHT and E2 with the activity in the presence of E2 alone to determine the degree to which the presence of 4-OHT inhibits (or in some cases stimulates) E2-driven transcription (Table II). The addition of 4-OHT led to significant reductions in the reporter activity levels induced by cotreatment of wt ERα/H9251.

**Fig. 3.** Transcription activation properties of F domain mutants of the ERα. The pEREBLCAT reporter was transiently cotransfected into HeLa cells along with the pSG5-HEGO expression vector encoding the wt or mutated hERα, exposed to ethanol vehicle control (nh), E2 (A), 4-OHT (B), or 10^{-7} M 4-OHT in combination with the indicated concentration of E2 (C). The resulting CAT activity was measured as described under “Experimental Procedures.” The results have been calculated by dividing the activity of the reporter induced by the wt or mutant ER at each concentration of E2 by the activity of the reporter induced by the corresponding receptor in the absence of ligand and are expressed as “fold over control”; this allows us to specifically examine ligand-stimulated activity of the mutant and wt receptors. The y axis in A is divided at 30-fold stimulation. The results shown are the means ± S.E. of 3–7 independent experiments, each carried out in duplicate. wt, black; S554stop, light blue; S559A/E562A, red; G556L/G557L, green; Q565P, dark blue.
transfectants with \(10^{-10}\) to \(10^{-7}\) E2, but was slightly stimulatory in the presence of \(10^{-6}\) M E2 (Table II). Similarly, 4-OHT-mediated antagonism was ameliorated by increasing concentrations of E2 in the S554stop, G556L/G557L, and S559A/E562A mutants (Table II). Indeed, the presence of 4-OHT with 10\(^{-7}\) M E2 was slightly stimulatory to not only the wt but the S554stop and G556L/G557L mutants as well (Table II). By contrast, for the S559A/E562A mutant, even though \(10^{-6}\) M E2 stimulated transcription greatly in the presence of 4-OHT, transcription was still reduced 24% compared with E2 alone (Table II), indicating that the repressive effect of 4-OHT on the activity of this mutant was not completely overcome. Finally, transcription by the Q565P mutant was strongly inhibited by 4-OHT at all concentrations of E2 used; even at \(10^{-6}\) M E2, transcription was still inhibited by 65% (Table II).

By using the data in Table II, we then calculated the concentration of E2 that would overcome 50% of the inhibition produced by \(10^{-7}\) M E2 (Table III). For example, the presence of 4-OHT reduced transcription activation by the wt protein by 66% at an E2 concentration of \(10^{-7}\) M but stimulated transcription by 22% at 1 \(\mu\)M E2 (Fig. 2, A and C; Table II); we calculate 50% of the inhibition would be overcome at an E2 concentration of 0.16 \(\mu\)M (Table III). The S554stop mutant required less E2 to overcome 4-OHT inhibition than the wt protein, 0.008 \(\mu\)M (Table III). The G556L/G557L mutant exhibited similar sensitivity to E2 as the wt protein (Table III). The S559A/E562A mutant required slightly more E2, 0.32 \(\mu\)M, to overcome 50% of the inhibition by 4-OHT (Table III). The Q565P mutant was the least responsive to E2, requiring 320 \(\mu\)M E2 to overcome 50% of the inhibition by 4-OHT (Table III). Clearly, the ability of E2 to overcome inhibition by 4-OHT can be increased (S554stop) or decreased (Q565P) by mutations in the F domain.

**Effect of F Domain Mutations on E2 Binding by the ERα**—We next investigated the effects of mutations in the F domain on the affinity of the ER for estradiol, as well as the cooperativity of the interaction. The cooperativity of binding is measured by the Hill coefficient, \(n_H\) (20). A Hill coefficient near 1 is characteristic of a non-cooperative binding mechanism, that is either the protein is a monomer or binding of ligand to one subunit does not influence the binding of ligand to another subunit (26). A Hill coefficient greater than 1 indicates not only that there is more than one subunit but that binding of ligand to one subunit favors the binding of ligand to the other subunit(s) (26). Because the F domain has been implicated in receptor dimerization and could influence interactions between monomers of the receptor homodimer, we were especially interested in the effect of mutations on the cooperativity of binding. The S554stop (ΔF) mutant ERα has an increased affinity for E2 relative to that of the wt receptor, 0.05 ± 0.007 \(\mu\)M compared with 0.64 ± 0.41 \(\mu\)M (Table IV; Fig. 4). Deleting the F domain of ERα had no detectable effect on the cooperativity of hormone binding, as the Hill coefficients for the binding of \[^3\text{H}\]estradiol to the wt and the S554stop mutant were ~1.6, and the Scatchard plots were curved (Table IV; Fig. 4). By contrast, the affinity of the Q565P ERα mutant for E2 was similar to that of the wt protein, 0.23 ± 0.01 \(\mu\)M versus 0.64 ± 0.41 \(\mu\)M, yet the cooperativity of E2 binding as measured by the Hill coefficient was quite different, 0.94 ± 0.1 versus 1.58 ± 0.18 (Table IV); the Scatchard plot of this mutant was linear (Fig. 4). Thus, this point mutation eliminated the positive cooperative E2-binding mechanism of the ERα (Fig. 4 and Table IV). These results show that although the subunit-subunit interactions necessary for the positive cooperativity of E2 binding do not require the presence of the F domain, a point mutation within a predicted α-helix in the F domain can interfere with them.

**DISCUSSION**

We, like others, have shown that deleting the extreme carboxyl-terminal region of hERα (F domain) did not reduce E2-stimulated transcriptional activation, yet eliminated the agonist activity of 4-OHT (3–5, 10) (Fig. 3). Moreover, we have shown that deleting the F domain actually increased the affinity of the hERα for E2 (Fig. 4). We also investigated the effects of mutations targeted to a predicted α-helix within the F domain. The Q565P mutation, which was designed to distort the predicted α-helix, decreased the response of the hERα to E2 (Fig. 3A) and eliminated the cooperativity of E2 binding (Fig. 4; Table IV). Although many mutations have been reported that affect the transcription function of the hERα and its affinity for E2, this is the first report of a mutant that exhibits a non-cooperative hormone-binding mechanism. By combining the binding and transcription data, we assessed whether the S554stop and Q565P mutations alter the preference of the ER for the agonist-bound versus the 4-OHT-bound conformation. The S554stop mutant exhibited an increased affinity for E2 and required less E2 to override 4-OHT inhibition than the wt protein. This suggests that the S554stop mutant preferentially adopts the agonist-bound, rather than the 4-OHT-bound, conformation. By contrast, the Q565P mutant exhibited non-coop-
erative E₂ binding, a reduced response to E₂ in transient transfection assays, and required substantially more E₂ than the wt to override the inhibition by 4-OHT. This suggests that the Q565P mutant has a reduced ability to adopt the fully active, agonist-bound conformation and that it preferentially adopts a 4-OHT-bound conformation.

The G556L/G557L and S559A/E562A mutants were designed to have more subtle effects on ER function than the S554stop and Q565P mutants and display more complex phenotypes. Gly-556 and Gly-557 at the start of the F domain (Fig. 1) occupy a predicted turn/coil region that could act as a flexible linker between the ER LBD and the rest of the F domain. Mutation of the glycines to alanine was predicted to disrupt the potential turn and/or reduce the local flexibility of this region. Because the mutated glycines are part of a consensus sequence for arginine methylases, RGG (27, 28), we also cannot exclude the possibility that this mutation alters post-translational modification of hERα. In transactivation assays, impaired function of this mutant relative to the wt was observed only at the highest concentration of each ligand, 10⁻⁶ m (Fig. 3, A–C). Thus, mutating these residues, which lie outside the predicted α-helix, had a relatively minor effect on transactivation in response to E₂ and 4-OHT.

The S559A/E562A mutant was designed to test whether hydrogen bonding by Ser-559 and Glu-562 at the start of the predicted α-helix is important to ER function. The side chains of these residues could hydrogen-bond with the peptide backbone and stabilize the start of the predicted helix (Fig. 2); mutation to alanine would then destabilize, but not eliminate, helix formation (29–31). The sequence SVEE is also a casein kinase II consensus sequence (28); phosphorylation of Ser-559 could enhance helical stability (32–34). In transactivation assays, mutating these residues did not blunt the response to E₂; indeed, at the highest concentrations, the response of the S559A/E562A mutant to E₂ was nearly three times the response of the wt protein. However, this mutant responded to 4-OHT as an agonist only at the highest concentration, 10⁻⁶ m (Fig. 3B). Also, although E₂ could stimulate transcription in the presence of 4-OHT, inhibition was not entirely overridden (Table II) (3). Thus, this mutant exhibited a strong response to E₂, impaired agonist activity of 4-OHT, but a slightly increased antagonist activity of 4-OHT. This suggests that the predicted helical region is necessary for the agonist activity of 4-OHT and that mutation of this region enhances the antagonist activity of 4-OHT.

The F domain has been proposed to inhibit receptor dimerization (5). The effects of the S554stop and Q565P mutations on hormone binding are consistent with altered dimerization and/or subunit–subunit interactions. However, if the role of the F domain were solely to inhibit dimerization, one would predict that if the activity in response to one ligand were increased because of increased dimerization, then the constitutive activity and the activity in response to other ligands would also be increased. Our results show that a mutation (S559A/E562A) can increase the response to E₂ while eliminating the agonist activity of 4-OHT. Thus, the agonist activity of E₂ can be uncoupled from the agonist activity of 4-OHT. In addition, two other mutants have lost the agonist activity of 4-OHT, yet the antagonist activity of 4-OHT has been reduced in one (S554stop) and increased in the other (Q565P) (Table III). Therefore, mutations targeting the F domain can uncouple the agonist and antagonist activities of 4-OHT as well. It would appear that the F domain has other functions in addition to modulating dimerization.

The results of digestion of the ER by proteases are consistent with our modeling studies. The region of the F domain carboxy-terminal to the predicted α-helix is highly sensitive to proteolysis (35). The core of the ER in tryptic digests extends to residue 571, corresponding with the carboxyl-terminal end of the predicted helix, although other enzymes can cut within the predicted α-helix in limit digests (35). It is tempting to speculate that the vulnerability of the F domain to proteolytic attack, and the subsequent alterations in the response of the hERα to E₂ and 4-OHT, may play a role in breast cancer progression and response to anti-hormone therapy.

Overall, our results provide strong support for the idea that the F domain of the hERα contains an α-helix, “helix 13.” They also show that mutations of this region alter not only the response to E₂, the affinity for hormone, and the interaction between receptor subunits, but they can uncouple the agonist and antagonist activities of 4-OHT as well. These results suggest that the F domain modulates the activity of the estrogen receptor-α through multiple mechanisms.

| Table IV |
The binding of [³H]estradiol to the wild type, S554stop (ΔF), and Q565P mutant hERα

| Receptor | Affinity, Kₐ | Hill coefficient, nH |
|----------|--------------|---------------------|
| wt hERα  | 0.64 ± 0.41  | 1.58 ± 0.18         |
| S554stop (ΔF) | 0.05 ± 0.007 | 1.65 ± 0.36         |
| Q565P    | 0.23 ± 0.01  | 0.94 ± 0.1          |

**Fig. 4. 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 Sf9 insect cell extracts containing baculovirus-expressed receptor as described under “Experimental Procedures.” These data are representative of two independent experiments, each carried out in duplicate. Receptor inactivation was less than 10% in each experiment. Left, non-transformed saturation binding data used to calculate the affinity and Hill coefficient; the lines are the best fit by non-linear regression to the Hill equation. The x axis is divided at 1.5 nM E₂ to show better the data at the lowest [³H]E₂ concentrations. Right, Scatchard plot of the same data.
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