Different positioning of the ligand-binding domain helix 12 and the F domain of the estrogen receptor accounts for functional differences between agonists and antagonists

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The estrogen receptor is capable of binding a diverse set of ligands that are broadly categorized as agonists or antagonists, depending on their abilities to induce or interfere with transcriptional responsiveness. We show, using a fusion protein assay for ligand-binding which does not rely on transcriptional responsiveness, that agonists and antagonists differently position the C-terminus of the ligand-binding domain (helix 12) and the F domain. Upon antagonist binding, the F domain interferes with the fusion protein activity. Mutational disruption of helix 12 alters the position of the F domain, imposing interference after agonist or antagonist binding. Genetically selected inversion mutations where only agonists, but not antagonists, induce interference are similarly reliant on helix 12 and F domain positioning. Our results demonstrate that agonists and antagonists differently position helix 12 and implicate the F domain in mechanisms of antagonist action.

Keywords: activation function AF-2/codon substitution mutagenesis/FLP recombinase fusion protein/steroid receptor

Introduction

Estrogens regulate target cell proliferation and gene transcription through a pathway of molecular events initiated by binding to the estrogen receptor (ER), a member of the steroid hormone receptor class of nuclear receptors (Gronemeyer and Laudet, 1995; Beato *et al.*, 1996). A diverse group of antagonistic ligands have been identified which interfere with ER activation (Dauvois and Parker, 1993; McDonnell *et al.*, 1995; Tonetti and Jordan, 1996). Prominent among these are the clinically relevant antagonists, 4-hydroxytamoxifen (Z-OHT), raloxifene and ICI 182,780. Biochemical and functional work on the mechanisms of antagonist action has led to the recognition that they induce different conformations of the 240 amino acid ER ligand-binding domain (domain E) than agonists and show a spectrum of activities, presumably a reflection of the multiple steps involved in the ER activation pathway and the diversity of responses elicited (see McDonnell *et al.*, 1995; Metzger *et al.*, 1995). However, the molecular basis of the differences between agonist and antagonist activities remains to be elucidated.

Steroid receptors are modular proteins possessing two highly conserved domains, termed the C and E domains (Gronemeyer and Laudet, 1995; Beato *et al.*, 1996). They are joined by an unstructured and variable D domain sequence (Gronemeyer and Laudet, 1995). The E domain binds ligand and can be fused onto certain other proteins to impose ligand dependency on protein activity (Picard, 1994). Here we implement our observation that steroid regulation can be imposed on the enzyme activity of a site specific recombinase, FLP, by expressing FLP/steroid receptor fusion proteins (FLP–LBDs) (Logie and Stewart, 1995; Nichols *et al.*, 1997). FLP–LBDs are inactive as recombinases in the absence of a cognate ligand and respond to both agonists and antagonists in a titratable manner (Nichols *et al.*, 1997). Thus FLP–LBDs faithfully reflect the initially repressed, unliganded state of steroid receptors, but do not discriminate between agonists and antagonists. Hence the differences between these two classes of ligands must occur after ligand-induced release from the initially repressed state (Nichols *et al.*, 1997; C.Logie, M.Nichols, K.Myles, J.W.Funder and A.F. Stewart, submitted). Consequently, FLP–LBDs present assays where ligand activities are measured directly, by site specific recombination, and the multiple steps and specificities involved in ligand-mediated transcriptional responses are circumvented. Here, an FLP–estrogen-binding domain fusion protein assay in yeast, where no transcriptional cofactor has been found which interacts with the steroid hormone receptor AF-2 function (Berry *et al.*, 1990), is used to address conformational differences induced by agonists and antagonists.

Results

An FLP–E/F estrogen-receptor fusion protein binds, but is not activated by, antagonists

The recombination substrate for chromosomal excision in yeast, shown in Figure 1A, expresses the URA3 selectable marker and a suppressor tRNA required for expression of the ADE2 gene. Recombination deletes these two genes and was assessed either by a change in colony color due to loss of ADE2 expression or, for quantification, by Southern blotting. FLP–ER fusion proteins were expressed from single-copy CEN plasmids by galactose induction where, up to 10 h, very similar amounts of the different FLP–ERs are produced and the kinetics of ligand induced recombination are linear (Nichols *et al.*, 1997; data not shown). As shown before, a FLP–ER fusion protein that included the ER D, E and F domains (FLP–D/E/F) responded to all ligands tested, whether agonist or antagonist (Nichols *et al.*, 1997). This demonstrates that both ligand classes release ER from its initially repressed state and that the known differences between their activities

Fig. 1. (A) Diagram of the FLP excision recombination strategy. Before recombination, the constitutively active ADH1 promoter expresses the URA3 selectable marker which lies between FLP recombination targets (shown as triangles). Also between the recombination targets is a SUP11 suppressor tRNA gene, transcribed in the opposite direction. After recombination, the URA3/SUP11 region is excised and lost. Activity of the endogenous Ade2 protein relies on SUP11 ochre suppression, so the yeast cells grow white before recombination and red afterward. The diagram also outlines the Southern strategy employed, where a 5.6 kb fragment is reduced by recombination to a 4.0 kb fragment. (B) Diagram of the FLP-ER expression plasmid displaying its functional elements. The gene for the FLP-ER fusion protein is transcribed from the GAL10 promoter on a single-copy centromeric plasmid in yeast. (C) Schematic representation of various forms of the estrogen receptor fused to FLP. In these constructs, the D domain starts with hER amino acid 251, the E domain with amino acid 304 and the F domain deletion omits amino acids 552–595. Positions of the helix 10 mutation (L508R) and the helix 12 mutation (L540P) near the C-terminal end of the E domain are also shown as asterisks. IC₅₀, the amount of cold ligand competitor needed to reduce 1 nM ³H-estradiol bound in the absence of cold steroid to 50% is shown for each of the FLP-ER forms and ligands. None of the mutations or deletions shown significantly affect binding by any of the ligands. E2 = estradiol, ZOHT = 4-hydroxytamoxifen, RAL = raloxifene, ICI = ICI 182,780.

are caused by differing specificities later in the pathway of transcriptional activation.

In contrast, the FLP-E/F protein, which has no D domain, was activated by agonists but not by antagonists (Figure 2A; Nichols *et al.*, 1997; data not shown). This was unexpected since all studies with nuclear receptors

show that the E domain is a modular entity that entirely encompasses the function of ligand-binding (Wurtz *et al.*, 1996). Consistent with previous studies, binding experiments using yeast extracts containing FLP-E/F and FLP-D/E/F proteins showed that both fusion proteins bound all agonists and antagonists with affinities close to those of the native estrogen receptor (Figure 1C; Nichols *et al.*, 1997). Therefore the lack of activation of FLP-E/F by antagonists was not due to a lack of binding.

Differences between agonist and antagonist activation are partly dependent on the F domain

Insight into the molecular basis underlying this difference between binding and activation was found from experiments which examined the role of the estrogen receptor F domain. In contrast to the FLP-E/F fusion protein, which was not activated at all by antagonists, the FLP-E fusion protein, derived from FLP-E/F by deletion of the F domain, was partially activated by Z-OHT (Figure 2A, compare FLP-E/F and FLP-E). Similarly, with FLP-D/E, the absence of the F domain also improved activation by Z-OHT (Figure 2B, compare FLP-D/E/F with FLP-D/E). These results demonstrate that the difference between antagonist binding and activation was due in part to interference by the F domain. This interference was increased by removal of the D domain, moving the E and F domains closer to FLP and its tetrameric reaction intermediate (Chen *et al.*, 1992). In contrast, the agonist bound conformation did not result in F domain interference.

Repositioning of helix 12 by mutation invokes F domain interference upon agonist binding

The X-ray crystal structures of the unliganded retinoid X receptor (RXR) and agonist-bound retinoic acid receptor (RAR) E domains led to a 'mouse trap' model of ligand-binding by nuclear receptors, which invokes a large repositioning of the C-terminus of the E domain, helix 12 (Bourguet *et al.*, 1995; Renaud *et al.*, 1995). It is important to note that so far, no X-ray structures of ligand-binding domains include the F domain. F domain interference upon antagonist binding could reflect different positioning of helix 12, and consequently the F domain. If so, then altering the position of helix 12 by mutation should invoke F domain interference upon agonist binding. This proved to be the case. Regardless of the inclusion of the 'spacer' D domain or not, mutational disruption of helix 12 by proline (L540P) impaired agonist activation [Figure 2A, FLP-E(L540P)/F and B, FLP-D/E(L540P)/F]. This impairment was due, in part, to F domain interference, since deletion of the F domain from either helix 12 mutant protein restored activation [Figure 2A, FLP-E(L540P) and B, FLP-D/E(L540P)].

Genetically selected activation mutants relied on selection pressures imposed by the positions of helix 12 and the F domain

In an independent series of experiments to determine why the FLP-E/F protein was resistant to activation by antagonists, we used the connection between recombination and ligand activation to select mutants that switched from agonist activation/antagonist resistance to antagonist activation/agonist resistance. The selection procedure is

Fig. 2. The activity of FLP-ER fusion recombinases is affected by the presence of the D and F domains, as well as helix 12 integrity. The figure shows Southern blots used to measure recombinase activity in ligand titration experiments with either estradiol (E2) from 10^{-6} to 10^{-10} M or 4-hydroxytamoxifen (Z-OHT) from 10^{-5} to 10^{-9} M. Control samples were cultured in glucose (gl, first lane) or galactose without ligands (-). (A) shows experiments with FLP-ERs without the D domain; with (FLP-E/F) or without (FLP-E) the F domain; or including the L540P mutation with [FLP-E(L540P)/F] or without [FLP-E(L540P)] the F domain. (B) shows the equivalent FLP-ERs which contain the D domain.

outlined in Figure 3A. FLP-E/F was heavily mutated between amino acids 506 and 527 by codon substitution mutagenesis (Glaser *et al.*, 1992; Cormack and Struhl, 1993). This region was chosen because it includes helix 11, which is important in agonist contacts in the RAR and thyroid hormone receptor (TR) crystal structures (Renaud *et al.*, 1995; Wagner *et al.*, 1995), and ER amino acid 521, the site of ligand selective ER mutants (Danielian *et al.*, 1993). FLP-E/F mutations that were resistant to

activation by estradiol were selected by culturing the mutant libraries in the presence of estradiol, while selecting for expression of the URA3 gene and hence against recombination. Survivors were then cultured in the presence of an antagonist (Z-OHT or raloxifene) without selection, to permit growth after recombination, and then spread on plates to identify single recombinants by a red colony color. Red colonies identify those mutations between amino acids 506 and 527 that are resistant to activation by estradiol but are activated by the antagonists Z-OHT or raloxifene. Candidate FLP-mutant E/F expression plasmids were isolated from red colonies and retested in the recombination reporter host (Figure 1A) by a colony color assay. Yeasts carrying the candidate expression plasmid and the unrecombined recombination reporter were spread as a lawn on a plate to which ER ligands were applied (Figure 3B). The diameter of the circle of red yeast, induced by diffusing ligand, directly reflects the relative activation of the recombinase fusion by that ligand (Figure 3B; data not shown). Here the plate assay shows the agonist-only activation phenotype of the parent FLP-E/F protein, and for one of the mutants isolated (FLP-E(508E)/F), it shows the inversion phenotype of resistance to agonists, activation by antagonists.

Unexpectedly, no helix 11 mutations were recovered in this screen, although 17 independent mutations of the region were identified (Table I). As a control, the known estradiol-resistant, antagonist-inducible G521R helix 11 mutation (Danielian *et al.*, 1993) was tested directly as a FLP-E(G521R)/F protein and was virtually inactive in this protocol (Figure 3C). We attribute this to the fact that the G521R mutation is both a loss of binding mutation for estradiol ($>10\,000$ -fold) and a loss of binding mutation for Z-OHT (~ 100 -fold, Figure 3C; data not shown) therefore making it too insensitive to ligands to be identified by this analysis. On the other hand, if the D domain is included [FLP-D/E(G521R)/F], the recombinase activity is induced by antagonist and not agonist, as expected (Figure 3C), and is consistent with a model of reduced interference with increased spacer sequence. Most of the mutations identified affected helix 10 by introducing charged or polar residues into a very hydrophobic part of the α -helix (Table I). None showed substantial differences in binding of either agonists or antagonists (Figure 1C; data not shown). These mutations therefore affected estradiol activation, not binding, and so were conceptually similar to the case of antagonists and FLP-E/F (Figure 2A). Consequently, we tested a representative helix 10 mutation to establish whether the F domain was also responsible for interfering with activation.

FLP-E(L508R)/F was partially activated by Z-OHT and not significantly by estradiol (Figure 4A) as expected from the mutagenesis and the selection protocol used. Activation by both agonists and antagonists was improved when the F domain was deleted [Figure 4A, FLP-E(L508R); data not shown]. Disordering helix 12 by mutation had a slight effect on estradiol activation in the presence of the F domain [Figure 4A, compare FLP-E(L508R)/F with FLP-E(L508R, L540P)/F]. However, the combination of the helix 12 mutation with the removal of the F domain fully restored estradiol activation [Figure 4A, FLP-E(L508R, L540P)]. Plate assay experiments, such as shown in Figure 3B, also confirm the results and

Fig. 3. (A) Isolation of mutations which show altered specificity of ligand induced recombination. In step 1, libraries of FLP-ER mutagenised between ER amino acids 506–527 were grown in the presence of estradiol to activate FLP-ER recombination and in the absence of uracil to select against recombination. Thereby FLP-ER mutants not activated by estradiol were enriched. In step 2, the surviving cells were collected and grown in the presence of a second ligand (Z-OHT or raloxifene) in the presence of uracil to permit recombination and then screened on plates for red colonies indicating recombination (Figure 1A). Plasmids containing candidate mutations were rescreened to verify estradiol resistance and antagonist inducibility. (B) Colony color plate assay to evaluate ligand responsiveness of FLP-ERs. The plasmids were retested in the parent strain to confirm differential inducibility by a set of ligands, as shown, in plate assays. E2 = estradiol, Z-OHT = 4-hydroxytamoxifen, RAL = raloxifene, DES = diethylstilbestrol, TAM = tamoxifen, HEX = hexestrol. FLP-E/F is activated only by agonists (E2, HEX, DES) and a representative helix 10 mutation [FLP-E(L508E)/F] is activated only by antagonists (Z-OHT, RAL, TAM). (C) The figure shows Southern blots used to measure recombinase activity in ligand titration experiments as in Figure 2. A comparison of the relative ligand activities for mutation LBDs from our screen and a previously identified mutation with agonist/antagonist reversed activity.

conclusions drawn from the Southern assays (data not shown). Since none of these mutations had any significant effect on agonist or antagonist binding, this demonstrates that the helix 10 mutations identified in the selection protocol imposed interference on estradiol activation by structurally repositioning helix 12 and the F domain.

Discussion

In previous work with FLP-steroid receptor fusion proteins, we observed that all ligands, whether agonists or antagonists, served to release the cognate FLP-fusion protein from its initially repressed condition. This is true

both in yeast (Nichols *et al.*, 1997) and mammalian cells (Logie and Stewart, 1995; C.Logie, M.Nichols, K.Myles, J.W.Funder and A.F.Stewart, submitted). Therefore, release from the initially repressed condition is concomitant with ligand-binding and any differences in activities between agonists and antagonists must lie downstream. However, we also observed that estrogen receptor fusion proteins that omitted the D domain FLP-E/Fs, were activated by agonists but not by antagonists, in spite of the fact that both ligand classes were bound with near wild type affinities (Figure 1; Nichols *et al.*, 1997; C.Logie, M.Nichols, K.Myles, J.W.Funder and A.F.Stewart, submitted). In this paper, we describe work to understand this

Table I. Mutations recovered by selection for agonist insensitivity/antagonist activation are shown underneath the amino acid sequence of the C-terminus of the ER ligand-binding domain, E

helix 10			helix 11			helix 12 (AF-2)		
510			520			530	540	550
HQRLAQ	LLILSHIRHMS	NKGM	EHLYSMKCK	NNV	PLYD	LLLEMLDA	HRLH	
E								
R								
K								
Q								
I	E							
R			L					
DR								
D			K					
VK								
H			K					
RD			Y					
RH	G							
SA	V							
VGS								
RA	T							
A	T	D						
VQ	A	C						
HQRLAQ	LLILSHIRHMS	NKGM	EHLYSMKCK	NNV	PLYD	LLLEMLDA	HRLH	
510			520			530	540	550

The positions of helices 10, 11 and 12 are indicated, based on the consensus alignment (Wurtz *et al.*, 1996) and the ER structure (Brzozowski *et al.*, 1997).

difference between binding and activation. We found that antagonist activation can be partially restored by removal of the F domain, arguing that antagonist binding positions the F domain so that it interferes with FLP recombination. Repositioning the F domain by mutating helix 12 resulted in F domain interference with agonist binding. Genetically selected mutations that inverted agonist activation/antagonist resistance to agonist resistance/antagonist activation were similarly dependent on the positioning of helix 12 and the F domain. Thus, three aspects important to the difference between ligand-binding and activation were identified — the presence of the F domain, the positioning of helix 12 and the proximity of domain E to FLP recombinase in the fusion protein.

Taken together, the data can only be simply explained by steric differences induced by binding of agonists or antagonists, or the mutations employed. In particular, we show that distancing domains E and F from FLP in the fusion protein, by including the D domain in between, dilutes interference in all cases. This discounts simple explanations that rely on intermolecular interactions with other components present in yeast and strongly favors intramolecular interactions within the fusion protein, reflecting steric differences in the estrogen receptor moiety of the fusion proteins. Figure 5 presents a simplified explanation of the steric differences observed. In the case of the wild-type estrogen receptor, agonist binding positions helix 12, and consequently the F domain, in an organized conformation for cofactor binding and transcription activation by AF-2. Mutating helix 12 (L540P) disorganizes the positioning of helix 12 and the F domain, causing interference. Interference is also caused by antagonists bound to the wild-type estrogen receptor, an effect that is partly relieved by deletion of the F domain. Helix 10 mutations (at 508) disorganize the positioning of helix

12 and the F domain upon agonist binding yet permit, in part, an organized positioning of helix 12 and the F domain upon antagonist binding, leading to activation (Figure 4A). Deletion of the F domain relieves interference in all cases.

Repositioning helix 12 by either antagonist binding or mutation could cause it and the F domain to be mobile; thus, interference with FLP recombinase would be explained by dynamic steric clashes. Alternatively, interference could be caused by static, inappropriate positioning of helix 12 and the F domain. In either case, we favor a model based on an organized positioning of helix 12 and the F domain by agonist binding. Previous work on ligand-binding by nuclear receptors has implicated the correct positioning of helix 12 in agonist action (Danielian *et al.*, 1992; Bourguet *et al.*, 1995; Mahfoudi *et al.*, 1995; Renaud *et al.*, 1995; Heery *et al.*, 1997). Our work extends these implications to demonstrate that the different positions of helix 12 adopted upon agonist or antagonist binding can have a dominant effect in a functional assay. Our work also highlights a role for the F domain in the differences between agonist and antagonist action.

Inversion mutations of nuclear receptors reposition helix 12 and the F domain

Several studies based on transcriptional assays have identified mutations in ER, and other steroid receptors, that convert antagonists into agonists (Vegeto *et al.*, 1992; Lanz and Rusconi, 1994; Mahfoudi *et al.*, 1995; Montano *et al.*, 1995, 1996). All of these mutations occur near to, or in, helix 12 and can, in the context of the work presented here, be evaluated as conformational mutations that disorder helix 12 and reposition the F domain. In one study, the ER helix 12 mutation, L540Q, inverted transcriptional activity so that antagonists induced activity

Fig. 4. F domain interference causes agonist inactivity for helix 10 inversion mutations. The figure shows Southern blots used to measure recombinate activity in ligand titration experiments as in Figure 2. (A) shows experiments with the L508R inversion mutant without the D domain; with [FLP-E(L508R)/F] or without [FLP-E(L508R)] the F domain; or including the L540P mutation with [FLP-E(L508R,L540P)/F] or without [FLP-E(L508R,L540P)] the F domain. (B) shows the equivalent FLP-ERs which contain the D domain as additional controls for the ligand-binding properties of these mutations and to illustrate the proximity dependence of interference.

but estradiol did not. Deletion of the F domain from the L540Q mutant ER restored estradiol-induced, and diminished antagonist-induced, transcriptional activity (Montano *et al.*, 1996). This indicates that, by this mutation, the F domain is differently positioned upon agonist and antagonist binding in a transcriptional activity assay. Work on the glucocorticoid receptor (GR) mutation, I747T

which lies between helices 11 and 12, documents a complementary observation of a difference between near normal binding of agonists and impaired transcriptional activity due, we suggest, to altered helix 12 position and F domain interference (Roux *et al.*, 1996). In other work with GR, mutations in helix 12, combined with a deletion of two amino acids just C-terminal to helix 12, lead to

Fig. 5. Schematic diagram of various agonist and antagonist bound forms of the FLP-ER showing positioning of helix 12 and the F domain which alters interference with the FLP recombinase activity. Helix 10 is signified by the thick bar inside the ligand-binding domain (shaded region) and helix 12 and the F domain are depicted outside the shaded region as a thick bar and thin line, respectively.

potent activation by RU486 and none by dexamethasone in transcription (Lanz and Rusconi, 1994). We also suggest that different positioning of the F domain will differently affect cofactor binding. By analogy to the local steric effects on FLP recombination, we reason that a mispositioned helix 12 and F domain can interfere with local protein-protein interactions relevant to steroid receptor action. Of particular interest are interactions with steroid receptor transcriptional cofactors. Recent work has indicated that a variety of nuclear receptor cofactors exist (Horwitz *et al.*, 1996 and references within; Heery *et al.*, 1997). It may be possible that different agonists and antagonists, by inducing different helix 12 and F domain positions, differently influence cofactor interactions. By this means, selectivity among cofactor binding directed by different ligands may be achieved. We also note that the same considerations of local protein-protein interactions may also influence receptor homo- or heterodimerization. Further work is required to establish the degree to which these speculations describe the transcriptional activities of the steroid receptors.

The F domain length is conserved

The F domain, here defined as the amino acids following the consensus helix 12 of domain E, may not be as inert for steroid receptor function as previously thought. It has not been included in any X-ray structures so far, for technical reasons, and yet abuts helix 12, important for ligand activated AF-2 function (Danielian *et al.*, 1992; Heery *et al.*, 1997). An examination of F domains from all available steroid receptor sequences revealed a striking observation — namely, the lengths of the F domains have been largely conserved (Table II). ER α has the longest F domain, 50 amino acids in all known higher vertebrates. Fish ER α F domains differ somewhat in that they are

Table II. The F domain amino acid length, but not sequence, is conserved

Receptor	Species	Length (amino acids)
Estrogen α	Human	50
	Mouse	50
	Rat	50
	Chicken	50
	Pig	50
	Zebra finch	50
	<i>Xenopus</i>	49
	Japanese eel	48
	Rainbow trout	64
	Atlantic salmon	64
	Medaka fish	71
	<i>O. aureas</i> (fish)	84
Estrogen β	Human	34
	Mouse	34
	Rat	34
Progesterone	Human	19
	Mouse	19
	Chicken	19
	Rabbit	19
Androgen	Human	19
	Mouse	19
	Rat	19
	Rabbit	19
Mineralocorticoid	Human	19
	Rat	19
	Tree shrew	19
	<i>Xenopus</i>	19
Glucocorticoid	Human	19
	Mouse	19
	Rat	19
	<i>Xenopus</i>	19
	Guinea pig	19
	Rainbow trout	25

For this list, the 'F' domain is considered to be the number of amino acids following the last one of the ligand-binding domain helix 12, as defined by the consensus alignment in Wurtz *et al.* (1996).

usually even longer, up to 84 amino acids. All three known ER β have F domains of 34 amino acids in length. Remarkably, all other steroid receptors have F domains of 19 amino acids in length, with one exception (trout glucocorticoid receptor has 25). Since there is little sequence conservation, especially for the ER versions, the conservation of F domain lengths is very unlikely to be coincidental. We reason that there are functional constraints that bear upon the F domain and that one of these constraints, as identified here, is the organized positioning of the F domain upon agonist binding.

The crystal structures of the ER LBD confirm helix 12 (and F domain) positions

During review of this paper, the X-ray crystal structures of the estrogen receptor hormone binding domain bound by estradiol or raloxifene were published (Brzozowski *et al.*, 1997). The structures show a very large repositioning of the helix 12 by raloxifene, relative to the estradiol bound form. Hence our data confirms this aspect of the X-ray structures with functional data, and the X-ray structures confirm our basic conclusions. In addition, the

F domain position, though not included in the X-ray structures for technical reasons, would be vastly different when the ER LBD is bound by raloxifene instead of estradiol, as also inferred from the studies presented here. The helix 10 inversion mutations identified here occur along the dimerization interface, though the exact position of 508 and other helix 10 residues was not presented in the crystal structures (note: helix 10 and 11 are contiguous in the ER LBD and are simply referred to as helix 11 in the recent structure; Brzozowski *et al.*, 1997). It is plausible that the helix 10 mutations we identified impair or alter dimerization in such a way as to relax or alter the steric positioning of the ER LBD with respect to the FLP recombinase. However, secondary alterations to the overall conformation of the ER LBD, independent of effects on dimerization, could also explain, or contribute to, the inversion phenotype observed. Finally, it is worth noting that mutations that impair dimerization would also impair transactivation in a conventional transcription assay and would not distinguish between agonists and antagonists. The non-transcriptional basis of the assay employed here permitted a description of agonist- and antagonist-specific steric differences.

Materials and methods

Strains and chemicals

The *Saccharomyces cerevisiae* strain used for these experiments [*MATa leu2-3, 112 his3-11, 15 ura3-52 trp1-1:: (TRP1 URA3 SUP11) ade2-1ochre can1-100*] was derived from RS453 (R.Serrano, Valencia, Spain) by integrating the target of recombination (Figure 1A) at the *trp1* locus. Transformation of yeast by the standard lithium acetate method was performed as described (Ausubel *et al.*, 1995). Transformed yeasts were grown and maintained by selection for leucine and tryptophan in glucose- or galactose-supplemented synthetic media from BIO 101, Inc. The hormones and antihormones were purchased from Sigma, except 4-hydroxytamoxifen (Research Biochemicals International), and ICI 182,780 (a gift from Dr A.Wakeling, Zeneca Pharmaceuticals).

Southern assays and ligand titration experiments

Transformed yeasts, containing the GAL10 promoter, FLP-ER gene on pRS315 (Sikorski and Hieter, 1989), were grown in synthetic glucose medium lacking leucine and tryptophan to OD₆₀₀ = 1.5. Equal volumes of cultures were collected and resuspended in medium containing 2% galactose with or without ligands, which was dissolved in ethanol as a 1000- or 10 000-fold stock solution. The 'no hormone' samples received an equal volume of ethanol. Cells were collected at times noted and DNA was prepared by standard procedures using a zymolyase 20T (ICN) incubation, SDS lysis, followed by potassium acetate precipitation as described (Ausubel *et al.*, 1995). About 10 µg of DNA per lane was digested with *Pst*I and loaded onto 0.7% gels in 1× TAE buffer. Gels were treated with 0.25 M HCl for 10 min, 0.4 M NaOH for 2× 30 min, 20× SSC for 30 min, and then blotted to Qiagen nylon plus filters with 20× SSC. After baking the filter at 80°C for 2 h, they were probed at 72°C with a riboprobe, made from the 1.2 kb *Sca*I-*Bst*WI fragment of the *Escherichia coli* LacZ gene, in a buffer containing 250 mM sodium phosphate pH 7.2, 7% SDS and 1 mM EDTA. Washes were performed in 25 mM sodium phosphate pH 7.2, 1% SDS and 1 mM EDTA at 72°C. Recombination was calculated as a ratio of [counts in the recombined band/(counts in recombined + unrecombined bands)] and was therefore not affected by minor variations in the amount of DNA loaded.

Ligand-binding assay

Ligand-binding experiments were performed to measure estradiol, 4-hydroxytamoxifen, raloxifene and ICI 182,780 binding by the various FLP-ER fusion proteins. The protein extracts were made from FLP-ER transformed yeast, grown in galactose without hormones to OD₆₀₀ = 1. The resuspended yeast pellets were lysed using a glass bead procedure (Ausubel *et al.*, 1995) in a buffer containing 20 mM Tris pH 7.9, 10 mM MgCl₂, 1 mM EDTA, 5% glycerol, 1 mM DTT, 420 mM KCl and

protease inhibitors. The ligand-binding experiments were done in 300 µl volume with a fixed concentration (1 nM) of radiolabeled ³H-estradiol (84 Ci/mmol; Dupont NEN), which was pre-mixed in the respective tubes with zero or increasing amounts of unlabeled ligand (1–1000 nM). The binding was at 4°C for 16–18 h in buffer (PMMG) containing 8.5 mM Na₂HPO₄, 1.5 mM KH₂PO₄ pH 7.5, 10 mM sodium molybdate, 2 mM monothioglycerol, 20% glycerol and 1 mg/ml protein extract. After the binding incubation, unbound label was absorbed by adding 300 µl of DCC (0.5% charcoal Norit-A, 0.05% dextran T70) in PMMG buffer for 15 min at 4°C and then centrifuging at 12 000 r.p.m. for 5 min in a microfuge. Equal volumes of supernatant were quantified by liquid scintillation counting. Binding values (IC₅₀) are expressed as the amount of unlabeled ligand competitor needed to reduce to 50% the ³H-estradiol bound in the absence of unlabeled steroid.

Color plate assay

Yeast containing the integrated SUP11 recombination substrate (Figure 1A) were transformed by plasmids containing the various FLP-ER genes. Cultures were grown in glucose with leucine selection and then plated at high density on synthetic galactose plates lacking leucine and tryptophan. A 2 µl drop of ethanol containing each ligand was placed on the plate, as shown (Figure 3B). An adenine marker in yeast gives a color phenotype (red) if the target gene has been deleted by the recombinase. The plates were grown at 30°C for 4 days to maximize red color formation.

Generation of mutated FLP-ER libraries by codon substitution mutagenesis (CSM)

CSM was performed as described (Cormack and Struhl, 1993) on amino acids 506–527 of the human estrogen receptor.

Acknowledgements

We thank V.Adam for the production of CSM oligonucleotides, M.Meredith and A.P.Monaghan for comments and Dr A.Wakeling for providing ICI 182,780. This work was supported by funds from the USAMRMC Breast Cancer Research Program, Fort Detrick, MD, grants # DAMD17-94-J-4103 and DAMD17-94-J-4249 and the EU BIOMED 2 programme — PL 950181.

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Received October 7, 1997; revised November 12, 1997;
accepted November 14, 1997