Ligand-independent activation of the oestrogen receptor by mutation of a conserved tyrosine

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The oestrogen receptor is a member of the nuclear receptor family of transcription factors which, on binding the steroid hormone 17β-oestradiol, interacts with co-activator proteins and stimulates gene expression. Replacement of a single tyrosine in the hormone-binding domain generated activated forms of the receptor which stimulated transcription in the absence of hormone. This increased activation is related to a decrease in hydrophobicity and a reduction in size of the side chain of the amino acid with which the tyrosine is replaced. Ligand-independent, in common with ligand-dependent transcriptional activation, requires an amphipathic α-helix at the C-terminus of the ligand-binding domain which is essential for the interaction of the receptor with a number of potential co-activator proteins. In contrast to the wild-type protein, constitutively active receptors were able to bind both the receptor-interacting protein RIP-140 and the steroid receptor co-activator SRC-1 in a ligand-independent manner, although in the case of SRC-1 this was only evident when the receptors were pre-bound to DNA. We propose, therefore, that this tyrosine is required to maintain the receptor in a transcriptionally inactive state in the absence of hormone. Modification of this residue may generate a conformational change in the ligand-binding domain of the receptor to form an interacting surface which allows the recruitment of co-activators independent of hormone binding. This suggests that this tyrosine may be a target for a different signalling pathway which forms an alternative mechanism of activating oestrogen receptor-mediated transcription.

Keywords: co-activators/conserved tyrosine/ligand independent/oestrogen receptor

Introduction

The oestrogen receptor α (ER) is a member of a large family of nuclear hormone receptors which are characterized by a highly conserved DNA-binding domain and function as transcription factors in target cells (Parker, 1993; Beato et al., 1995; Mangelsdorf et al., 1995). The N-terminal region of the ER contains a transcriptional activation function termed AF1 while the C-terminal region is required for high affinity ligand binding and contains a ligand-inducible transactivation function termed AF2 (Lees et al., 1989; Tora et al., 1989). It has been proposed from structural studies of the ligand-binding domains of three different nuclear hormone receptors, the retinoic X receptor α (RXRα) (Bourguet et al., 1995), the retinoic acid receptor γ (RARγ) (Renaud et al., 1995) and the thyroid hormone receptor β (TRβ) (Wagner et al., 1995), that ligand binding results in an alteration in conformation of the ligand-binding domain to generate a novel surface required for the formation of AF2. This results in part from the reorientation of an α-helix, helix 12 (H12), across the ligand-binding pocket which is stabilized both by the ligand itself and by interactions between H12 and helices 3 and/or 4. The amphipathic character of the amino acids which form H12 is conserved in the majority of members of the nuclear hormone receptor superfamily (Danielian et al., 1992; Saatcioglu et al., 1993; Baretino et al., 1994; Durand et al., 1994) and this helix forms the AF2 activation helix, AF2 AH. The amino acids predicted to form H12 in the ER (Wurtz et al., 1996) are completely conserved in primary sequence between different species, and mutations in either the charged or hydrophobic amino acids result in receptor proteins which bind oestradiol but are impaired in ligand-dependent transcriptional activation (Danielian et al., 1992).

In addition to ligand binding, a number of the nuclear receptors may also be activated by different signalling pathways, including those stimulated by the neurotransmitter dopamine (Power et al., 1991; Smith et al., 1993), growth factors such as epidermal growth factor (EGF), transforming growth factor-α (TGF-α) and insulin-like growth factor 1 (IGF-1) (Ignar-Trowbridge et al., 1992, 1996; Aronica and Katzenellenbogen, 1993; Bunone et al., 1996), and by activators of protein kinase A (Aronica et al., 1994). The molecular mechanisms for such cross-coupling between nuclear receptors and other signalling pathways have yet to be elucidated but may be mediated in part by receptor phosphorylation. The ER has been demonstrated to be phosphorylated by the MAPK pathway in response to EGF, resulting in stimulation of AF1 (Kato et al., 1995; Bunone et al., 1996). Other studies have demonstrated that the ER (Auricchio et al., 1987; Arnold et al., 1995c; Pietras et al., 1995), TRβ (Lin et al., 1992), RARγ (Rochette-Egly et al., 1992), glucocorticoid receptor (Rao and Fox, 1987) and the orphan receptor HNF-4 (Ktistaki et al., 1995) are also targets for tyrosine phosphorylation. A specific tyrosine phosphorylation site has been identified within the AF2 domain of the human ER (hER) at amino acid 537 (Y537) (Castoria et al., 1993; Arnold et al., 1995a). This tyrosine is located immediately N-terminal of the AF2 AH sequence and is conserved in all known ER sequences from diverse species including the novel receptor ERβ (Kuiper et al., 1996; Mosselman et al., 1996).

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A number of proteins have been identified which interact with the ligand-binding domains of nuclear hormone receptors in the presence of ligand and have been proposed or demonstrated to act as co-activators in hormone-regulated gene transcription. These include RIP-140 and RIP-160 (Cavailles et al., 1994, 1995), ERAP-140 and ERAP-160 (Halachmi et al., 1994), TIF1 (Le Douarin et al., 1995), TRIP1/SUG-1 (Lee et al., 1995; vom Baur et al., 1996) TIF2 (Voegel et al., 1996) and a series of SRC-1 isoforms (Onate et al., 1995; Kamei et al., 1996). In addition, interactions have been demonstrated between SRC-1, nuclear hormone receptors and the co-activator CBP/p300 (Chakravarti et al., 1996; Kamei et al., 1996; Yao et al., 1996), and overexpression of CBP/p300 has been shown to potentiate ligand-dependent transcription (Chakravarti et al., 1996; Smith et al., 1996). The results of mutagenesis studies and structural predictions suggest that the interaction of these proteins with receptors may depend on the integrity of the AF2 AH (Danielian et al., 1992; Bourguet et al., 1995) and its realignment following ligand binding (Renaud et al., 1995; Wagner et al., 1995).

In view of the close proximity of the conserved tyrosine in the ERs relative to AF2 AH and its identification as a site of phosphorylation, we have investigated whether it may be required or involved in the modulation of AF2 activity in the ER. Point mutations have, therefore, been introduced at tyrosine 541 (Y541) in the mouse ER, and the resulting proteins have been analysed for their ability to interact with potential co-activator proteins in vitro in the presence and absence of DNA, and to transactivate in transient transfection studies when expressed in mammalian cells. In contrast to the wild-type protein, we find that some mutations at this position activate the receptor both to stimulate transcription and to interact with co-activator proteins in the absence of added ligand. We propose, therefore, that activation of the ER by modification of a tyrosine residue may generate a conformational change in the ligand-binding domain which facilitates the binding of specific co-activators required for the regulation of gene expression.

Results
Modification of a conserved tyrosine increases ligand-independent activity
The basic organization of functional domains within the ER is shown in Figure 1A together with the position of the conserved amphipathic α-helix AF2 AH. A comparison of AF2 AH sequences from ERα and ERβ from different species, including the position of the conserved tyrosine, is shown in Figure 1B. Initially Y541 in the mouse ER (mER), which corresponds to Y537 in the hER, was replaced by phenylalanine (Y541-F), an amino acid with a hydrophobic side chain similar in size and structure to tyrosine but which lacks a hydroxyl group and cannot be modified by phosphorylation. Mutants were also generated in which Y541 was altered to either the negatively charged amino acids aspartic acid (Y541-D) and glutamic acid (Y541-E) or to alanine (Y541-A). In transient transfection studies in HeLa cells, the wild-type receptor (MOR 1–599) stimulated transcription from an ERE-TK-luciferase reporter gene 3- to 4-fold and this was increased ~3-fold further in the presence of oestradiol (Figure 1C). The transcriptional activity of the mutant receptor Y541-F was similar to that of the wild-type receptor in the presence of oestradiol, although the activation observed in the absence of added hormone was found consistently to be slightly reduced. The receptors Y541-D, Y541-E and Y541-A were also able to transactivate to transcription to levels similar to the wild-type in the presence of oestradiol, demonstrating that the tyrosine residue...
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Table I. The dissociation constants of oestrogen receptors

<table>
<thead>
<tr>
<th>Receptor</th>
<th>$K_d$ (nM)</th>
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<tbody>
<tr>
<td>MOR 1–599</td>
<td>0.35</td>
</tr>
<tr>
<td>Y541-F</td>
<td>0.5</td>
</tr>
<tr>
<td>Y541-D</td>
<td>0.65</td>
</tr>
<tr>
<td>Y541-E</td>
<td>0.5</td>
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<tr>
<td>Y541-A</td>
<td>0.35</td>
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Extracts prepared from transfected COS-1 cells were analysed for ligand-binding activity. The $K_d$ for ligand binding for each mutant was determined by Scatchard analysis.

4-hydroxytamoxifen, demonstrating that ligand-dependent and ligand-independent activation of transcription may occur, in part, by a common mechanism.

To determine if the increase in transactivation by the mutant receptors was derived from activation of AF2 or required the presence of AF1, the point mutations described above were introduced into a chimeric receptor consisting of the ligand-binding domain fused to the DNA-binding domain of GAL4 and expressed together with a GAL4 reporter gene in HeLa cells (Figure 2A). The wild-type GAL4-AF2 receptor stimulated transcription from the reporter up to 100-fold in the presence of oestradiol. GAL4-AF2 Y541-F stimulated transcription in the presence of oestradiol, but was slightly reduced in activity compared with the wild-type AF2 in the absence of hormone. Chimeric proteins containing the point mutations Y541-D, Y541-E and Y541-A all showed enhanced levels of transcription in the absence of oestradiol compared with GAL4-AF2, and their activities were equivalent to that of GAL4-AF2 following hormone treatment. Similar results were obtained in COS-1 cells (our unpublished data). Expression of increasing amounts of the chimeric proteins in HeLa cells (Figure 2B) demonstrated that the enhanced hormone-independent activity of the mutant receptors occurred over a range of receptor concentrations and was significantly greater than that derived from the wild-type protein at equivalent levels of expression. The results of transient transfection experiments demonstrate that the conserved tyrosine residue N-terminal to AF2 AH is not required for the oestrogen-dependent activation of AF2 but is necessary to maintain AF2 in an inactive state, since mutations which reduce hydrophobicity and the size of the amino acid side chain at this position result in partial activation of AF2 combined with activation of AF1 by other signalling pathways.

**Mutation of tyrosine 541 does not affect ligand binding or DNA binding**

Expression of the wild-type and mutant receptors was verified by Western blotting and immunofluorescence (our unpublished data). All receptor proteins bound ligand with similar affinities (Table I) and bound to DNA as dimers in a gel retardation assay (Figure 3). In all cases, the mobility of the receptor–DNA complexes was slightly increased by the addition of oestradiol and retarded by the ER-specific antibody MP16. Therefore, mutant recep-
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**Fig. 3.** Tyr541 mutant receptors bind to DNA with affinity similar to the wild-type receptor. Wild-type (MOR 1–599) and mutant forms of the full-length mER were transiently expressed in COS-1 cells. Equal amounts of receptor were analysed for DNA binding activity in a gel retardation assay using a \(^{32}P\)-labelled oligonucleotide containing a single oestrogen response element (ERE) corresponding to the sequence from the vitellogenin A2 gene. Prior to electrophoresis, the samples were treated with either ethanol vehicle alone, oestradiol (E2) at \(1 \times 10^{-7} \text{M}\) or the ER-specific antiserum MP16. Protein–DNA complexes were resolved on 6% polyacrylamide gels in 1 T/2 Tris/glycine buffer and detected by autoradiography. The increase in transcriptional activity of the mutant receptors observed in the absence of oestradiol described above may result from a hormone-independent realignment of AF2 AH, resulting in the recruitment of receptor-interacting proteins.

**Differential interaction of RIP-140 and SRC-1 with activated receptors in vitro**

A number of proteins have been identified which interact with the ligand-binding domain of nuclear hormone receptors in the presence of ligand and have been proposed to act as co-activators in hormone-regulated gene transcription. The interaction of these proteins with receptors has been shown to depend on the integrity of AF2 AH and its realignment following ligand binding. The increase in transcriptional activity of the mutant receptors observed in the absence of oestradiol described above may result from a hormone-independent realignment of AF2 AH, resulting in the recruitment of receptor-interacting proteins.

The wild-type and mutant ERs were therefore analysed for their ability to interact with RIP-140 and SRC-1 in vitro using GST pull-down experiments (Figure 4). \(^{35}\text{S}\)Methionine-labelled RIP-140 and SRC-1 bind to the ligand-binding domain of the wild-type receptor (GST-AF2) in an oestrogen-dependent manner. Similarly, the interaction of GST–AF2 Y541-F with SRC-1 and RIP-140 was also oestrogen dependent; however, GST fusion proteins of the AF2 mutants Y541-D, Y541-E and Y541-A interact with RIP-140 in both the absence and the presence of ligand. In contrast, the ability of these mutant receptors to bind SRC-1 was still oestrogen dependent, with no interaction observed in solution in the absence of oestradiol. The difference in the binding affinities of RIP-140 and SRC-1 in the absence of ligand may reflect their interaction with distinct surfaces on the ligand-binding domain.

We next examined the ability of RIP-140 and SRC-1 to interact with the wild-type and mutant receptors pre-bound to DNA. The receptors were overexpressed in COS-1 cells, purified on an immobilized oestrogen response element and incubated with \(^{35}\text{S}\)methionine-labelled RIP-140 or SRC-1 in the absence and presence of 17β-oestradiol (Figure 5). When pre-bound to DNA, the wild-type mER and the Y541-F mutant interact with both RIP-140 and SRC-1 in an oestrogen-dependent manner whereas the constitutive mutants Y541-D, Y541-E and Y541-A interact with both proteins in the absence of hormone. Thus, the recruitment of SRC-1 to the activated mutant receptors may depend on the presence of additional factors in the cell lysates or a further conformational change in the ligand-binding domain which appears to occur upon DNA binding.

**Co-activator proteins enhance ligand-independent transactivation**

The effect of the overexpression of RIP-140 and SRC-1 on the AF2 activity was analysed using co-transfection experiments in HeLa cells. Overexpression of RIP-140 (Figure 6A) resulted in a reduction in ligand-dependent
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Fig. 6. (A) Effect of RIP-140 expression on transactivation by the oestrogen receptor. A constant amount of either the wild-type chimeric receptor GAL4-AF2 of the mutant GAL4-AF2 Y541-A was expressed in HeLa cells together with a GAL4 reporter gene GSE1BCAT, the luciferase expression vector pGL2control (Promega) as an internal control for transfection efficiency and increasing amounts of RIP-140. Samples were maintained with ethanol vehicle alone (j) or with oestradiol at 1×10^{-8} M (r). Cell extracts were prepared and assayed for CAT and luciferase activities. Normalized values are expressed as percentage activity compared with the wild-type receptor in the presence of oestradiol. The results shown were determined from a minimum of three independent experiments with all samples assayed in duplicate. (B) Effect of SRC-1 expression on transactivation by the oestrogen receptor. A constant amount of either the wild-type chimeric receptor GAL4-AF2 or the mutant GAL4-AF2 Y541-A was expressed in HeLa cells together with increasing amounts of SRC-1, and maintained in the presence and absence of hormone and assayed as described in (A).

Transactivation by GAL4-AF2, as previously described (Cavailles et al., 1995), and also reduced transactivation by GAL4-AF2 Y541-A in both the absence and presence of oestradiol. Expression of SRC-1 (Figure 6B) enhanced the ligand-dependent activity of GAL4-AF2 and GAL4-AF2 Y541-A by ~2- to 3-fold and stimulated the ligand-independent activity by ~4-fold. Although expression of SRC-1 stimulated the ligand-independent activity of the wild-type receptor, its overall activity, in contrast to that of the Y541-A mutant, was almost entirely dependent on the addition of ligand.

**Ligand-independent activity requires AF2 AH**

Mutations of the hydrophobic amino acids in AF2 AH have been demonstrated to inhibit the ligand-dependent activity of the mER in transfection studies (Danielian et al., 1992) and also to prevent the binding of receptor-interacting proteins such as RIP-140 in vitro (Cavailles et al., 1995). The role of AF2 AH in ligand-independent activation was therefore examined, in the context of the full-length receptor MOR 1–599, by combining the mutations at Y541 with the mutations in H12. The effect of the combined mutations was analysed in transient transfection studies in HeLa cells (Figure 7). Mutations in hydrophobic residues in AF2 AH inhibited ligand-dependent transactivation. The combined mutant (Y541-D, M547-A, L548-A) was also inactive, demonstrating that AF2 AH is required for the ligand-independent activity observed with MOR 1–599 Y541-D. Co-expression of RIP-140 or SRC-1 has no effect on either a hydrophobic mutant alone or the combined mutant receptor. Therefore, the ability of the ER to activate transcription in the absence

![Fig. 7. AF2 AH is required for ligand-independent activity. HeLa cells were transfected with oestradiol receptors in which mutations had been introduced in the hydrophobic residues in helix 12 or at Tyr541 (Y541-D) or in combination Y541-D, M547-A, L548-A. Following transfection, cells were treated with ethanol vehicle alone (■) or oestradiol at 1×10^{-8} M (□). After 48 h, cell extracts were prepared and assayed for luciferase and β-galactosidase activities. Normalized values were determined from a minimum of three independent experiments with all samples assayed in duplicate.](image-url)
of ligand may be modulated by co-activator proteins and requires the formation of an interacting surface similar to that which is generated as a result of ligand binding.

Discussion

The main conclusion from this study is that a tyrosine at the N-terminus of a conserved helix, which forms a major part of the ligand-dependent activation function in the ER, is required to maintain the receptor in a transcriptionally inactive state in the absence of hormone. The conserved helix corresponds to helix 12 (H12) of the ligand-binding domain of nuclear receptors (Wurtz et al., 1996). In the absence of ligand, amino acids at the N-terminus of H12 appear to interact with residues in the loop between helices 2 and 3 (Bourguet et al., 1995). It is proposed that following ligand binding this interaction may be disrupted, allowing helices 10 and 11 to rearrange to form one continuous helix while a shortened H12 realigns over the ligand-binding pocket (Renaud et al., 1995; Wagner et al., 1995). This alteration of the position of H12 involves the introduction of a kink or break in the helical structure at proline residue(s) which are highly conserved at this position in the nuclear hormone receptors. The realignment of H12 against the core of the ligand-binding domain in combination with helices 3 and/or 4 may result in the formation of a novel interacting surface to which co-activators bind (Parker and White, 1996; Henttu, et al., 1997).

It has been proposed based on sequence homology that the helical structure of the ligand-binding domain may be conserved as a common fold for all nuclear hormone receptors, including the ER (Wurtz et al., 1996). It is conceivable, therefore, that Y541 in the mER, which is at the N-terminus of the realigned H12, is involved in hydrophobic interactions with the loop between helices 2 and 3 in the ligand-binding domain in the absence of oestradiol and that these are maintained or strengthened when this residue is replaced by phenylalanine, an amino acid with a planar structure similar to tyrosine. Reduction in either the hydrophobicity or the size of the side chain of the amino acid at this position with charged amino acids or a neutral amino acid such as alanine, would reduce the stability of the interactions, resulting in the release of H12 from its inactive conformation. Interestingly, it is the introduction of alanine, an amino acid with a small alkyl group side chain, which results in the greatest hormone-independent activation of AF2 when it is fused to a heterologous DNA-binding domain. Comparison of the primary sequence of the ER with other nuclear hormone receptors reveals that although a tyrosine is conserved in the ERX from diverse species and in the related isoform ERβ, a number of other receptors contain a hydrophobic residue in a similar position immediately N-terminal to either the charged amino acid or the proline at the predicted start of H12. It is conceivable, therefore, that the ligand-binding domains of a number of receptor proteins may be maintained in an inactive conformation by similar hydrophobic interactions. The lack of conservation of this tyrosine may reflect a mechanism of differential signalling specific for the ER.

The ER has been demonstrated to be phosphorylated on serine residues (Ali et al., 1993; Lahooti et al., 1994; Le Goff et al., 1994; Arnold et al., 1995b) and on tyrosine residues (Auricchio et al., 1987; Arnold et al., 1995a,d; Pietras et al., 1995), suggesting that it may be a target for different signalling pathways (Kato et al., 1995; Bunone et al., 1996). In addition, although it has been reported that dimerization and DNA binding of the hER are regulated by phosphorylation at Y537 (Arnold et al., 1995d), we find that these properties are unaffected in any of the mutants tested here. Furthermore, it has been shown that the C-terminal region of the ER, including the tyrosine residue at position 541, can be deleted without disrupting either ligand-binding, dimerization or DNA-binding properties of the receptor (Fawell et al., 1990a). In particular, the lack of an absolute requirement for phosphorylation at this site is demonstrated by the ability of all the mutant receptors to transactivate to a similar level to the wild-type protein in the presence of oestradiol. Phosphorylation at this site, however, would decrease the potential for hydrophobic interactions between the tyrosine and other parts of the ligand-binding domain and therefore may be an alternative means of receptor activation.

The mechanism of ligand-independent activation may depend on a combination of two processes. Firstly, modification of the receptor by the action of a tyrosine kinase might facilitate the disassembly of aporeceptor complexes. This could result from a reduced association with DNA/Hsp70 family members and the loss of interaction with Hsp90, generating a pool of unliganded active receptor similar to that proposed from studies of steroid receptor function in ydj1 mutant strains of yeast (Bohen et al., 1995; Caplan et al., 1995; Kimura et al., 1995). Preliminary studies, however, indicate that the activated mutant receptors used in this study retain the ability to interact with Hsp90 in vitro (our unpublished data). Secondly, it is possible that ligand-independent activation may result from a conformational change which generates an interacting surface that is capable either of recruiting different co-activators required for receptor-mediated transcription, or altering the binding affinity of potential co-activator proteins for the ligand-binding domain. The demonstration that anti-oestrogens inhibit both ligand-dependent and ligand-independent transcriptional activity probably reflects their ability to interfere with the correct realignment of H12 with helices 3/4 and thereby prevent the recruitment of co-activators. These results, together with the analysis of receptors in which mutations at Y541 have been combined with mutations in the hydrophobic amino acids of AF2 AH, demonstrate that both the ligand-dependent and the ligand-independent activity of the ER result from a common mechanism.

Since mutations in AF2 AH prevent interaction between the ligand-binding domain of the receptor and potential co-activator proteins (Cavaillès et al., 1994), the activated receptors were analysed for their interaction with two different types of co-activator in vitro. The ability of the Y541 mutants to interact with RIP-140 but not SRC-1 in a hormone-independent manner in the GST pull-down assay, in contrast to both co-activators being able to interact when receptors are bound to DNA, implies that different surfaces of the AF2 domain may be involved in the interaction with these proteins and that a conformational change is induced in the receptor on DNA binding. It is conceivable, therefore, that RIP-140 may associate
with the receptor prior to the binding of more potent core-activators such as SRC-1 as a result of a difference in the relative affinities of these proteins for the receptor in the presence and absence of DNA. Alternatively, additional factors, or modification of the receptors in the cell lysate in which they are expressed, may facilitate the binding of SRC-1 in the presence of DNA.

It has been proposed from studies using either in vitro translated proteins (Castoria et al., 1993), or from breast tumour samples reported to be devoid of ligand-binding activity (Castoria et al., 1996), that phosphorylation of the conserved tyrosine results in the conversion of a non-hormone-binding form of the ER to a hormone-binding form. Since receptors in which this tyrosine has been phosphorylated (Castoria et al., 1993), or modified by mutagenesis as described in this study, bind ligand with an affinity similar to the wild-type receptor, it is conceivable that phosphorylation at this site may alter the ligand-binding domain to a more active conformation which has an enhanced ability to stimulate transcription or promote cell growth in an ER-dependent but ligand-independent manner. The demonstration that the hER may be phosphorylated in vitro on Y537 by src-family tyrosine kinases (Arnold et al., 1995a), combined with the mutagenesis studies described here, suggests that tyrosine phosphorylation may result in the recruitment of co-activators to the ligand-binding domain of the receptor. The observation that oestradiol may also act directly in a receptor-dependent way to stimulate a tyrosine kinase/p21ras/MAP-kinase pathway in MCF-7 breast cancer cells (Migliaccio et al., 1996) indicates a convergence of multiple signalling pathways in the function of this nuclear hormone receptor. The ligand-independent alteration in receptor conformation which promotes the interaction with co-activator proteins may, therefore, provide an alternative mechanism of activating ER-mediated transcription.

Materials and methods

Plasmids

The following plasmids have been characterized previously: GAL4-AF2 and GST-AF2 (Cavalliès et al., 1994), pMT2 MOR (Lahooti et al., 1995), pBluescript-RIP-140 and pEBFS/RIP-140 (Cavalliès et al., 1995), pERE-TK-LUC (White et al., 1993), or modified by at 450 V and 250 μl in the presence of 20 μg of plasmid DNA. Cells were then plated out in DMEM containing 10% DCFBS and grown for 48 h. Whole cell extracts were prepared in buffer containing 0.4 M KCl, 20 mM HEPES (pH 7.4), 1 mM dithiothreitol and 20% glycerol. The protein content of cell extracts was determined using a colorimetric method (Bio-Rad).

GST pull-down assays

The ligand-binding domains of wild-type and mutant receptors were expressed as GST fusion proteins in Escherichia coli as has been described previously (Cavalliès et al., 1994). For GST pull-down assays, bacterially expressed GST or GST fusion proteins were bound to glutathione-Sepharose 4B beads (Pharmacia Biotech). RIP-140 and SRC-1 cDNAs in pBluescript vectors were used to generate [35S]methionine (Amersham International) labelled proteins using the TNT-coupled in vitro translation system (Promega). The 35S-labelled proteins were incubated with beads containing either GST or GST fusion proteins in the absence or presence of 10–7 M 17β-oestradiol, and the antibody MP16 specific to ERs (Fawell et al., 1990b) was added as indicated. Receptor–DNA complexes were separated from unbound DNA in non-denaturing polyacrylamide gels and visualized by autoradiography.

Ligand-binding analysis of the wild-type and mutant receptors was performed as described previously (Fawell et al., 1990a) using [3H]oestradiol (Amersham International). Scatchard analysis was performed over the range 0.3–30 nM labelled steroid in the absence or presence of 100-fold excess of unlabelled oestradiol.

Protein–protein interaction assays in the presence of DNA

The analysis of interactions between co-activators and receptors bound to DNA was carried out according to the method of Kurokawa et al. (1995), pERE TK-LUC (White et al., 1993), or modified by at 450 V and 250 μl in the presence of 20 μg of plasmid DNA. Cells were then plated out in DMEM containing 10% DCFBS and grown for 48 h. Whole cell extracts were prepared in buffer containing 0.4 M KCl, 20 mM HEPES (pH 7.4), 1 mM dithiothreitol and 20% glycerol. The protein content of cell extracts was determined using a colorimetric method (Bio-Rad).

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