Molecular mechanisms of steroid hormone action

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Introduction

The ability of oestrogens and androgens to stimulate the growth of a number of endocrine cancers is well established and several endocrine therapies are widely used to reduce the availability of the hormones or to block their action. These include non-steroidal hormone antagonists such as tamoxifen and flutamide, steroidal compounds which include ICI 182780 and cyproterone acetate, and both steroidal and non-steroidal aromatase inhibitors. Although we have learned a great deal about the molecular mechanism of steroid hormone action, it is still unclear how hormones stimulate the proliferation of tumour cells and how hormone antagonists function. In part this is a result of the ability of oestradiol and testosterone to regulate the expression of many proteins implicated in the control of cell proliferation making it difficult to identify the crucial targets. Some of these targets are growth factors and/or their receptors which suggests that the mitogenic effects of steroids may be mediated by indirect autocrine or paracrine mechanisms (Clarke et al. 1991, Roberts & Sporn 1992). Alternatively since steroids regulate the expression of certain cyclins or kinase inhibitors (Musgrove & Sutherland 1994, Altucci et al. 1996) they may control cell cycle progression directly. Recent work suggests that as well as the cyclin D1 gene, cyclin D1 itself may be a crucial target (Zwijnen et al. 1997) but additional proteins could also be important in different subsets of tumours.

The identification of target genes for steroid hormones is further complicated by the observation that receptor signalling is cross-coupled with that of other signalling pathways. It used to be thought that signalling by receptors was relatively straightforward compared with most other signalling pathways, since the receptor itself is a transcription factor. It is now clear, however, that growth factors, neurotransmitters and other hormones are able to modulate the activity of steroid hormone receptors (Power et al. 1991, Aronica & Katzenellenbogen 1993, Ignar-Trowbridge et al. 1993). This means that alterations in the activity of receptors and the expression of individual target genes involved in cell proliferation is determined not only by hormonal signals but also by changes in other signalling pathways, which undoubtedly take place during breast and prostate cancer progression. Receptors are also capable of regulating the activity of a number of other transcription factors, either directly or indirectly, and thereby modulate the ability of other signalling pathways to control gene transcription (Shemshedini et al. 1991, Philips et al. 1993, Stein & Yang 1993, Webb et al. 1995).

Although the precise role of steroid hormones in cell proliferation is still ill-defined, tremendous progress has been made in elucidating the role of hormones in receptor activation and the mechanism by which hormone antagonists block this activity. In particular two recent advances have been made which provide new insights into steroid hormone action and the function of nuclear receptors in general. First, models for the three-dimensional structure of the ligand binding domain of several receptors have been determined (Bourguet et al. 1995, Renaud et al. 1995, Wagner et al. 1995, Brzozowski et al. 1997) and secondly, novel proteins have been identified (Cavaillès et al. 1994, Halachmi et al. 1994) which interact with receptors in a ligand dependent manner and may play a role in gene transcription. These developments are helping to provide a better understanding of the mechanism of action of both hormones agonists and antagonists and the links between nuclear receptors and other signalling pathways. In this review we will outline these advances, with reference to the oestrogen receptor (ER), and discuss their relevance to anti-oestrogen therapy and tamoxifen resistance.

Steroid hormone receptors are members of the nuclear receptor family

Steroid hormone receptors are members of the nuclear receptor family, a large group currently totalling approximately 150 different proteins, which function as transcription factors in many different species including both invertebrates and vertebrates. In addition to steroid hormone receptors, the nuclear receptor family consists of receptors for retinoids, thyroid hormone, fatty acids and prostaiglandins and a number of so-called orphan receptors.
Figure 1 (A) Organisation of functional domains in the nuclear receptors. DBD and LBD refer to the positions of the DNA binding domain and the ligand binding domain respectively. The relative positions of the two transactivation domains AF1 and AF2 are also shown. (B) Schematic diagram of steroid hormone receptor binding to DNA. The receptors bind as homodimers to palindromic sequences. The specific sequences of simple response elements for the ER and other steroid hormone receptors are shown.
receptors, the ligands of which have yet to be identified (Parker 1993, Mangelsdorf et al. 1995). They are highly related in both primary amino acid sequence and the organisation of functional domains suggesting that many aspects of their mechanism of action are conserved. Thus, progress in our understanding of steroid hormone action has been facilitated by studies of many nuclear receptor family members.

The sex steroid receptors are not only expressed in sex accessory tissues but in many other types of cells including liver, bone, pituitary and cardiovascular cells. Androgen receptors are encoded by a single gene whereas there are two genes for ERs, ERα and ERβ (Kuiper et al. 1996, Mosselman et al. 1996). Our current knowledge of the distribution of ERβ (Kuiper et al. 1997) is based on mRNA analysis either by RT-PCR or by in situ hybridisation and the relative levels of ERα and ERβ protein levels have yet to be determined. Analysis of mice made devoid of ERα by targeted gene disruption (Bocchinfuso & Korach 1997) indicates that this receptor is essential for uterine growth and mammary gland development but not for mediating the inhibitory effects of oestrogens in vascular injury (Jafrati et al. 1997). Thus, it is possible that ERα and ERβ have distinct functions in some tissues but not in others. Undoubtedly the role of these individual receptors will be confirmed in the future by gene knock-out experiments to generate mice lacking either both receptors or one isoform only and analysing the resulting phenotypes.

A simplified diagram of the organisation of functional domains in nuclear receptors is shown in Fig. 1A. The receptors are characterised by a highly conserved DNA binding domain and a moderately conserved ligand binding domain which also functions in dimer formation and transcriptional activation (Parker 1993, Mangelsdorf et al. 1995). A second dimerisation domain is located within the DNA binding domain itself and the receptors contain two transcriptional activation functions by which they are able to regulate the expression of target genes; AF1, which is located in the N-terminal or A/B domain, and AF2 in the ligand binding domain. When activated by hormone binding, the receptors may act directly as transcription factors by binding to specific DNA sequences, termed hormone response elements, found in the vicinity of target genes (Evans 1988, Beato 1989). The sequences of simple binding sites for the steroid receptors are shown in Fig. 1B. Composite response elements have also been identified which bind receptors in addition to other transcription factors such as AP-1 (Philips et al. 1993, Webb et al. 1995) and NF-kB (Stein & Yang 1995). To date most of the analysis which has been carried out has been to determine the mechanism by which receptors function as DNA dependent transcription factors but it is likely that the transcription of many genes is regulated indirectly by non-classical mechanisms.

**Nuclear receptors function as ligand dependent transcription factors**

In the absence of hormone, steroid hormone receptors exist as inactive oligomeric complexes with a number of other proteins including chaperon proteins, namely the heat shock proteins Hsp90 and Hsp70 and cyclophilin-40 and p23 (Smith & Toft 1993, Pratt & Toft 1997). The role of Hsp90 and other chaperons may be to maintain the receptors folded in an appropriate conformation to respond rapidly to hormonal signals. Whether different types of receptor complexes occur in cells, each containing a subset of chaperons and able to respond to different types of signal, or whether receptors are folded into a mature complex containing chaperons in an assembly line type of process is as yet unclear (Bohen et al. 1995, Pennisi 1996). Following hormone binding, the oligomeric complex dissociates allowing the receptors to function either directly as transcription factors by binding to DNA in the vicinity of target genes or indirectly by modulating the activity of other transcription factors.

Nuclear receptors may be classified into four types based on their dimerisation and DNA binding properties. Steroid hormone receptors bind to DNA as homodimers either to simple response elements comprising short palindromic sequences or to composite response elements with other transcription factors. Oestrogen response elements consist of inverted repeats of the sequence A/GGTCA separated by three nucleotides, whereas the receptors for androgens, glucocorticoids and progestins bind to inverted repeats of the sequence AGA/GACA, also separated by three nucleotides. In contrast, a second group of nuclear receptors, including the retinoic acid receptor (RAR), thyroid hormone receptor (TR) and vitamin D receptor, bind to DNA as heterodimers in combination with the retinoid X receptor (RXR) (Mangelsdorf et al. 1995). The binding sites for these receptors consist of direct repeats of the sequence A/GGTCA separated by up to five nucleotides with the DNA binding specificity of different receptors being determined to some extent by the spacing of the repeats. In addition RXR itself and some orphan receptors bind to direct repeats as homodimers while a final group of orphan receptors are also able to bind as monomers to single copies of the extended binding site sequence TCAAGGTCA. The major part of the dimerisation interface is formed from sequences within transcription factors such as AP-1 (Philips et al. 1993, Webb et al. 1995) and NF-kB (Stein & Yang 1995).
the ligand binding domain of the receptors while a second dimerisation function is located within the DNA binding domain itself (Kumar & Chambon 1988, Fawell et al. 1990).

Transcriptional activation by steroid receptors is mediated by at least two distinct activation functions; one, referred to as AF1, is located in the N-terminal domain and a second, AF2, is in the hormone binding domain (Lees et al. 1989, Tora et al. 1989). In the ER, and probably in other receptors as well, the absolute and relative activities of these two domains vary depending on the target promoter and the cell type. However, while the two activation domains have the potential to act independently, they appear to interact with each other in the context of the intact receptor in a way which has yet to be determined.

The N-terminal activation function, AF1, varies considerably in both size and primary amino acid sequence in different steroid hormone and nuclear receptors. A number of studies have shown that this domain is a target for phosphorylation by other signalling pathways (Ali et al. 1993, Kato et al. 1995, Bunone et al. 1996). The second activation function, AF2, is induced by hormone binding and a short sequence encoding an amphipathic α-helix in the C-terminal part of the ligand binding domain, which is conserved in all transcriptionally active nuclear receptors, has been shown to be

**Figure 2** Diagrams of models of the structure of the ligand binding domains of nuclear receptors to demonstrate the effect of agonist binding. Panel A shows the arrangement of sequence elements for three different receptors, human RXRα in the absence of ligand and human RARγ and rat TRα in the presence of their respective ligands. The numbers 1 to 12 correspond to separate α-helices and s1 to s4 to β-strands. The position of amino acids which are in close contact with ligand are marked and are found in helices 3, 5, s1 and s3, the loop between helices 6 and 7 and helices 11 and 12. Panels B and C show diagrams of the folded structure in the absence and presence of ligand and the relative positions of helices 1 to 12 (H1-H12). This shows the realignment of helices 10 and 11 and the alteration in position of helix 12 to form a lid over the ligand binding pocket. This repositioning results in the formation of a new surface on the ligand binding domain, indicated by the white circles, which is believed to be required for the interaction with coactivator proteins.
essential in receptor function (Danielian et al. 1992, Saatcioglu et al. 1993, Baretino et al. 1994, Durand et al. 1994). However, it is doubtful whether this short sequence functions autonomously since mutation of conserved residues in other parts of the ligand binding domain, which do not affect ligand binding or dimerisation, also generate transcriptionally defective receptors suggesting that AF2 is derived from a number of different elements (O’Donnell & Koenig 1990, Henttu et al. 1997).

Structure of the ligand binding domain of nuclear receptors

A major advance in our understanding of how nuclear receptors function has been the determination of models for the ligand binding domain based on crystal structures of three different receptors either in the absence or presence of their cognate ligands. The secondary structure of all three nuclear receptors is shown in Fig. 2A and schematic diagrams of models of the structures obtained in the absence and presence of ligand in Fig. 2B and C respectively. The structure of the ligand binding domain of RXRα crystallised in the absence of ligand, consists of 12 α-helices and two β-strands arranged in three layers to form an antiparallel α-helical sandwich (Bourguet et al. 1995). This receptor was crystallised in the form of a dimer with the dimer interface formed mainly from helices 9 and 10. Subsequently, crystals of the ligand binding domains of RARγ (Renaud et al. 1995) and TRα (Wagner et al. 1995) generated in the presence of their respective ligands were shown to have a similar overall structure, but with one major exception. The C-terminal helix, helix 12, which protrudes beyond the core of the ligand binding domain in the unliganded RXRα, is folded back across the core of the domain in both RARγ and TRα. It has therefore been proposed that in the case of RARγ, ligand binding results in a realignment of helices 10 and 11 which rearrange to form a continuous helix. This creates a shortened helix 12, corresponding to the amphipathic α-helix described earlier, which is released from the core of the domain to fold back over the top of the ligand binding pocket where it forms both contacts with the ligand and a salt bridge in helix 4. Helix 12 is similarly aligned in the case of the TRα although a salt bridge is not formed. Protein sequence comparisons between different receptors suggest that their overall helical structure is conserved (Wurtz et al. 1996) and unpublished results indicate that the structures of peroxisome proliferator activated receptor (PPAR) and ERα are consistent with this prediction. Thus it appears that a major role for ligand binding is an alteration in the conformation of the ligand binding domain to form a novel surface. This may then allow the binding of coactivators and other regulatory proteins.

Recruitment of receptor interacting proteins

Gene transcription depends on the formation of a pre-initiation transcription complex that includes basal transcription factors and one of the roles of transcriptional activators, including steroid hormone receptors, is thought

<table>
<thead>
<tr>
<th>Group</th>
<th>Name(s)</th>
<th>Protein</th>
<th>Predicted size (kDa)</th>
<th>Receptor binding</th>
<th>Function(s)</th>
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<td>ER, RAR, TR, PR</td>
<td>Chromatin remodelling?</td>
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AR, androgen receptor; PR, progesterone receptor; VDR, vitamin D receptor.

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to be the stabilisation of this complex. Although receptors have been shown to bind directly to basal transcription factors \textit{in vitro} the significance of these interactions is unclear since they are unaffected by mutations in the receptor that destroy transcriptional activity (Sadovsky \textit{et al.} 1995). The involvement of additional targets is suggested by the observation that AF2 activity is inhibited when receptors are overexpressed suggesting that limiting downstream target proteins, required for gene transcription, are being sequestered (Tasset \textit{et al.} 1990). A number of candidate target proteins have been detected in extracts from mammalian cells which bind to the ligand binding domain of receptors in a ligand dependent manner (Cavaillès \textit{et al.} 1994, Halachmi \textit{et al.} 1994). Since these proteins fail to interact with mutant receptors which are transcriptionally inactive it has been proposed that they may have a role in ligand dependent transcriptional activation by AF2.

Receptor interacting proteins may be classified into two groups (Table 1). The first group consists of two families of proteins which appear to stimulate transcription and represent bona-fide coactivators. One family, originally identified as proteins of 160 kDa, consists of a number of related proteins; the steroid receptor coactivator proteins (SRC-1a and SRC-1c) (Onate \textit{et al.} 1995, Kamei \textit{et al.} 1996), transcription intermediary factor TIF2 (Voegel \textit{et al.} 1996) (independently identified as GRIP-1 (Hong \textit{et al.} 1996)) and CREB binding protein (CBP) interacting protein (p/CIP) (Torchia \textit{et al.} 1997). The second family comprises CBP and p300, which were originally shown to function as coactivators for cAMP response element binding protein (CREB), the transcription factor that mediates responses to protein kinase A stimulation. Subsequently, however, CBP/p300 were shown to function as coactivators for many other transcription factors and may play a central role in many signalling pathways (Janknecht & Hunter 1996, Shikama \textit{et al.} 1997).

The second group contains a number of unrelated proteins which also interact with the ligand binding domain but their role in receptor function is still unclear. This group includes a 140 kDa protein termed RIP 140 (Cavaillès \textit{et al.} 1995), TIF1 (Le Douarin \textit{et al.} 1995), ARA70 (Yeh & Chang 1996), which has been shown to stimulate transactivation mediated by the androgen receptor, and TRIP1/SUG1 (Lee \textit{et al.} 1995, vom Baut \textit{et al.} 1996). RIP 140 stimulates receptor mediated transcription in yeast (Joyeux \textit{et al.} 1996) and contains an ‘activation domain’ which is functional in mammalian cells (L’Horset \textit{et al.} 1996) but it is still unclear whether it is a bona-fide coactivator. TIF1 has been shown to interact with proteins associated with heterochromatin and may play a role in chromatin remodelling (Le Douarin \textit{et al.} 1996). All these proteins have been shown to contain one of more copies of a short sequence motif LXXLL which is necessary and sufficient to mediate the binding of ligand bound nuclear receptors. This motif has been described as a signature sequence for the interaction of proteins with nuclear receptors (Heery \textit{et al.} 1997, Torchia \textit{et al.} 1997).

Other proteins have also been isolated which interact with other regions of the ligand binding domain. For example two related proteins, N-CoR and SMRT, which function as repressors for RXR and TR in the absence of ligand (Horlein \textit{et al.} 1995, Heinzal \textit{et al.} 1997, Nagy \textit{et al.} 1997), have been reported to bind to oestriadiol and progesterone receptors in the presence of antagonists (Jackson \textit{et al.} 1997, Smith \textit{et al.} 1997). A detailed description of all these proteins, both activators and repressors, is beyond the scope of this article; they are reviewed in detail elsewhere (Horwitz \textit{et al.} 1996, Heery \\& Parker 1997) and therefore we will focus on the importance of coactivators in steroid hormone action together with potential interactions between receptors and other transcriptional regulators.

\textbf{Structure and function of coactivators for nuclear receptors}

To date, it appears that the p160 proteins and CBP/p300 are the most important of the receptor interacting proteins in terms of their ability to potentiate the transcriptional activity of receptors. The p160 proteins SRC1, TIF2 and p/CIP share significant sequence homology, most notably in a central region found to be required for interaction with the ligand binding domain of nuclear receptors. They show ligand dependent binding to nuclear receptors \textit{in vitro} but fail to bind to transcriptionally inactive receptors in which mutations have been introduced in the conserved hydrophobic residues in helix 12 (Kamei \textit{et al.} 1996). Similarly mutations in conserved residues in helix 3 which also impair AF2 also prevent binding of these coactivators (Henttu \textit{et al.} 1997). This suggests that, in the presence of ligand, helices 3 and 12 form a composite surface recognised by the p160 proteins, a hypothesis which is consistent with the models of the holo ligand binding domains. Similarly, CBP/p300 have been reported to bind to the ligand binding domains of nuclear receptors in a ligand dependent manner and increase AF2 activity in transfected cells (Chakravarti \textit{et al.} 1996, Hanstein \textit{et al.} 1996, Kamei \textit{et al.} 1996, Yao \textit{et al.} 1996). This binding is also dependent on the integrity of helix 12, implying that these proteins recognise a similar if not identical binding site (Heery \textit{et al.} 1997). The p160 and CBP/p300 family members also interact directly with one another (Hanstein \textit{et al.} 1996, Kamei \textit{et al.} 1996, Yao \textit{et al.} 1996) but we have little information about the stoichiometry of binding of any of these proteins to receptors. For example, it is unclear whether the proteins bind to receptors
independently or as complexes, whether they bind sequentially or simultaneously, which may pose steric problems given their relative sizes, and whether there is redundancy or receptor selectivity. These questions are being tackled by many laboratories at the present time.

When coactivators are recruited to receptors they may serve at least two distinct roles. First, they may stabilise the formation of a pre-initiation transcription complex of DNA polymerase and basal transcription factors, and secondly they may remodel chromatin by destabilising the binding of nucleosomes. Regions required for transcriptional activation have been found in both p160 and CBP/p300 family members. These have been identified by fusing fragments of these proteins to a DNA binding domain and testing their ability to stimulate transcription of reporter genes in transfected cells. In SRC1, one corresponds to the region involved in CBP binding and may reflect the recruitment of CBP and/or p300 to the promoter. In CBP, an activation domain corresponds to a region that binds to TATA-box binding protein (TBP) and so may directly interact with the basal transcription machinery. Both families of proteins also encode histone acetyl transferases that acetylate lysine residues on specific histones which may destabilise nucleosomes to promote gene transcription (Ogryzko et al. 1996). In addition, they are also reported to be capable of recruiting P/CAF, another histone acetyl transferase (X-J Yang et al. 1996). What emerges from these studies is that co-activators are multidomain proteins that presumably function either independently or as complexes and the goal is now to determine the precise roles and relative importance of each of these domains when they are recruited by different receptors to target genes. A model showing some of the functions of coactivator proteins is shown in Fig. 3.

**Role of hormonal agonists and antagonists in coactivator recruitment**

Although crystal structures have yet to be solved for the ligand binding domain of any one receptor in the liganded and unliganded state, the overall similarity of the structures for apo-RXRα and holo-RARγ and the high level of sequence conservation in the nuclear receptor family, allows a model to be proposed for the mechanism of action of hormone agonists and antagonists. The realignment of helix 12 as a lid over the ligand binding pocket provides a potential molecular explanation for the disparate activities of different ligands. Ligands which function as agonists would be expected to allow the formation of an interacting surface made up from residues in helices 3 and 12. In contrast, ligands that fail to allow the correct realignment of helix 12 are likely to function as antagonists. Interestingly, although the structure of the ligand appears to determine its affinity for binding to the receptor it does not seem to be as important in determining AF2 activity, implying that the role of agonists may be simply to release helix 12 from an inactive conformation rather than to promote its correct realignment. This may be true in the case of the ER since many environmental oestrogens that bind relatively poorly still function as agonists (Soto et al. 1991, White et al. 1994, Jobling et al. 1995).

Hormone antagonists may be classified into two basic types, either mixed agonists/antagonists or pure antagonists, exemplified by the antioestrogens tamoxifen and ICI 182780 respectively. The binding site in the receptor for both types of compound overlaps with that of oestradiol and so they act as competitive inhibitors of oestrogen action (Dauvois & Parker 1993). In vitro assays and transient transfection studies suggest that tamoxifen binding allows the receptor to dimerise and bind to DNA with high affinity but blocks transcriptional activity mediated by AF2. The agonist activity is thought to be derived primarily from AF1, which is active even when tamoxifen is bound to the receptor (Berry et al. 1990, Danielian et al. 1993, Tzukerman et al. 1994). Therefore tamoxifen has the potential to act as an agonist when the transcriptional activity of the receptor is mediated by AF2 but as an agonist on promoters or in cell types where AF1 is active. The pure antioestrogens, however, seem to block the activity of both AF1 and AF2. Evidence is emerging that part of the mechanism of antagonism may be a result of the recruitment of corepressors to the receptor (Horwitz et al. 1996, Jackson et al. 1997, Smith et al. 1997). The lack of AF2 activity in the presence of

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**Figure 3** Interactions between receptors and coactivator proteins. Activated receptors bind p160 proteins and proteins of the CBP/p300 family in a ligand dependent manner. This DNA bound complex may then stimulate the expression of hormone responsive genes by a number of possible mechanisms including the remodelling of chromatin, interaction with other transcription factors and the stabilisation of the pre-initiation complex which consists of DNA polymerase and basal transcription factors.
either type of antioestrogen is likely to reflect the incorrect realignment of helix 12. This has recently been confirmed by the determination of models for the structure of the ligand binding domain of the ER both in the presence of oestradiol and in the presence of the antagonist raloxifene (Fig. 4). The major difference in the two structural models is an alteration in the positioning of helix 12 which is a consequence of the need to accommodate the long, bulky side chain of the antioestrogen in the ligand binding cavity (Brzozowski et al. 1997). The failure to correctly reposition helix 12 and the inability to form an appropriate surface for the binding of coactivator proteins in the presence of hormone antagonists may be analysed in vitro.

Addition of either tamoxifen or ICI 182780 prevents binding in assay systems in which coactivator proteins are analysed for their ability to associate with the ligand binding domain of the ER (Henttu et al. 1997). Similarly these antagonists inhibit the stimulatory effects of coactivators such as SRC1 on receptor mediated transcriptional activation in mammalian cells. Finally it may be significant that many steroid antagonists, e.g. tamoxifen, ICI 182780 and RU486, are of greater size than the natural ligand suggesting that interference with the realignment of helix 12 may be a common mechanism of hormone antagonism.

In addition to their effects on the realignment of helix 12 pure antioestrogens have been found to suppress the action of ERs in other ways. They clearly reduce the intracellular concentration of ERs by reducing their half life (Gibson et al. 1991, Dauvois et al. 1992). In the presence of ICI 182780, receptors accumulate in the cytoplasm as a consequence of a block in nuclear uptake of the protein (Dauvois et al. 1993) and it is conceivable that this aberrant accumulation and/or conformation triggers degradation. It has also been proposed that ICI 182780 blocks receptor dimerisation and, as a consequence, inhibits DNA binding (Fawell et al. 1990b). These observations, however, were based on in vitro experiments and have been questioned by a number of workers analysing the effects of antioestrogens in intact cells (Reese & Katzenellenbogen 1991, Metzger et al. 1995).

Although it may be possible to assign all antioestrogens as either mixed agonists/antagonists or pure antagonists it is unlikely that their mechanism of action will be precisely the same as either tamoxifen or ICI 182780. Partial agonists are particularly interesting because their agonist activity is cell specific. Thus, both raloxifene and tamoxifen act as antagonists in breast cancer and agonists in bone while their action differs in the endometrium where only raloxifene is an antagonist (Draper et al. 1996, N N Yang et al. 1996). This difference must reflect distinct conformations induced in the receptor by the two antioestrogens which might, for example,
differentially affect the sensitivity of the N-terminal activation domain to phosphorylation by other signalling pathways or alter interactions between AF1 and AF2.

**Ligand independent stimulation of ERs**

The transcriptional activity of the ER has been reported to be modulated by a number of other signalling pathways involving different protein kinase cascades. The best characterised of these is the ability of epidermal growth factor (EGF) to modulate the activity of the ER. It was initially observed that the effects of oestrogen on the mouse uterus can be mimicked by the administration of EGF to animals and that this is a result of the activation of the ER in an oestrogen independent manner (Ignar-Trowbridge *et al.* 1992). These results have been confirmed by analysing the uterotrophic response in ER knock-out mice, where the oestrogen-like effects of EGF are absent, demonstrating that these responses are dependent on the expression of the ER (Curtis *et al.* 1996). The activation of ERs by EGF has been analysed in cell lines (Ignar-Trowbridge *et al.* 1993, 1996) and shown to be mediated by the MAP kinase pathway which phosphorylates a serine residue at position 118 in AF1 (Ali *et al.* 1993, Kato *et al.* 1995, Bunone *et al.* 1996). Phosphorylation of this residue seems to be required for AF1 activity but little is known about its role in transcriptional activation. Since AF1 is functional in the presence of tamoxifen it is likely to be important in the agonist effects of this antioestrogen. Cell specific variations in AF1 activity probably reflect differences in the stimulation of the MAP kinase pathway in response to growth factors and this may account for the cell-specific differences observed for the agonist activity of tamoxifen. Agents that increase cyclic AMP levels and stimulate protein kinase A activity also increase transactivation by the ER, an effect which was first demonstrated by treating cells with the neurotransmitter dopamine (Power *et al.* 1991). However, it is not known whether this is achieved by phosphorylation of the receptor itself or other proteins involved in transcriptional activation.

![Figure 5](image_url)

**Figure 5** Activation of the oestrogen receptor. The receptor may be activated by ligand binding and in addition by stimulation with growth factors acting via MAP kinase (MAPK) to phosphorylate AF1. The receptor may also be a target for tyrosine kinase(s) resulting in activation of AF2. Partial antagonists such as tamoxifen block ligand dependent activity but not growth factor stimulation of AF1. Pure antagonists prevent ligand dependent activation and may inhibit other pathways by completely blocking the action of the receptor protein.
The ER is also modified by phosphorylation on tyrosine residues, in particular at Y537 in the human protein (Arnold et al. 1995), which is located at the N terminus of helix 12 in the hormone binding domain. Phosphorylation of this tyrosine residue is not absolutely required for AF2 activity since it can be replaced with phenylalanine without affecting oestrogen dependent activation (White et al. 1997). However, analysis of other mutations introduced at this position suggests that phosphorylation may represent an alternative mechanism for ligand independent activation of the ER for the following reason. Replacement of the tyrosine residue with charged residues, either aspartic acid or glutamic acid, which might mimic tyrosine phosphorylation, generates constitutively active receptors (Weis et al. 1996, White et al. 1997). We think that this is likely to occur as a consequence of the realignment of helix 12 to form the interacting surface required to recruit coactivators. Surprisingly an alanine replacement or a serine at this position has a similar effect. One interpretation of these results is that the tyrosine residue takes part in hydrophobic interactions which maintain the receptor in an inactive state and that activation is achieved by disruption of these interactions allowing the release of helix 12, analogous to the conformational change that occurs as a result of ligand binding. The importance of this residue, and the possibility that phosphorylation may represent a means for activating the receptor, is suggested from the sequence conservation between both ERα and ERβ, which have been analysed from a number of different species all of which have a tyrosine residue in this position. However, the identification of a growth factor/kinase pathway which both phosphorylates and activates the receptor at this position remains elusive. A summary of the various pathways which may be involved in the activation of the receptor is shown in Fig. 5.

Interactions between nuclear receptors and other transcription factors

Interactions may also occur within the cell nucleus between receptors and other transcription factors, for example AP-1. This transcription factor is implicated as a critical target for many signalling pathways that regulate cell differentiation, proliferation and transformation (Angel & Karin 1991). It is important as a target for growth factors which stimulate the phosphorylation of Fos/Jun family members and may also play a role in growth inhibition by retinoids which have been shown to block AP-1 activity (Pfahl 1993, Chen et al. 1995). Oestrogens, which stimulate the proliferation of MCF-7 breast cancer cells, increase growth factor induced AP-1 activity whereas antioestrogens, which inhibit growth of these cells, inhibit the activity of AP-1 (Philips et al. 1993, Webb et al. 1995). A number of alternative mechanisms have been described to explain how nuclear receptors may modulate the activity of other transcription factors. These include the binding of receptors to DNA at composite response elements in association with other factors, e.g. the control of proliferin gene expression by the glucocorticoid receptor and AP-1 (Diamond et al. 1990), and the oestrogen regulation of ovalbumin gene expression (Gaub et al. 1990). The recent discovery of the ability of receptors to associate with proteins such as CBP/p300, which themselves have the potential to make multiple contacts with other transcription factors, suggests a possible mechanism for the integration of different signalling pathways at target gene promoters. For example, it has been reported that the ability of receptors to repress AP-1 activity results from the sequestering of CBP (Kamei et al. 1996) although other factors, as yet unidentified, have also been proposed (Saaticioglu et al. 1994, 1997).

The problem of tamoxifen insensitivity and tamoxifen resistance

There is no a priori reason why ER positive breast tumours should all be sensitive to tamoxifen treatment since their proliferation may be completely independent of the activity of the ER. Similarly, tumours may become tamoxifen resistant as a consequence of further genetic changes that allow them to proliferate in the absence of functional ER. However, a relatively high proportion of tamoxifen resistant tumours are still sensitive to alternate endocrine therapies suggesting that the proliferation of a subset of tumours is receptor dependent.

The molecular basis for the tamoxifen resistance of this type of tumour is unknown but several possibilities have been proposed. A point mutation has been identified at amino acid 537 in the human ER from a breast tumour sample in which a tyrosine residue, previously shown to be a site of protein modification by phosphorylation, as described earlier, is altered to asparagine. This generates a constitutively active protein which appears to be insensitive to the affects of antioestrogens (Zhang et al. 1997). However, mutation of this residue to a number of other amino acids, including alanine and serine, results in receptor proteins which although constitutively active are inhibited by treatment with either tamoxifen or ICI 182780 (Weis et al. 1996, White et al. 1997). Receptor mutations (Jordan et al. 1995) and variants (Fuqua 1994, Fuqua et al. 1995) have also been proposed to account for tamoxifen insensitivity. In particular, a variant lacking the oestrogen binding domain has been reported to exhibit constitutive activity and stimulate the proliferation of breast cancer cells in vitro in the presence of tamoxifen. We have been unable to confirm this observation when the expression of this variant was induced in cell lines and we
conclude that changes in other signalling pathways must also be necessary (Rea et al. 1996). One candidate is the MAP kinase pathway, which potentiates the activity of the N-terminal activation domain, AF1, even in the presence of tamoxifen. Tamoxifen, like oestrogen, seems to promote the binding of the receptor to stimulate the expression of target genes and it is conceivable that AF1 mediated transcription may therefore be increased in cells with enhanced MAP kinase activity. These target genes, however, may remain sensitive to alternative endocrine treatments. For example, ER dependent transcription would remain sensitive to aromatase inhibitors because there would be insufficient available oestrogen to activate the receptor. Similarly, ICI 182780 treatment would not only reduce ER levels but may inhibit both activation domains. Thus, the ability of alternative endocrine therapies to block tumour growth in tamoxifen resistant patients might reflect the failure of the receptor to activate a subset of target genes even when the N-terminal domain AF1 is stimulated by other signalling pathways.

**Future prospects**

An understanding of the molecular mechanisms of action of the ER is not only beginning to provide an explanation for the function of clinically useful antioestrogens but is also suggesting novel therapeutic targets. These include, for example, the interactions between coactivators and receptors which can be disrupted by specific peptides in vitro (Heery et al. 1997, Torchia et al. 1997). It may therefore be possible to prevent these interactions in vivo and block the agonistic effects of oestrogens. The two areas in which progress has been relatively slow in the past, namely the identification of the crucial oestrogen regulated target genes involved in cell proliferation and the mechanism of tamoxifen resistance, remain very active areas of research. Hopefully, with a better understanding of both the mammalian cell cycle and alternate intracellular signalling mechanisms, more progress will continue to be made in the future.

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