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I. Steroid Receptors and New (Anti-)Steroidal Agents

MODULATION OF OESTROGEN RECEPTOR ACTIVITY BY OESTROGENS AND ANTI-OESTROGENS

STEPHEN GREEN
Imperial Chemical Industries. Central Toxicology Laboratory, Alderley Park, Macclesfield, Cheshire SK10 4TJ, England

Summary—The oestrogen receptor is a member of a supergene family that includes receptors for steroid and thyroid hormones, vitamin D3, and retinoic acid. A number of additional members of the family have been cloned where the putative ligand remains to be identified. The oestrogen receptor is a ligand-activated transcription factor that modulates specific gene expression by binding to short DNA sequences (oestrogen response elements) located in the vicinity of oestrogen-regulated genes. Regions of the receptor responsible for hormone-binding, DNA-binding and activation of transcription, have been identified. The anti-oestrogen, tamoxifen (Nolvadex), behaves as a weak oestrogen agonist. A model, based upon our current understanding of the molecular mechanism of oestrogen action, will be presented to explain the cell and gene specific effects of some anti-oestrogens.

INTRODUCTION

The oestrogen receptor (ER) is a member of a supergene family, known as the nuclear hormone receptor family, that includes receptors for steroid and thyroid hormones, vitamin D3, and retinoic acid [1, 2]. A number of additional members of the family have been cloned where the putative ligand remains to be identified. The ER is a ligand-activated transcription factor that modulates specific gene expression by binding to short DNA sequences, termed oestrogen response elements (ERE), located in the vicinity of oestrogen-regulated genes. The ERE is an enhancer since it exerts its action irrespective of its orientation and when positioned at a variable distance upstream or downstream from a variety of promoters [3, 4]. Therefore the ER represents an inducible enhancer factor that contains regions important for hormone binding, ERE recognition and activation of transcription.

The ER is detectable in more than half of human breast tumours. Approximately two-thirds of these ER-positive tumours respond favourably to either the withdrawal of oestrogens or to anti-oestrogen therapy compared to only 5% of ER-negative tumours [5]. This correlation suggests that the ER plays an important role in breast cancer by mediating the mitogenic action of oestrogens. Similarly, oestrogens are mitogenic in the breast cancer cell line, MCF-7, that contains functional ER [6]. Although we have yet to understand how oestrogens act as mitogens in breast cancer we are now beginning to uncover the complexity of oestrogen-regulated gene transcription and to identify those features of the regulated genes that confer responsiveness. In order to determine the role of oestrogens in breast cancer and evaluate the potential of anti-oestrogen therapy it is important to understand the molecular mechanism of oestrogen action.

The primary amino acid sequence

The human ER (hER) cloned from cDNA libraries prepared from the MCF-7 breast cancer cell line encodes a 595 amino acid protein of 66 kDa [7]. Comparison of the primary amino acid sequence of the human and chicken ERs indicates 6 regions (A–F) of differing homology [8]. There are three highly conserved regions, A (87%), C (100%), and E (94%) and three that have been less well conserved, B (56%), D (38%), and F (41%). The DNA-binding domain (region C) and the hormone-binding domain (region E) are the most highly conserved regions.

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Receptor activation

The unoccupied ER migrates as an 8S form on low salt sucrose gradients but can be dissociated with high salt to give a 4S form. Hormone binding "transforms" or "activates" the receptor so that its affinity for DNA is increased and the receptor–hormone complex becomes tightly associated with the nuclear compartment. Receptor activation appears to involve several concomitant events including transformation from the 8S form to a 4S form; dimerization, which may represent the 5S nuclear form of the ER [9]; and increased affinity for the nuclear compartment and specific EREs.

DNA binding experiments, using mixtures of wild type and truncated ERs, have indicated that in the presence of oestradiol two ER monomers can dimerize in solution and bind to a palindromic ERE [10]. Removal of the A/B region has no effect on DNA binding. However, removal of the hormone binding domain greatly reduces the ability of the ER to bind to the palindromic ERE, suggesting the presence of a dimerization domain in this region. Similarly, this truncated receptor fails to bind to a half site ERE, suggesting that a weaker dimerization domain exists in or near to the DNA binding domain [10]. A dimerization domain has recently been identified in the C-terminal of region E [11]. The region contains a heptad repeat that is functionally distinct from the leucine zipper motif [12] and is conserved amongst the members of the nuclear receptor family. This may indicate that this region has a similar function in other receptors and that heterodimers may occur in some cases.

Hormone-induced transformation of the 8S receptor to the 4S form appears to reflect loss of an associated 90 kDa heat shock protein, hsp90 [13, 14]. Although it is not clear whether the ER is associated with hsp90 *in vivo*, formation of an ER–hsp90 complex in the absence of hormone could explain why the unoccupied ER fails to bind to the ERE. Since the ER binds to the palindromic ERE more stably as a dimer [10] then DNA binding could be reduced if interaction with hsp90 prevents dimerization. Alternatively, or in addition, hsp90 may mask the DNA binding domain thereby preventing recognition of the ERE. Interestingly, the hormone binding and dimerization activities overlap and can be functionally separated by point mutations [11]. These results indicate that steroid binding is not necessary *in vitro* to promote dimerization and support a model in which one role of the hormone is to allow dissociation of the heat shock protein so as to unmask the dimerization domain. A role for hsp90 in preventing the unoccupied receptor from binding to its target genes is favoured by experiments demonstrating that partially purified steroid receptors that have lost hsp90 bind to their cognate hormone response element *in vitro* in the absence of hormone [15, 16]. In addition, deletion of the hormone binding domain produces a truncated receptor (amino acids 1–281) that fails to interact with hsp90 [14] but binds weakly to the ERE and constitutively activates transcription at a level that is 5–10% that observed with the full length ER [17].

Multiple distinct regions important for transcriptional activation

Once bound to the ERE the hormone–receptor complex activates transcription. Functional dissection of the hER using mutants obtained by site-directed mutagenesis indicates there to be a transcriptional activation function (TAF) within both the A/B region (TAF-1) and hormone binding domain (TAF-2) of the receptor [17–22].

Truncation of the hER by removal of the hormone binding domain produces a constitutively-active transcription factor with approx. 5% wild type activity in HeLa or COS cells [17, 24] but with 60–70% activity in chicken embryo fibroblasts (CEF) and 100% activity in yeast [19,21]. These results indicate several points. (1) TAF-2 represents the major trans-activation activity of the receptor in HeLa and COS cells; (2) that there is an additional trans-activation function, TAF-1, contained within the truncated receptor and (3) the activity of TAF-1 and TAF-2 is dependent upon additional cell specific factors.

Mechanisms of oestrogen antagonism

Anti-oestrogens such as tamoxifen (Nolvadex), 4-hydroxy-tamoxifen, and the pure anti-oestrogen, ICI 164,384, antagonize oestrogen action by competing with oestrogens for receptor binding [25]. Since these compounds bind to the receptor it is pertinent to ask whether anti-oestrogens invoke or inhibit conformational changes in the receptor in order to suppress full oestrogen activity. Using gel retardation and methylation interference assays it has been demonstrated that the ER recognizes an ERE specifically when either oestradiol or hydroxy-tamoxifen is bound [10, 18, 22]. However, the

receptor fails to bind to DNA when bound to ICI 164,384 (V. Kumar, M. Berry, and P. Chambon, unpublished results; S. Fawell and M. Parker, unpublished results). This suggests that *in vivo* the ER recognizes the same target genes irrespective of whether hydroxy-tamoxifen or oestradiol is bound but does not bind DNA when bound to ICI 164,384. It is interesting to note that in the gel retardation assay the receptor–ligand–DNA complex migrates slightly slower in the presence of hydroxy-tamoxifen than it does in the presence of oestradiol [10, 18, 22], suggesting that the structure of the ER is different when bound to tamoxifen. Differences in ER structure when bound to either oestradiol or tamoxifen have also been noted when using monoclonal antibodies against the ER [26].

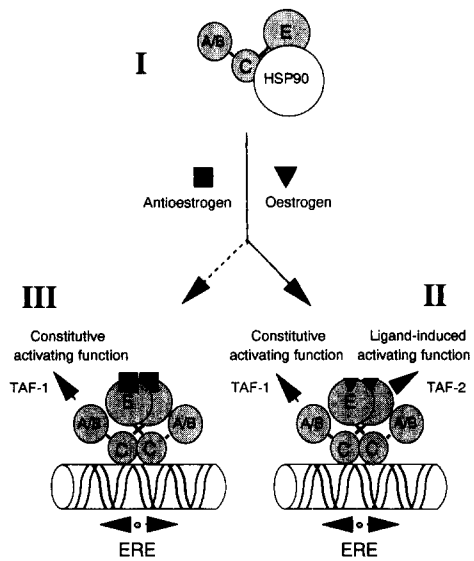

Fig. 1. Model for the mechanism of oestrogen and anti-oestrogen action. The receptor is schematically shown as three domains representing regions A/B, the DNA binding domain (C) and the hormone binding domain (E). The unoccupied receptor (I) is unable to bind strongly to the ERE possibly because of the presence of the heat shock protein hsp90 which may prevent dimerization and/or recognition of the ERE. Addition of oestrogen (II) promotes dimerization involving strong interaction between the hormone binding domains (E) and weak interactions between the DNA binding domains (C). The receptor dimer binds tightly to the ERE and activates transcription via a ligand-inducible transcriptional activating function (TAF-2) present in the hormone binding domain and a constitutive transcriptional activating function (TAF-1) in region A/B. Anti-oestrogens, such as tamoxifen, promote dimerization and DNA binding (III) whereas others, such as the pure anti-oestrogen ICI 164,384, do not. Tamoxifen appears not to activate the oestrogen-inducible transcriptional activating function in region E (TAF-2) whereas the constitutive TAF-1 region remains active. The agonistic potency of tamoxifen depends upon the ability of TAF-1 to synergize with other transcription factors that bind to the promoter and therefore is both gene and tissue specific.

Although the receptor binds to an ERE equally well in the presence of either oestradiol or hydroxy-tamoxifen, it activates transcription less well when bound to the anti-oestrogen. For example, the ER or the GAL-ER chimaera (constructed from the DNA binding domain of the yeast GAL4 transcription factor and the hormone binding domain of the hER) stimulate transcription in HeLa cells in the presence of oestradiol but not in the presence of hydroxy-tamoxifen [20]. These results imply that oestrogens, but not anti-oestrogens, promote a structural change in the ER hormone binding domain that is necessary for complete activation of a functional receptor.

Similar experiments compared the ability of the GR and GAL-GR (a chimaera consisting of the GAL4 DNA binding domain and the GR hormone binding domain) to activate transcription in the presence of either the synthetic glucocorticoid, dexamethasone, or the anti-glucocorticoid, RU486 [20]. Interestingly, RU486 behaved as a complete antagonist with GAL-GR but as a partial agonist with the intact GR demonstrating 20% the activity seen with dexamethasone. RU486 may act as a GR antagonist by promoting DNA binding in the absence of activation of the hormone binding domain trans-activation function [20]. A trans-activation function, representing approx. 20% of the activity seen with the intact receptor, has been identified in the GR A/B region [27]. Because the A/B region is absent in GAL-GR but present in the GR it is speculated that anti-hormones may function as partial agonists by promoting DNA binding thereby allowing the A/B region of the receptor to activate transcription (Fig. 1; Ref. 1).

The potency of tamoxifen as an oestrogen antagonist depends upon the tissue and response examined [28, 29]. The ability of hydroxy-tamoxifen to function as a partial oestrogen agonist appears to result from its ability to promote DNA binding of an ER in which TAF-1 is active but TAF-2 is not. A variable degree of agonist activity expressed by tamoxifen may be explained by a variation in the activity of TAF-1 in different species and tissues and also by the nature of the oestrogen-responsive target gene. For example, specific trans-activating factors may functionally co-operate with TAF-1 and the presence of these factors could be species and tissue specific (see above). Thus it can be demonstrated that hydroxy-tamoxifen is a potent ER agonist and

strongly activates transcription of the pS2 promoter in either CEF cells or yeast where TAF-1 accounts for a major proportion of the receptor activity (M. Berry, D. Metzger, and P. Chambon, unpublished results). Similarly, the contribution made by TAF-1 to target gene transcription is dependent upon other transcription factors that can bind to the promoter [30]. For example, although TAF-1 is unable to homo-synergize, it can synergize with transcription factors that possess acidic activation domains [21].

The above results have important implications for the role of tamoxifen in breast cancer therapy. Circulating levels of oestrogens are believed to play an important role in promoting the growth of ER positive breast tumours [31]. The ability of tamoxifen treatment to achieve remission in patients with oestrogen-responsive tumours is thought to be due to the role of tamoxifen as an oestrogen antagonist that competitively binds to the ER. Because tamoxifen may also act as a partial agonist it is possible that tamoxifen may partially activate transcription of some of the oestrogen-responsive genes involved in the regulation of tumour growth. Pure anti-oestrogens, such as ICI 164,384, may therefore be expected to completely suppress expression of these genes and therefore be of greater efficacy in controlling hormone-dependent breast cancer.

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