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Tamoxifen resistant breast cancer: coregulators determine the direction of transcription by antagonist-occupied steroid receptors ☆

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Abstract

Pharmacological antagonists of steroid receptor action had been thought to exert their effects by a passive mechanism driven principally by the ability of the antagonist to compete with agonist for the ligand binding site. However, recent analyses of antagonist-occupied receptor function suggest a more complex picture. Antagonists can be subdivided into two groups, type I, or pure antagonists, and type II, or mixed antagonists that can have variable transcriptional activity based upon differential dimerization and DNA binding properties. This led us to propose that receptor antagonism may not simply be a passive competition for the ligand binding site, but may, in some cases, involve active recruitment of corepressor or coactivator proteins to produce a mixed transcriptional phenotype. We used a yeast two-hybrid screen to identify proteins that interact specifically with antagonist-occupied receptors. Two proteins have been characterized: L7/SPA, a ribosome-associated protein that is localized in both the cytoplasm and nucleus, but with no known extranucleolar nuclear function; and hN-CoR, the human homolog of the mouse thyroid receptor corepressor mN-CoR. In in vivo transcription assays we show that L7/SPA enhances the partial agonist activity of type II mixed antagonists, and that N-CoR and the related corepressor, SMRT, suppresses it. The coregulators do not affect agonists or pure antagonists. Moreover, the net agonist activity seen with mixed antagonists is a function of the ratio of coactivator to corepressor. Based upon these results, we proposed that in breast tumors the inappropriate agonist activity seen with therapeutic antagonists such as tamoxifen is responsible for the hormone-resistant state. To confirm this, we are quantitating coactivator/corepressor ratios in breast tumor cells lines and clinical breast cancers. Results should provide new insights into the mechanisms underlying the progression of breast cancer to hormone resistance, and may suggest strategies for delaying or reversing this process. © 1999 Elsevier Science Ltd. All rights reserved.

1. Introduction

Nuclear receptors belong to a large superfamily of proteins that regulate gene expression in a ligand-dependent manner [1,2]. The ligands for the steroid/thyroid receptor family members are known; however, there is also a large and growing family of orphan receptors for which no ligands have been identified. Because of the vast number of homeostatic and developmental processes known to be regulated by this nuclear receptor superfamily, understanding the mechanisms mediating ligand-dependent gene activation and repression is fundamental to identifying therapeutic targets and formulating treatment strategies.

Until recently, models of activated gene transcription were relatively simple. That is, upstream activators were thought to enhance basal transcription by binding to basal factors (TFIID, TFIIB, etc.) either directly, or indirectly through interaction with one or more unknown 'adaptor' proteins. However, utilization of improved biochemical and genetic screens for pro-

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tein–protein interactions has recently led to the identification of a bewildering array of coregulatory proteins that interact with promoter-bound nuclear receptors to modulate their transcriptional responses ([3,4] and references therein). Functional effects obtained by overexpressing these proteins in transient transfection assays have led to a modification of our view of transcription activation [5–9]. Namely, that optimal activation requires the assembly of the upstream activator protein with several coregulatory proteins present at rate-limiting levels, that serve ultimately to stabilize formation of the preinitiation complex. It has even been proposed that non-DNA-binding cointegrator proteins such as CBP/p300 serve as a platform onto which coregulatory proteins, mediating responses from different signaling pathways, converge [4]. Coregulatory/cointegrator proteins are also multifunctional, as several of these factors possess intrinsic histone acetyltransferase and deacetylase activities, suggesting a potential regulatory role in chromatin remodeling [10,11]. The nuclear receptor coregulatory proteins thus far identified can be classified according to their ability to impart either activator (e.g. SRC-1/TIF2/GRIP1, RIP140, and TIF1) or repressor (e.g. N-CoR and SMRT) functions on the DNA-bound upstream activator [3,4]. The mechanisms by which these coregulators activate and repress transcription appear to be distinct, since coactivators associate with the liganded, DNA-bound receptors whereas, until now, corepressors have been shown to bind constitutively only to the unliganded, DNA-bound receptors of the retinoic acid/vitamin D/thyroid hormone receptor subfamily, and to dissociate upon ligand binding.

Our laboratory has been interested in the mechanisms by which pharmacological antagonists of steroid receptor action exert their effects. Since antagonists directly compete with agonists for the ligand binding site of the receptors, it has been suggested that agonist activity is inhibited principally by this competitive mechanism. However, more extensive, recent analyses of antagonist-occupied receptor function has suggested a more complex picture. One group of agents, including the antiestrogen, tamoxifen, and the antiprogestin/antiglucocorticoid, RU486, induce dimerization and DNA binding and have mixed agonist/antagonist transcriptional profiles that are tissue and promoter specific [12–14]. They are often referred to as type II antagonists. Tamoxifen is a prototypical agent in this respect as it has been shown to act as an antagonist in breast but as an agonist in uterus and bone [15–17]. Such tissue-specific estrogenic ligands have recently been termed selective estrogen receptor modulators, or SERMs. A second group of antagonists, called type I, includes the antiestrogen, ICI164,384, and the antiprogestin, ZK98299. They may not induce appropriate dimerization or DNA binding and produce only the antagonist transcriptional profile [14,18,19]. The fact that antagonists can be subdivided into two transcriptional groups based on differential dimerization and DNA binding properties, raised the possibility that inhibition may not be a passive, competitive process as originally thought, but may, for some antagonists, involve active recruitment of corepressor or coactivator proteins, to produce the mixed responses.

In recent studies we have focused upon the inappropriate agonist-like effects elicited by antagonists such as tamoxifen on estrogen receptors (ER) and RU486 on progesterone receptors (PR) and glucocorticoid receptors (GR). Tamoxifen is of particular interest, since its agonist-like effects in breast cancers would be misconstrued as 'tamoxifen resistance'. We speculated that antagonist-occupied, DNA-bound steroid receptors inadvertently recruit corepressor proteins to produce the suppressed transcriptional phenotype, or recruit coactivator proteins to produce the activated or agonist-like transcriptional phenotype. Binding of these proteins to antagonist-occupied steroid receptors is 'inadvertent', since they would ordinarily have no role to play on either unliganded steroid receptors, or in physiological settings involving natural steroidal agonists.

We used a yeast two-hybrid screen to identify PR-interacting proteins that specifically bind the antagonist-occupied receptors. For this, the yeast cells were purposely treated with RU486 in order to bias the screen. Several proteins have been isolated, two of which were identified: L7/SPA, originally described as a ribosome-associated protein that is localized in both the cytoplasm and nucleus, but with no known extranucleolar nuclear function [20,21]; and hN-CoR, the human homolog of the mouse thyroid receptor corepressor mN-CoR [22]. mN-CoR had been shown to interact with unliganded thyroid and retinoic acid receptors, but not with unliganded or agonist-occupied steroid receptors. More significantly however, mN-CoR had not been tested with antagonist-occupied steroid receptors prior to our discovery [23,24].

Using in vivo transcription assays we show that L7/SPA enhances the partial agonist activity seen with mixed steroid receptor antagonists like tamoxifen and RU486, and that N-CoR or the related corepressor, SMRT, suppresses it. Interestingly, L7/SPA and the corepressors do not affect the transcriptional activity of agonists, or of pure type I antagonists. The net agonist activity seen with mixed antagonists is shown to be a function of the ratio of coactivator to corepressor. These results have led us to propose that in breast tumors the inappropriate agonist-like activity seen with therapeutic antagonists such as tamoxifen accounts for the hormone-resistant state — a condition under which breast tumor cells proliferate with the continued administration of the antagonist [25]. This leads to the prediction that progression to hormone resistance is associated with an increase in the ratio of antagonist-

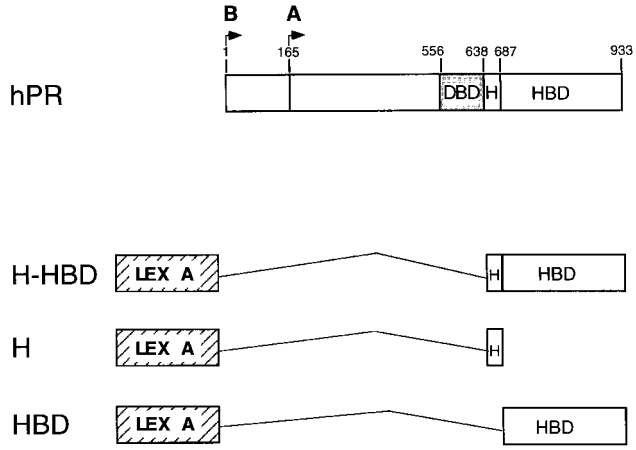

Fig. 1. Human progesterone receptor (hPR) fusion proteins used in the yeast assay. Shown are schematic representations of: hPR, B and A isoforms; DBD-DNA binding domain; H-HBD-hinge (H) and hormone binding (HBD) domains fused to LexA; H-hinge domain fused to LexA; HBD-hormone binding domain fused to LexA.

specific coactivators to corepressors in the tumor cells. This prediction has been preliminarily tested in pilot measurements of coactivator/corepressor levels in cultured breast tumor cells and breast tumors taken from patients.

2. Results

2.1. L7/SPA

Having first determined that the transcriptional response of PR to agonists and antagonists in transformed yeast cells is identical to that obtained with transiently transfected mammalian cells (Jackson et al., unpublished data), we proceeded to use the two-hybrid screen to isolate proteins that interact with antagonist-occupied PR. The hinge region (H) and hormone binding domain (HBD) of PR (see Fig. 1) were fused to the LexA DNA binding (DBD) and dimerization domains, and used as bait to screen a HeLa cDNA library. The yeast cells were treated with the type II antiprogestin, RU486. Approximately 10 million recombinants were screened, of which 28 independent positives were identified that interact with RU486-occupied H-HBD. These clones are still being evaluated. One clone, however, TJ48, was sequenced and found to be identical to nucleotide 54 to 744 of the L7/SPA cDNA, which encodes a 27 kDa protein originally defined as a potent autoantigen associated with the large ribosomal subunit [21]. The N-terminus of L7/SPA contains a basic region leucine zipper domain through which it forms stable homodimers that bind to RNA and double-stranded DNA [26]. The protein is detectable in the cytoplasm and nuclei of human cell lines and the transcript is expressed in a variety of adult mouse tissues and in human T47D breast cancer and HeLa cervicocarcinoma cell lines ([21]; Tung et al., unpublished data). It has no known extranucleolar nuclear function. However, transient expression of L7/SPA fused to green fluorescent protein in HeLa cells results in considerable accumulation not only in nucleoli, but also in other nuclear compartments (Fig. 2). To further map the L7/SPA-PR interaction, the PR H-HBD, H or HBD fused to LexA (see Fig. 1) were coexpressed with the original GAL4 AD-L7/SPA library fusion protein lacking 18 N-terminal amino acids. The cells were treated or not with RU486, and β-galactosidase activity driven by a LexA-regulated promoter was measured. Our data suggested that L7/SPA binds to the hinge domain (H), which is ordinarily blocked by the HBD. However, this inhi-

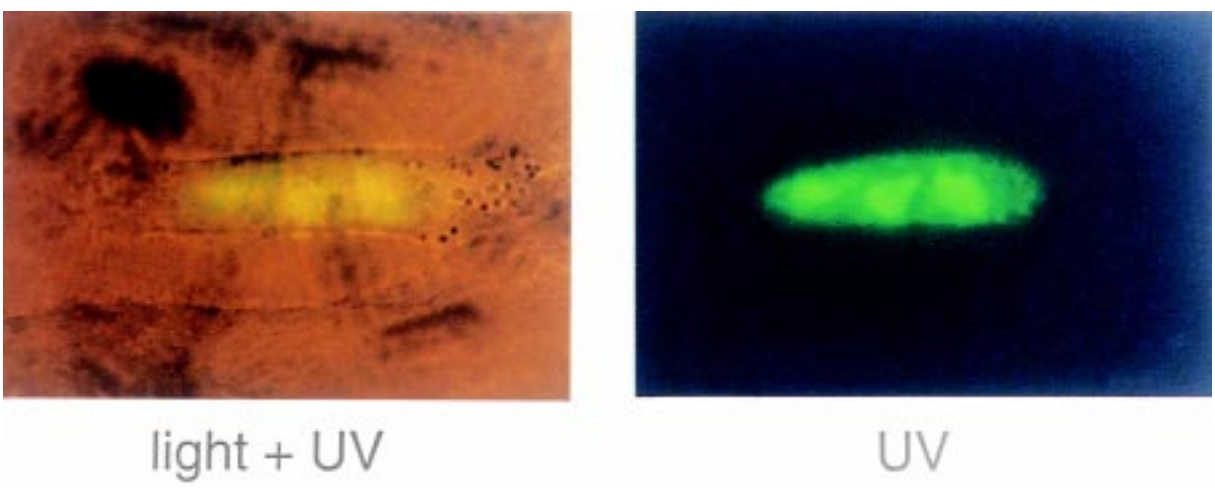

Fig. 2. Subcellular localization of L7/SPA by fluorescence microscopy. L7/SPA cDNA, inserted downstream of the green fluorescent protein in the mammalian expression vector, pEGFP- C1, was transiently transfected into HeLa cells. The cells were fixed in 4% paraformaldehyde in PBS and visualized by phase contrast microscopy. They were exposed to UV light at 450–490 nm. Note in the right photo that the fluorescence has a speckled appearance throughout the nucleus with more dense fluorescence associated with the nucleoli.

bition can be relieved by RU486 occupancy of the HBD. L7/SPA does not bind to ZK98299-occupied H-HBD. Similarly, L7/SPA binding to H-HBD of ER is dependent upon occupancy by the antiestrogen, tamoxifen, whereas the pure antiestrogen, ICI 164,384 does not promote the L7/SPA-ER interaction.

To test the effect of L7/SPA on PR-mediated transcription in mammalian cells, a C-terminal fragment containing the PR DBD-H-HBD was cotransfected into HeLa cells with a PRE_2-$TATA_{tk}$-CAT reporter [27]. Transcription by PR in the presence of the synthetic progestin agonist, R5020, was completely unaltered by overexpression of L7/SPA. However, the partial agonist activity seen with the antagonist, RU486, was enhanced by overexpression of L7/SPA. This extensive up-regulation can be completely squelched by the PR hinge domain, H, which is consistent with the yeast interaction assay showing that L7/SPA binds to this region of PR. Identical results were obtained with RU486-occupied GR and tamoxifen-occupied ER suggesting that the antagonist-specific coactivator activity of L7/SPA is a general steroid receptor phenomenon. L7/SPA had no effect on steroid receptors occupied by type I antagonists lacking partial agonist activity, such as ZK98299 for PR and ICI164,384 for ER, or on agonist-occupied steroid receptors. Interestingly, the effects of L7/SPA on ERα were quite different from ERβ. Unlike ERα, when occupied by tamoxifen, ERβ does not exhibit partial agonist activity and overexpression of L7/SPA has no coactivator effect (Horwitz et al., submitted). Moreover, when ERα and ERβ are coexpressed and treated with tamoxifen, ERβ acts as a dominant negative receptor to inhibit the tamoxifen-induced partial agonist activity, and the L7/SPA induced enhancement of partial agonist activity, of ERα. Thus, the ratio of ERα to ERβ in a tissue may, in part, determine whether tamoxifen is an agonist or an antagonist in that tissue.

2.2. N-CoR and SMRT

A second clone, TJ53, was identified in the yeast two-hybrid screen using RU486-occupied H-HBD as bait. Sequence analysis showed this partial cDNA to be the human homolog of the interaction domain (ID) of the mouse nuclear receptor corepressor, mN-CoR [22]. This was surprising since mN-CoR had no known function with respect to steroid receptors. Mapping studies using the yeast interaction assay showed that the HBD of PR but not the H region interacted with human (h) N-CoR ID in the presence of the antagonist RU486. Cloning of the full-length hN-CoR revealed a striking amino acid identity (>98%) with mN-CoR throughout the protein, with slightly greater sequence divergence in the two N-terminal repressor domains which had 96 and 80% identity. Interestingly, two splice variants were also identified, both of which contained deletions in the far N-terminal repressor domain. hN-CoR also shared 41% sequence identity with the related human corepressor, SMRT [28]. In vivo transcription studies using HeLa cells cotransfected with the PR B-isoform and the corepressors N-CoR or SMRT, showed that both corepressors completely suppressed the partial agonist activity of RU486. Identical results were obtained with RU486-occupied GR and with tamoxifen-occupied ER using either corepressor, suggesting that, like L7/SPA, the effect of the corepressors is general to members of the steroid receptor family when occupied by antagonists.

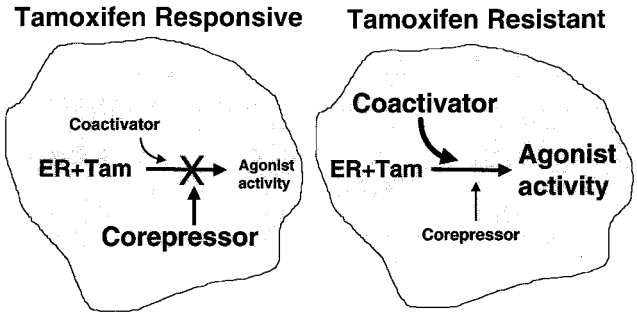

Fig. 3. Tamoxifen resistance in breast cancer. ER-estrogen receptor; Tam-tamoxifen. In tamoxifen responsive breast tumors there is a preponderance of corepressors that bind the antagonist-occupied receptors. Progression to tamoxifen resistance is characterized by cell proliferation in the presence of antagonist and a preponderance of coactivators bound to the antagonist-occupied receptors.

2.3. The corepressor to coactivator ratio determines the direction of transcription. Is it key to the development of hormone resistance?

Based on our findings with the antagonist-specific coactivator, L7/SPA, and the corepressors, N-CoR and SMRT, we postulated that the ratio of coactivators to corepressors that bind the type II antagonist-occupied steroid receptors determines the transcriptional phenotype. To test this hypothesis, HeLa cells were cotransfected with GR and either L7/SPA or SMRT, or with L7/SPA and SMRT together, and the cells were treated with RU486. As before, the partial agonist activity of RU486-occupied GR was enhanced by L7/SPA and repressed by SMRT, and when both coregulators were cotransfected in equivalent concentrations, an intermediate transcriptional phenotype was obtained, as we had predicted.

We therefore have formulated the following model of tamoxifen resistance in breast cancer (see Fig. 3). Namely, in tamoxifen responsive breast cancers, there is a preponderance of corepressors such as N-CoR and SMRT that bind antagonist-occupied receptors. Under these conditions tamoxifen has an inhibitory phenotype. Progression to tamoxifen resistance is characterized by cell proliferation in the presence of the antagonist. We

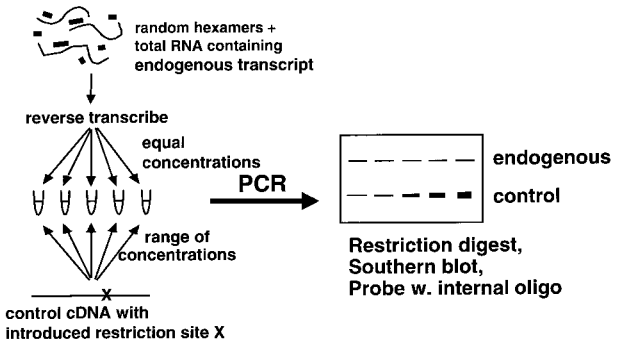

Fig. 4. Measurement of coregulator mRNA levels by quantitative PCR. Total tissue RNA containing the endogenous transcript is mixed with random hexamers and reverse transcribed. A series of PCR reactions are set up containing equal concentrations of the endogenous cDNA and a range of control cDNA concentrations. The control cDNA is identical to the coregulator cDNA except that it contains a unique restriction site inserted by site-directed mutagenesis. The cDNA mixture is amplified by PCR, digested with the appropriate restriction enzyme, and subjected to Southern blot analysis using an internal oligonucleotide probe. Gel bands are quantitated by densitometry, and the amount of endogenous mRNA is calculated from a regression analysis of the standard curve generated from the ratio of endogenous/control cDNA.

speculate that this occurs when coactivators like L7/SPA are the predominant coregulator bound to the antagonist-occupied receptors. This hypothesis suggests that the term 'tamoxifen resistance' may be a misnomer. Instead, the resistant tumor continues to respond to tamoxifen, albeit inappropriately.

2.4. Preliminary screen of breast cancer cell lines and breast tumors from hormone-dependent and resistant patients

To test our model that the coactivator/corepressor ratio dictates the progression of breast tumors to the hormone resistant state, we have initiated studies to measure coregulator transcript levels in cultured breast cells and in tamoxifen sensitive and resistant breast tumors. For this purpose, we are using a quantitative PCR assay (see Fig. 4) in which total tissue RNA containing the endogenous transcript is reverse transcribed using random hexamers, and aliquotted into several tubes. A control cDNA fragment, complementary to the endogenous transcript except that a unique restriction site has been introduced, is also added in a range of concentrations. The cDNA fragments are PCR amplified, digested with the restriction endonuclease for the introduced site to yield a smaller control fragment, and are subjected to Southern blot analysis using an internal oligonucleotide probe. Preliminary results indicate that levels of L7/SPA mRNA are higher in cultured malignant breast and HeLa cell lines than in 'normal' breast cells, and are higher in ER+/PR+ breast tumors than in ER+/PR− or ER−/PR−. This suggests that expression of antagonist-specific steroid receptor coregulators may be selectively dependent upon the growth characteristics and steroid hormone exposure of the tissue or tumor. Quantitation of SMRT mRNA in normal and malignant breast cell lines revealed the existence not only of a full-length transcript but also of splice variants. Interestingly, expression of the full-length transcript relative to the splice variants was considerably different between normal HBL100 breast cells and malignant breast cell lines. Analysis of several other breast cell lines showed substantial variation in the expression of these multiple transcripts. We are currently analyzing a panel of breast tumors for which we have samples at the hormone responsive state and after progression to hormone resistance.

3. Conclusions

Inhibition of steroid receptor function with pharmacological antagonists is not simply a passive process of agonist vs. antagonist competition for the ligand binding domain of the receptor. We suggest that it also involves the active recruitment of corepressor and coactivator proteins by the antagonist-occupied, promoter-bound receptors to produce a mixed transcriptional phenotype that manifests itself in a tissue and promoter specific manner. We have identified three proteins which include the coactivator, L7/SPA, and the corepressors, hN-CoR and SMRT, that bind adventitiously to antagonist-occupied steroid receptors to enhance or suppress the agonist activity seen with type II antagonists such as tamoxifen and RU486. In addition, our data suggest that the net agonist activity elicited by the type II antagonist-occupied receptors is dictated by the ratio of coactivators to corepressors. We propose that during steroid antagonist therapy for the treatment of breast tumors, the inappropriate expression of an agonist-like phenotype is characteristic of the hormone resistant state. Our retrospective study to measure coregulator levels in breast tumors should provide the data to determine whether this hypothesis is correct. Clearly, if overexpression of coactivators or underexpression of corepressors is associated with hormone resistance, then changes in levels of these coregulators during the course of hormone treatment may signal incipient development of resistance. This may allow timely cessation of therapy so that the antagonist can be reserved for future use. Additionally, if antagonist-occupied receptors bind specifically to certain coactivators, this binding could be used in a screening assay for new candidate antagonist ligands. Receptors occupied by ligands with mixed antagonist/agonist activities would bind such coactivators, while receptors occupied by pure antagonists would fail to bind them. This would allow rapid discrimination between type I and type II antagonists.

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