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Development of peptide antagonists that target estrogen receptor–cofactor interactions

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Abstract

We have developed a series of high-affinity peptide antagonists that inhibit the transcriptional activity of both subtypes of the human estrogen receptor (ERα and ERβ). We believe that it will be possible to develop these peptides, or corresponding peptidomimetic derivatives, into pharmaceuticals for use in the treatment of breast cancer and other estrogenopathies. It is anticipated that drugs of this type could be used in combination with classical antiestrogens, such as tamoxifen, to achieve a complete blockage of ER-transcriptional activity. Although ER has been the primary target of our studies to date, it is likely that the insights gained from this work will apply to other nuclear receptors and transcription factors.

Keywords: Peptide antagonists; Estrogen; Receptor–cofactor interaction; Transcriptional activity

1. Introduction

Until recently, it was considered that all the biological actions of estrogens and antiestrogens were manifest through a single estrogen receptor (ER) subtype that was biochemically identical in all cells [1]. However, the discovery of a second ER subtype (ERβ) significantly increased the biological complexity of estrogen action [2]. Upon binding hormone, these receptors regulate target gene transcription by binding directly to specific estrogen response elements (EREs) located within the regulatory regions of target genes. The impact of the receptor on transcription is determined by promoter context, the nature of the bound ligand and the expression level in different target cells of receptor-associated co-activator and co-repressor proteins [3–5]. Support for this model of ER action was provided in 1995 when the first bona fide steroid receptor co-activator protein, SRC-1, was identified [6]. Subsequently, a large number of potential co-activator proteins have been found, although not all have been as well validated as SRC-1 [7]. These proteins bind to agonist-activated ER and facilitate the assembly of a large complex of proteins on target gene promoters. Since some of these co-activator proteins possess intrinsic histone acetylase activity, their recruitment to target gene promoters can lead to the acetylation of histones and a subsequent decondensation of chromatin in the vicinity of the DNA-bound receptor complex [8]. The biological significance of these proteins was revealed when it was demonstrated that overexpression of co-activators like SRC-1 enhanced the transcriptional activity of ER [9,6,10]. In addition, the recent demonstration that the genetic knockout of SRC-1 in mice leads to a mild form of resistance to steroid hormones confirms the role of this co-activator in hormone signaling [11]. Of equal importance, however, was the observation that overexpression of SRC-1 enhanced the partial agonist activity of tamoxifen in target cells [10]. It is possible that the ability of tamoxifen to manifest partial agonist activity in some cells is a reflection of the expression level of SRC-1 (or another co-factor) in a particular cell, and that epigenetic changes in the expression of these cofactors may explain how cells become resistant to the antiestrogenic actions of tamoxifen over time.

In the past 3 years, it has become clear that, in addition to co-activators, steroid receptor function can also be regulated by co-repressors, proteins that sup-

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press ER activity [12,13]. To date, two nuclear co-repressor proteins, NCoR and SMRT, have been shown to be important in ER pharmacology. These co-repressor proteins bind to ER in the absence of hormone, or in the presence of antagonists, and deacetylate histones within the promoter of target genes, facilitating chromatin condensation and a subsequent inhibition of gene transcription. The potential importance of the co-repressors in ER pharmacology was suggested by studies that demonstrated that passage of human breast tumors in mice from a state of tamoxifen sensitivity to an insensitive state was accompanied by a decrease in the expression level of the co-repressor NCoR [14]. Thus, it is likely that alterations in the relative expression of co-activators and co-repressors is a key determinant of the relative agonist/antagonist activity of ER ligands such as tamoxifen.

2. There is an unmet medical need for novel antiestrogens

The antiestrogen tamoxifen has been used successfully for the past 25 years as a treatment for metastatic breast cancer and as adjuvant chemotherapy [15]. From the first studies of tamoxifen in animal models of breast cancer, it appeared that it was the antiestrogenic actions of this drug that were required for its chemotherapeutic efficacy. However, tamoxifen has a complex pharmacology and it has taken a multitude of preclinical and clinical studies to establish a definitive link between antiestrogenicity and chemotherapeutic activity [16]. It is likely that this complexity is one of the reasons why, until recently, there has not been significant interest in ER as a drug target in breast cancers. A reflection of this observation is the fact that, in the past 25 years, only two antiestrogens (tamoxifen and toremifene) have been approved in the United States for the treatment of breast cancer [17,18]. In recent years, however, several factors have led to a renewed interest in developing novel antiestrogens. Specifically, these are: (a) the clinical success of tamoxifen and the validation of ER as a therapeutic target; (b) advances in our understanding of ER action that have indicated that it is possible to develop antiestrogens whose mechanism of action is distinct from tamoxifen; (c) the recent findings showing that antiestrogens may be effective as breast cancer chemopreventatives; and (d) the finding that most antiestrogens function as estrogens in bone and can be used for the treatment and prevention of osteoporosis [19–21,15,16,22,23]. Clearly, there is an unmet medical need for novel antiestrogens for use in the treatment of breast cancer and other endocrinopathies, and efforts towards accomplishing this goal are underway. To complement these initiatives, we have undertaken to develop ER antagonists that function by targeting specific protein–protein interactions in the estrogen-signal transduction pathway.

3. Development of peptide antagonists which target ER–co-activator interactions

All of the antiestrogens that have been developed to date bind directly to ERα or ERβ and competitively inhibit agonist binding [24]. In addition to functioning as competitive antagonists, it now appears that antiestrogens function also as 'active antagonists', altering the conformation of the receptor and modulating its ability to interact with different co-activators and co-repressors within the cell [25]. It is not surprising, therefore, that even within the same cell different ER–ligand complexes can manifest different biological activities and that alterations in the relative expression of co-activators and co-repressors can lead to a change in the biocharacter of a specific ER ligand. These findings may: (a) explain why breast cancer cells that are resistant to the antiestrogenic actions of tamoxifen are not cross-resistant to the pure antiestrogen ICI182,780 and; (b) confirm the validity of ER as a therapeutic target in both tamoxifen-sensitive and tamoxifen-resistant breast cancers. Not withstanding this position, we believe that it is unlikely that major improvements over existing therapies will be made by screening for new antagonists that competitively inhibit binding of 17β-estradiol to the receptor. Therefore, we have explored the possibility of developing specific peptide antagonists of ER that function by blocking the interaction of this receptor with required cofactors in target cells.

Upon binding estradiol, ERα undergoes a conformational change that alters the relative positioning of helices 3, 4, 5, and 12 within the hormone-binding domain and facilitates the formation of a hydrophobic co-activator binding cleft in the AF-2 region of the receptor [26]. Several co-activator-binding sites have been identified; however, the majority of the validated co-activators appear to utilize the AF-2 cleft [7]. Interestingly, all of the co-activators that bind to this cleft have at least one, and usually multiple, copies of the motif LXXLL [27,28]. Although most of the ER co-activator studies to date have focused on ERα, it is clear that the major conclusions of these studies apply also to ERβ. The AF-2 domains in the nuclear receptor are closely related at the primary sequence and the structural levels [29]. Predictably, therefore, most of the known AF-2-dependent co-activators bind equally well to different receptors [7]. Due to a lack of apparent specificity, therefore, it was considered that this particular ERα–co-activator interaction would not be a bona fide drug discovery target. Recently, however, it has been shown, using the LXXLL sequences extracted from known co-activators, that not all LXXLL motifs

are the same [28]. We decided therefore to use phage display to identify LXXLL-containing peptides that could be used to disrupt specific ER–co-activator interactions. Phage display technology has been used in the past to identify and map surfaces on transcription factors and signaling molecules that may be involved in protein–protein interactions [30,31]. To our knowledge, this technique has not yet been applied to the study of the nuclear receptors.

Based on the observation that the LXXLL motif is highly conserved in the known AF-2 interacting co-activators (SRC-1, GRIP-1, AIB-1, etc.), we constructed and screened a 19-mer (X_7-LXXLL-X_7) 'focused' phage library. The phage screening protocol used for this analysis is outlined in Fig. 1 and described in detail elsewhere [32]. In brief, however, ERα was immobilized to streptavidin-coated plastic dishes in the absence of ligand, or in the presence of estradiol. The immobilized ERα was subsequently used to affinity purify specific M13 bacteriophage that interact with the receptor through specific LXXLL-containing peptides expressed on the viral pili. In these initial studies, purified, full-length ERα produced in baculovirus was used. These screens led to the identification of a large number of phage that bind to ERα with high affinity. Thus far, we have sequenced and characterized over 60 phage that interact with agonist-activated ERα. The sequence information obtained indicate that the peptides can be divided into three distinct classes: (I) SRLXXLL; (II) PLLXXLL and (III) SΨLXXLL (Ψ = L/I). The LXXLL sequences in SRC-1 and GRIP-1 do not cleanly fall into these categories. However, it must be stressed that our procedure selects for sequences that can interact with ERα as single copies, whereas the known co-activators usually contain multiple copies of these LXXLL motifs.

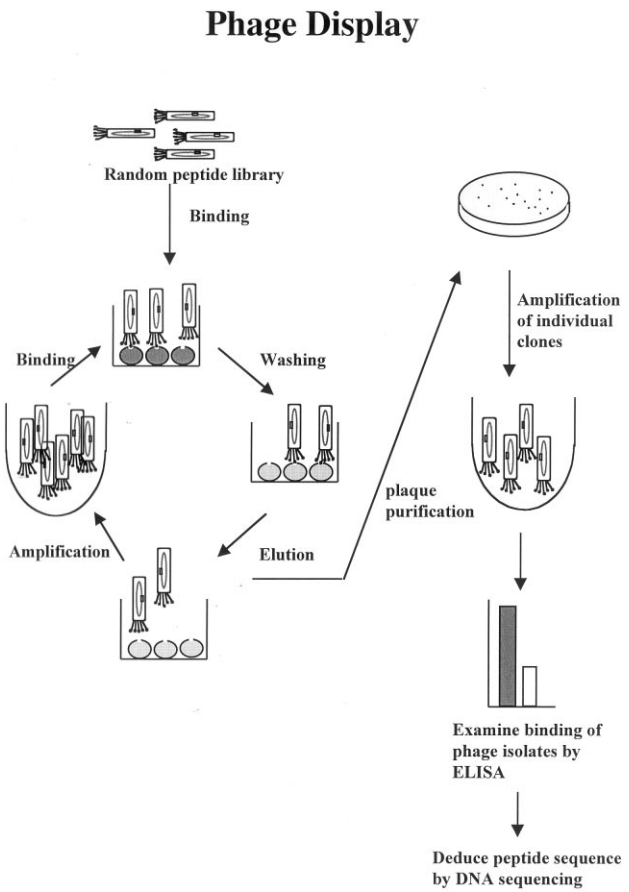

Fig. 1. Affinity selection of ER binding motifs using phage display technology. Baculovirus-expressed full-length ERα was treated with 10^{-6} M 17β-estradiol and immobilized on 96-well Immulon-4 plates. M13 phage-based random peptide libraries were incubated with target proteins, and ER binding phage were retained while the unbound phage were washed away. Bound phage were eluted using a low pH buffer, amplified in DH5αF′ cells and subjected to subsequent rounds of selection. The selection process was repeated two or three times to enrich for ER binding phage. Individual phage were plaque purified, amplified, and their binding characteristics examined by enzyme-linked immunosorbent assay. Phage that interacted specifically with estradiol-activated ER were selected and the peptide sequences were deduced by DNA sequencing.

As a first step in the characterization of these peptides, we wished to see if the sequence differences between the different classes of phage identified were manifest at a functional level on ERα. This was accomplished by performing a two-hybrid analysis using ERα–VP16 and a series of ERα–VP16 variants in which the AF-2 domain was altered (Fig. 2). As expected, all of the LXXLL sequences derived from our screens bound ERα in the presence of estradiol, as did fusion proteins containing the LXXLL domains of GRIP and SRC-1 (each domain contains three copies of the LXXLL motif). When the ability of the peptides to bind a series of ERα–AF-2 mutants was assessed, we were surprised to observe two distinct binding patterns. SRC-1, GRIP-1 (not shown), and most of the LXXLL peptides did not bind ERα when the AF-2 pocket was altered in any way. However, when the interaction studies were performed using a mutant ERα in which the AF-2 function was inactivated (ERα–3X), it was observed that the binding properties of the F6 peptide (Class III) was unaltered. It is important to note that ERα–3X was created by mutating the three charged amino acids within ERα–AF-2 [5]. One of these amino acids, Glu542, contributes to the formation of a 'charge clamp' that was demonstrated by crystallography to be required for co-activator binding [26]. The F6–LXXLL does interact with the AF-2 region of ERα, since mutation of the conserved hydrophobic amino acids within this domain (LL > AA) abolishes the binding of this peptide. This confirms that the integrity, but not the function, of this domain is required for LXXLL binding. We conclude, based on these results, that there are different functional classes of LXXLL motifs and that additional co-activators remain to be identified that share the ERα binding preferences of the F6/class III peptides.

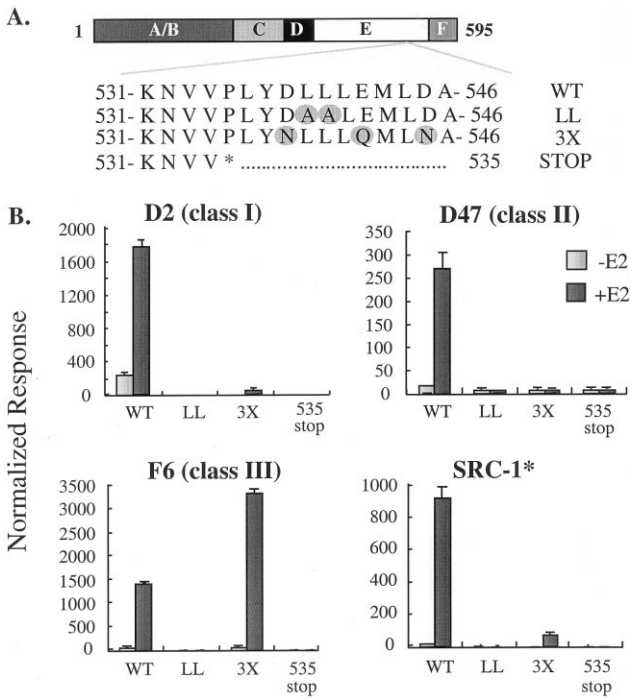

Fig. 2. Not all LXXLL peptide–ER interactions require a functional AF-2. (A) Mutant constructs used in the study. (B) The three classes of LXXLL-containing peptides (D2, D47 and F6) interact differentially with ER helix 12 mutants. Selected peptide sequences and different ER mutants were expressed as fusion proteins to the Gal4DBD and VP16, respectively. The binding activity of different peptides to ER mutants was assessed on a $5 \times$ Gal4Luc3 reporter construct. The SRC-1* construct contains the center three copies of a LXXLL motif (amino acids 621–765) fused to Gal4DBD. (Reprinted with permission from Ref. [32]. Copyright 1999, American Society for Microbiology.)

If LXXLL-containing peptides are able to block ER–co-activator interactions, then they should function as peptide antagonists of ER action when expressed in cells. To test this hypothesis, the LXXLL-containing peptides were expressed in ER-target cells as Gal4DBD fusions. This enabled us to monitor expression of the peptides using Western immunoblot analysis. Expression, in HeLa cells, of the F6–Gal4DBD peptide fusion did indeed decrease estradiol-induced ERα-dependent reporter gene expression to approximately 50% of that observed in the absence of the expressed peptide (Fig. 3, F6, open bars). Similar results were obtained when any of the high-affinity LXXLL peptides identified were evaluated in the same manner (data not shown). It has been suggested previously, that multiple copies of the NR boxes in GRIP-1 and SRC-1 can bind to ERα in a synergistic manner [27]. Thus, as expected, expression of the center three copies of the NR boxes from GRIP-1 permitted a more effective inhibition of ERα-mediated transcription than a single-copy peptide (Fig. 3, compare F6 and GRIP-1). Based on this result, we evaluated the inhibitory activity of a construct expressing two copies of the LXXLL motif on ERα transcriptional activity. The linker between the two copies was adapted from sequences found between the GRIP-1 NR box 2 and NR box 3. When analyzed in target cells, it was determined that fusion proteins containing two copies of the F6 peptide were more effective inhibitors of ERα transcriptional activity than those expressing a single copy. The $2 \times$ F6 was functionally comparable with the construct expressing the GRIP-1 NR boxes that contains three copies of the LXXLL motif (Fig. 3, $2 \times$ F6). The increased efficacy of $2 \times$ F6 as an inhibitor of ERα function required each of the two LXXLL motifs, since addition of the GRIP-1 linker sequence to a single copy of F6 did not increase its antagonist efficacy (data not shown).

4. Development of LXXLL-containing peptide antagonists that display ER subtype selectivity

Given the similarity in the sequence and structure of the co-activator binding pockets within the nuclear receptors, we were concerned that it might be difficult to develop peptides whose inhibitory activities were receptor specific. Although we did not consider specificity in our preliminary phage display screens, we decided to evaluate the specificity of the peptides identified to get an idea of how similar or different the LXXLL binding characteristics of ER were from other receptors. Our studies, and those of other workers, have

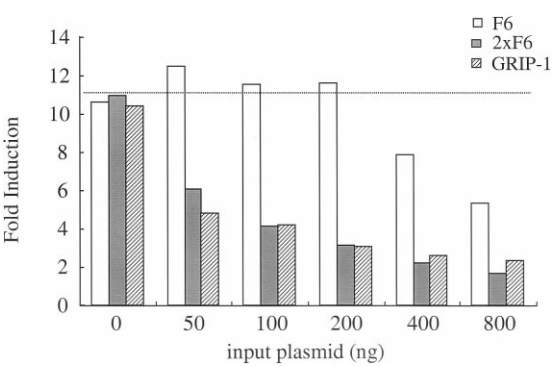

Fig. 3. LXXLL-containing peptides disrupt ERα transcriptional activity when overexpressed in target cells. HeLa cells were transfected with the ERα expression plasmid (pRST7ERa), 3XERE-TATA-Luc reporter, along with increasing amounts of a construct expressing the peptide–Gal4DBD fusions as indicated. F6 contains a single copy of the F6 peptide, $2 \times$ F6 contains two copies of the F6 peptide with 50 amino acids separating the two LXXLL motifs, and GRIP-1 contains the center three NR boxes from the co-activator GRIP-1. All these peptides were expressed as fusion proteins to Gal4DBD. In addition, a pCMVβgal plasmid was cotransfected to normalize for transfection efficiency. After transfection, cells were induced with 10^{-7} M 17β-estradiol for 16 h before assaying. Fold induction represents the ratio of estradiol-induced activity versus no-hormone control for each transfection. (Reprinted with permission from Ref. [32]. Copyright 1999, American Society for Microbiology.)

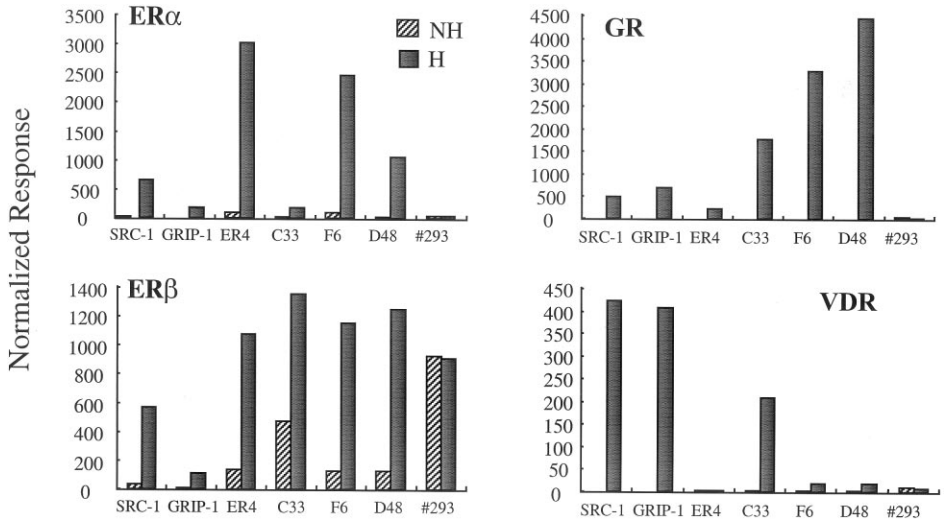

Fig. 4. Nuclear receptors have distinct preferences for different LXXLL motifs. The interactions between different LXXLL motifs and nuclear receptors were assayed using a mammalian two-hybrid system. Full-length receptors and selected peptides were expressed as VP16 and Gal4DBD fusion proteins, respectively. The magnitude of these interactions was measured using a $5 \times$ Gal4Luc3 reporter gene. Hormones used in this experiment: 10^{-7} M 17β-estradiol for ERα and ERβ, 10^{-7} M dexamethasone for GR, and 10^{-7} M 1,25-dihydroxyvitamin D3 for VDR. The luciferase activity was normalized to the activity of a co-transfected pCMVβgal.

shown that the receptor binding preferences of co-activators can be altered by changing the amino acid residues that flank the LXXLL motif in these proteins [32,33]. Thus, we next wished to define the sequences within the NR box that enable it to discriminate between receptors using the LXXLL-containing peptides identified. For this study, we selected representative members of each class of LXXLL identified from our focused library along with another LXXLL-containing peptide (#293), which was identified previously in screens of random peptide libraries for peptides that interacted with estradiol-activated ERβ. This specificity analysis was accomplished by performing mammalian two-hybrid assays, in which the LXXLL-containing peptides were fused to the Gal4DBD and the full-length receptors were expressed as VP16 fusion proteins. A complete profile of the NR–peptide interactions has recently been described [32]; however, the results of a representative experiment is shown in Fig. 4. Clearly, even in this limited analysis, it appears that receptor selectivity is manifest. Of particular importance was the observation that the #293 peptide interacts well with ERβ, weakly with thyroid hormone receptor α and retinoic acid receptor α (not shown), and not at all with ERα, vitamin D receptor, glucocorticoid receptor α or any other receptors tested. Thus, receptor specificity can be achieved by altering sequences flanking the core LXXLL motif. Predictably, when peptide #293 was expressed in target cells, it completely blocked ERβ transcriptional activity while having no effect on ERα signaling (not shown). Based on this result (and similarly compelling examples of specificity), we believe that it is possible to identify LXXLL-containing peptides that interact with and inhibit ERα or ERβ in a specific manner.

5. Identification of peptides that interact with ERα and ERβ at sites other than the LXXLL–co-activator binding pocket

it is now apparent from a large number of studies, using various technical approaches, that different ER agonists and selective estrogen receptor modulators and antagonists induce different structural alterations within the ER, and that there exists a close relationship between the structure of an ER–ligand complex and its biological function [34–36,3,37,38,26,24]. This has led to the hypothesis that different surfaces on ER are exposed in the presence of different ligands and that these surfaces may enable ER to interact with different subsets of cofactors (co-activators and co-repressors) within target cells [39,37,24]. Not surprisingly, therefore, it was possible to identify, using phage display, a series of peptides whose ability to interact with ER was influenced by the nature of the bound ligand [37]. The details of these studies, which have been discussed in detail elsewhere, led to the identification of a peptide (αII) that interacted with ERα in the presence of any ligand, and a series of peptides (α/βIII, IV and V) that interacted specifically with tamoxifen activated ERα or ERβ [40,37]. We were surprised with this latter result in view of the accepted hypothesis that tamoxifen functions as an antagonist because it reduces the affinity of ER for required co-activator proteins and that agonist activity is manifest when the expression level of these

co-activators rises to a point that it overcomes this reduced affinity [14,10]. Our data suggest that, in addition to this model, it must be considered that tamoxifen binding leads to the presentation of protein binding sites that are not physiologically relevant, but which may allow ER to interact in an ectopic manner with other transcription factors. These data provide compelling evidence that different ligands induce different structural alterations within ER, and that the ability of the cell to distinguish between these structures may explain the distinct pharmacology of these ligands observed in vivo. In addition, however, it suggests that, by targeting ligand-specific receptor–cofactor interactions, it may be possible to develop novel antiestrogens for use in the treatment of breast cancer and other endocrinopathies.

6. Different ER ligands induce distinct alterations in ER structure within the cell

We were concerned that the surfaces identified using phage display may only be exposed in vitro, when assayed using purified ERα, and that within the environment of the cell, these allosteric changes may not occur or may not be manifest. To address this issue, we used a two-hybrid assay to evaluate the ability of the peptide sequences identified to discriminate between different ERα–ligand complexes in target cells. The results obtained with some of the more interesting peptides are shown in Fig. 5. Using this assay, it was determined that the interaction of LXXLL-containing peptides, such as α/βI, occurs only in the presence of

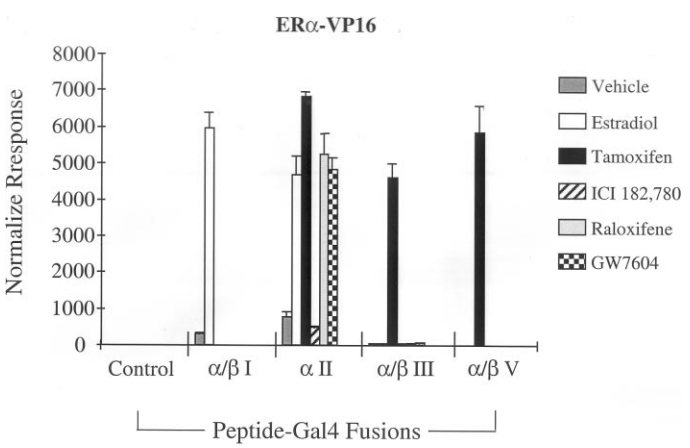

Fig. 5. ER–peptide interactions in mammalian cells. HepG2 cells were transiently transfected with expression vectors for ER–VP16, the peptide–Gal4 fusion proteins and a luciferase reporter construct under the control of five copies of a Gal4 upstream enhancer element. Transfection of the Gal4DBD alone is included as control. Cells were then treated with various ligands (100 nM) as indicated in the figure and assayed for luciferase and β-galactosidase activity. (Reprinted with permission from Ref. [40]. Copyright 1999, American Association for the Advancement of Science.)

17β-estradiol. The peptides α/βIII and α/βV bind in the presence of tamoxifen, and the αII peptide binds in the presence of any ligand tested. These results parallel closely the data generated in vitro and strongly support the hypothesis that there is a relationship between the structure of an ERα–ligand complex and its biological activity.

We have begun to map the surface(s) on ERα that interact with the various classes of peptides identified. These initial studies have indicated that they all bind within the ligand binding domain of ERα. Not surprisingly, α/βI interacts with the ERα–AF-2 core motif within ERα helix 12. In contrast, however, α/βIII, α/βV and αII bind at another site as deletion of helix 12 from ERα does not compromise their binding. These data suggest that tamoxifen binding to ERα presents a unique surface on ERα not presented by estradiol. Our ability to identify peptides that interact with this site suggests to us that there may be proteins within the cell that can bind this site. We initially tested whether this site may represent a co-repressor binding site. However, our preliminary data supports the hypothesis that the binding sites for NCoR and the tamoxifen-specific peptides that we have identified are distinct. Based on these results, we predicted that peptides that interacted with ERα at sites distinct from the AF-2 co-activator binding pocket would function as peptide antagonists of ER action.

7. Development of peptide antagonists that inhibit the partial agonist activity of tamoxifen

The identification of peptides that interact specifically with tamoxifen-activated ERα has prompted us to explore the possibility of developing them into antagonists of tamoxifen partial agonist activity. For this purpose, we have assessed the ability of each of the different classes of peptides identified to block estrogen and tamoxifen agonist activity when introduced into cultured HepG2 cells. The results of this experiment are shown in Fig. 6. In this system, tamoxifen manifests about 35% of the agonist activity of estradiol. As expected, expression of increasing amounts of the LXXLL α/βI–Gal4DBD fusion protein in the cell specifically inhibits estradiol-mediated transcription by about 30%. These initial studies were carried out using a single copy of the α/βI peptide. However, co-activators usually contain two or more copies of a LXXLL motif. Consequently, we tested fusion proteins containing two copies of the α/βI peptide and were able to inhibit estradiol-mediated transcriptional activity completely (data not shown). Similarly, we constructed αII, α/βIII, or α/βV–Gal4DBD fusions, and demonstrated that these proteins were capable of specifically inhibiting tamoxifen-activated transcription when introduced into target cells.

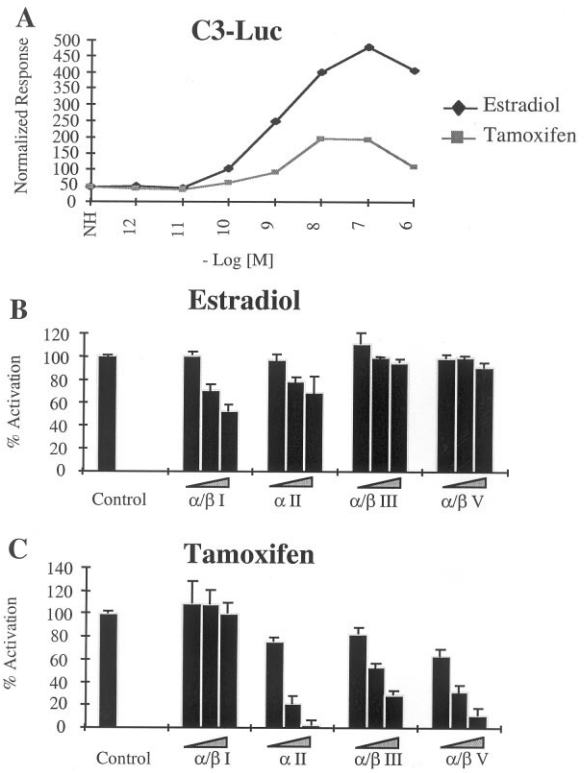

Fig. 6. Disruption of ER/ERE-mediated transcriptional activity. (A) HepG2 cells were transfected with the estrogen-responsive C3-Luc reporter gene along with an ER expression vector. Cells were induced with either estradiol or tamoxifen as indicated. NH, No hormone. (B) HepG2 cells were transfected as earlier except that expression vectors for peptide–Gal4 fusions were included as indicated. Control represents the transcriptional activity of estradiol (10 nM)-activated ER in the presence of the Gal4DBD alone and is set at 100% activity. Increasing amounts of input plasmid for each Gal4 peptide fusion is also shown (triangle) with the resulting transcriptional activity presented as percent activation of control. (C) As (B) except that 4-OH tamoxifen (10 nM) was used to activate the receptor. (Reprinted with permission from Ref. [40]. Copyright 1999, American Association for the Advancement of Science.)

It has been shown by several groups that, in addition to manifesting agonist activity through the classical ER/ERE-mediated pathway, tamoxifen and estradiol may also function as agonists by facilitating the interaction of ER with a promoter-bound AP-1 complex [41,42]. In this manner, both estradiol and tamoxifen can positively regulate AP-1-responsive genes. We reconstituted this AP-1 responsive pathway in HepG2 cells and were able to show that estradiol agonist activity in this system was blocked by expression of either the α/βI or the αII peptides [40]. When analyzed in a similar manner, it was demonstrated that tamoxifen agonist activity could be inhibited by expression of either the αII, α/βIII or α/βV peptide fusions (data not shown). These data suggest, but do not prove, that the surfaces presented on ER in the presence of either estradiol or tamoxifen are physiologically important and represent bona fide cofactor binding sites. Regardless, the ability of these peptides to inhibit ER-transcriptional activity in reconstituted transcription systems suggests that it may be possible to develop pharmaceuticals that target the surfaces exposed on ERα when it is occupied by different ligands.

8. Final comments

The studies we have performed to date indicate that it is possible to identify peptides that interact with agonist-activated ERα and/or ERβ, and that these peptides can be used to block ER-mediated transcriptional activity in target cells. We believe, therefore, that by targeting specific receptor–cofactor interactions, it will be possible to develop a novel class of ER antagonists. In the past, protein–protein interactions were considered to be poor targets for drug discovery, as it was technically difficult to obtain small molecules (drugs) that could mimic the interactions occurring between proteins or peptides. However, the recent identification of small molecule agonists and antagonists of the somatostatin, granulocyte-colony stimulating factor and growth hormone receptors, and molecules that block the ability of the epidermal growth factor and platelet derived growth factor receptors to interact with their intracellular target proteins, has indicated that complex protein–protein interactions can be targeted with small molecules [43–47]. If successful, this approach will provide a second class of pharmaceutical agents for the treatment of breast cancer and other estrogenopathies.

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