Combinatorial Phage Library Screening for Estrogen Receptor Interacting Peptides and Applications to the Study of Xenoestrogen Biology and Pharmacology

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The steroid hormone estrogen is a key regulator of growth, differentiation and function of a wide variety of target tissues of diverse function. Estrogen plays a critical role in the development of the female reproductive system, in directing neuroendocrine control of ovulation, and in regulating female sexual behavior. In addition, however, this hormone clearly has functions in tissues other than those related to reproduction. Specifically, estrogen is involved in the development and maintenance of skeletal integrity and is an important regulator of triglyceride and cholesterol homeostasis in both males and females.

The biological effects of estrogen are mediated through intracellular receptors, which function as ligand-inducible transcription factors in target cells. The human estrogen receptor (ER) belongs to the nuclear receptor superfamily whose members include the receptors for steroids, thyroid hormone, retinoic acid, vitamin D, and orphan receptors for which no ligands have yet been identified. The mechanism of action of ER is similar to that of other nuclear receptors. In the absence of hormone, the receptor is sequestered within the nuclei of target cells in a multiprotein inhibitory complex. The binding of ligand induces an activating conformational change within ER, an event that promotes homodimerization and high affinity binding to specific DNA response elements (EREs) located within the regulatory regions of target genes. The DNA-bound receptors contact the general transcription apparatus (GTA) either directly or indirectly via coactivator proteins. These interactions stabilize the formation of the transcription pre-initiation complex leading ultimately to an upregulation of gene transcription. In addition to the endogenous hormone, ER function can be modulated by endocrine disrupting chemicals termed xenoestrogens, which impact on estrogen biology by enhancing or blocking ER target gene transcription, thereby functioning as ER agonists or antagonists.

Estrogens and xenoestrogens manifest their biological activities through two distinct receptors

Until recently it was thought that all of the biological actions of estrogens and xenoestrogens were manifest through a single receptor located within target cell nuclei. However, the identification of a second estrogen receptor, ERβ, has indicated that estrogen signaling is more complex. The two ER subtypes, ERα and ERβ, share extensive amino acid similarity in their ligand- and DNA-binding domains, but minimal homology within their amino-terminal regions. Not surprisingly therefore, these receptors exhibit similar, but not identical, ligand binding characteristics and interact with the same DNA response elements. Both receptors contain a transcriptional activation function (AF-2) domain in the carboxyl-terminus that mediates the ligand-dependent transcriptional activity of the receptor through binding coactivator proteins. Analysis of estrogen signaling has revealed that the most obvious difference between the two receptors is that ERα is a more efficient activator of ERE-containing genes than ERβ under most circumstances. In addition, it has been noted that ERβ can interact in a constitutive manner with target promoters and can attenuate the ligand-activated transcriptional activity of ERα. Thus, in cells where both receptors are expressed, overall estrogen responsiveness is reduced. Notably, ERα and ERβ also display distinct transcriptional responses when bound by different xenoestrogen ligands, indicating that the two receptors play distinct roles in xenoestrogen biology.

Unfortunately, most of the functional differences between ERα and ERβ have been observed in reconstituted transcription systems *in vitro*, and their significance to ER biology *in vivo* remains to be established. Thus, there is a need for subtype-selective agonists and antagonists that will permit the transient manipulation of receptor function in intact animals. To complement the efforts of others who are engaged in screening for small molecules that interact with the ligand-binding pockets of ERα and ERβ, we undertook a novel approach to develop subtype-specific antagonists that inhibit ERβ action in a manner distinct from known antiestrogens.

Targeting ER-coactivator interactions to identify ER subtype-selective antagonists

All of the currently available ER antagonists function by (a) binding to the receptor ligand-binding domain thereby blocking agonist access and (b) inducing a conformational change within the receptor that prevents it from interacting efficiently with transcriptional coactivators such as SRC-1 and GRIP1. Specifically, it has been shown that agonist binding to the receptor induces a conformational change that permits the formation of a hydrophobic pocket, enabling the receptor to interact with the LXXLL (Leu-X-X-Leu-Leu) motif contained within the receptor interaction domains of most of the validated coactivators. The conformational changes induced in ER upon antagonist binding do not permit coactivator recruitment. Clearly, the most direct method of inhibiting ER function would be to develop drugs that bind directly to the coactivator-binding pockets within ERα or ERβ and block coactivator recruitment. Given that the coactivator-binding pockets in ERα, ERβ, and other nuclear receptors are structurally similar and that most of the known coactivators do not appear to demonstrate receptor selectivity, it was not obvious that the receptor-cofactor binding pocket was a *bona fide* drug target. However, recently we and others have shown that all LXXLL motifs are not functionally equivalent and that by altering sequences flanking the core LXXLL motif, some receptor selectivity can be obtained.

Combinatorial Phage Display Technology Screening for ERβ-interacting peptides

Building on these observations, we have screened combinatorial phage libraries, expressing peptides in the format X_7LXXLLX_7, for peptides that interact in a specific manner with ERβ. In this manner, a total of 70 phage were identified whose ability to interact with ERβ was confirmed using a secondary phage ELISA assay. Cross-screening revealed that 37 of the phage identified bound to both ER subtypes whereas 33 interacted selectively with ERβ. The latter subset of phage expressing LXXLL-containing peptides were brought forward for further analysis.

We next performed a mammalian two-hybrid assay to assess the ability of the peptides (EBIP= ERβ interacting peptide) to interact selectively with ERβ in intact cells. When analyzed in the two-hybrid assay, we found that 15 of the 33 peptides studied interacted with ERβ, but not ERα. Interestingly, several of the peptides were able to bind ERβ in the absence of ligand, suggesting either that a portion of the unliganded ERβ resides in an active conformation, or that the binding of LXXLL-containing motifs to the receptor may facilitate activation.

One of the major objectives of this research is to develop highly specific inhibitors that can be used to evaluate the relative contributions of the two ER subtypes in estrogen signaling. Consequently, we next examined the ability of the 15 ERβ-selective peptides to interact with other members of the nuclear receptor superfamily. This was accomplished using the mammalian two-hybrid assay to examine interactions of each peptide with 11 different nuclear receptors. No clear pattern emerged from these studies, as each receptor appeared to exhibit distinct peptide binding preferences. Based on these results, it is likely that most of the receptors will eventually be found to display different cofactor preferences. The most important result, however, was that the LXXLL-containing peptides EBIP-56 and EBIP-92 interacted exclusively with ERβ and did not interact with any other receptor under the conditions tested.

Cumulatively, these findings show that it is feasible to develop specific nuclear receptor antagonists by targeting receptor-coactivator interactions.

Evaluation of the antagonist properties of peptides that interact with ERβ in a specific manner

The antagonist efficacy of the ERβ-specific peptides, EBIP-56 and EBIP-92, was next evaluated. The nuclear receptor interaction regions of most of the well-validated coactivators have been shown to contain multiple LXXLL domains, which facilitate the interaction of these proteins with the AF-2 coactivator-binding pocket of their targeted receptor. Reflecting this observation, we created two copy-Gal4DBD fusions of our peptides. The two LXXLL motifs were separated by sequences corresponding to the linker region between LXXLL motifs 2 and 3 of the coactivator GRIP1. When expressed in mammalian cells, we observed that while the GRIP1 NR-box sequences inhibited the activity of ERα by 60%, the peptides 2xEBIP-56 and 2xEBIP-92 had no effect on transcriptional response. However, when tested on ERβ, it was found that the 2xEBIP-56 and 2xEBIP-92 peptides suppressed estrogen-stimulated transcriptional activity by 82% and 97%, respectively. Western immunoblotting was used to demonstrate that expression of the peptides did not alter cellular levels of ERβ. Thus, ERβ transcriptional activity can be inhibited in a specific manner by using LXXLL-containing peptides to block interactions with coactivators.

Comparison of the amino acid sequences of the ERβ -interacting peptides

Alignment of the two classes of ERβ-interacting peptides identified (ERβ-selective and ERβ-specific) was next performed (Table I). Surprisingly, minimal homology was observed outside of the conserved LXXLL motif between members within the same class. Therefore, it is likely that the residues flanking the LXXLL motifs are important determinants of higher order peptide structure. Interestingly, we observed that the two ERβ specific peptides, EBIP-56 and EBIP-92, contain a tryptophan (W) at position –5 relative to the central LXXLL motif, a residue shared by none of the other 68 ERβ-interacting peptides that were isolated in the primary screen. Therefore, we postulated that the tryptophan influenced the specificity of the peptide-ERβ interactions. To test this hypothesis, the tryptophan residue in the EBIP-92 peptide was converted to glutamine (an amino acid found at this position in many of the peptides identified). Analysis using a mammalian two-hybrid revealed that while the wild-type EBIP-92 interacted with ERβ in a hormone-dependent manner, a variant peptide in which the tryptophan residue at position –5 was mutated was unable to interact with ERβ. These studies demonstrate the importance of the sequences surrounding LXXLL motifs in determining receptor selectivity, and suggest that it may be possible to use site-directed mutagenesis to optimize the interactions of the peptides identified with their protein targets.

Table I: Comparison of the amino acid sequences of the ERβ interacting peptides.

ERβ-selective peptides

	-3 -2 -1	L X X L L	+1 +2 +3
Peptide EBIP-37	T G G G V S L	**L L H L L**	N T E Q G E S
Peptide EBIP-41	R R D D F P L	**L I S L L**	K D G A L S Q
Peptide EBIP-44	Y G L K M S L	**L E S L L**	R E D I S T V
Peptide EBIP-45	M S Y D M L S	**L Y P L L**	T N S L L E V
Peptide EBIP-51	F P A E F P L	**L T Y L L**	E R Q G M D E
Peptide EBIP-96	V E S E F P Y	**L L S L L**	G E V S P Q P
Peptide EBIP-49	V S S E G R L	**L I D L L**	V D G Q Q S E
Peptide EBIP-53	D T P Q S P L	**L W G L L**	S S D R V E G
Peptide EBIP-60	G G T Q D G Y	**L W S L L**	T G M P E V S
Peptide EBIP-66	S L P E E G F	**L M K L L**	T L E G D A E
Peptide EBIP-70	V M G N N P I	**L V S L L**	E E P S E E P
Peptide EBIP-76	V L V E H P I	**L G G L L**	S T R V D S S
Peptide EBIP-87	Q T P L	**L E Q L L**	T E H I Q Q G

ERβ-specific peptides

Peptide EBIP-56	G S **W** Q D S L	**L L Q L L**	N R **T** E L M A
Peptide EBIP-92	S V **W** P G P E	**L L K L L**	S G **T** S V A E

The ERβ-interactacting peptides were divided into two classes: ERβ-selective and ERβ-specific. In the latter class, consiting of peptides EBIP-56 and EBIP-92, the conserved tryptophan at position -5 relative to the LXXLL motif and conserved threonine at position +3 in these two sequences is shown in bold.

Consistent with our original objective, we have been successful using a novel approach, combinatorial phage display screening, to develop subtype-specific antagonists that inhibit ERβ action in a manner distinct from known antiestrogens. We are currently developing suitably formulated versions of these peptides that can be introduced into animals to study ERβ-action *in vivo*. This will enable us to identify target tissues of estrogen and xenoestrogen action and to determine the relative contributions of ERα and ERβ xenoestrogen biology.

ER-interacting peptides as tools to evaluate the estrogenicity of environmental toxicants

The interaction of LXXLL-containing coactivators such as SRC-1 or GRIP1 with ERα and ERβ has been shown to require agonist activation of the receptor and is blocked by antagonists. Therefore, we considered that binding of the LXXLL-containing identified in the phage library screening would demonstrate similar agonist specificity and perhaps could be used to evaluate the estrogenicity of uncharacterized environmental toxicants with potential endocrine disrupting functions. To determine the feasibility of a peptide-interaction assay to predict the agonist activity of ER ligands, a mammalian two-hybrid assay was used to examine the effect of different known ER ligands (agonists and antagonists) on peptide binding in intact cells.

Notably, the interaction of all peptides examined was enhanced by the addition of the agonists 17β-estradiol (estrogen) and genistein. However, administration of antiestrogens, in the absence of agonist, did not permit peptide binding and furthermore, antagonized the basal receptor-peptide interactions. Interestingly, peptide EBIP-92 appeared to interact more efficiently with genistein-activated ERβ than that activated by estradiol. These data, indicating that genistein and estradiol do not function in the same manner when assayed on ERβ, were interesting in light of the unique properties that have recently been ascribed to genistein. Similar differences in efficacy were noted when the experiments were repeated over a full range of ligand concentrations. Consistent with the results of recent crystallography studies, these findings indicate that estradiol and genistein induce unique conformational changes within ERβ, and that genistein-liganded ERβ may interact with cofactors in a different manner than the estradiol-activated receptor. We propose that the mechanistic differences between the two ER agonists underlies their discrete biological activities.

The observation that LXXLL-peptide binding was completely agonist-dependent prompted us to investigate the feasibility of the peptide binding assay to predict the estrogenicity of environmental toxicants. For these studies we used LXXLL peptides identified in the phage display screen that were capable of interacting with both ERα and ERβ to determine whether we could develop an assay for both receptor subtypes. In a blind study, we measured peptide binding to ERα or ERβ in the presence of 3 endocrine disrupting compounds (Toxicant X, Y and Z). Analysis of ERα in the assay revealed that peptide EBIP-52 interacted with ERα in the presence of each compound, albeit with different efficacies. Notably, the estrogenicity of each compound directly correlated with the quantitative measurement of peptide interaction. Interestingly, the pattern of peptide binding was distinct when tested on ERβ, suggesting that different compounds induce distinct conformational changes in the two receptors. As seen with ERα, however, the relative magnitude of peptide binding directly paralleled the ability of each toxicant to activate ERβ-mediated transcription. Overall, these results demonstrate that peptide binding correlates with the estrogenicity of different ligands, indicating that the interaction of peptides with ER bound to uncharacterized compounds can be used to predict their biological activities. This provides a novel functional screen with high-throughout capability to analyze the potential of environmental toxicants to disrupt estrogen biology.

Summary

The use of combinatorial phage library screening has led to the identification of a series of ER-subtype specific antagonists that inhibit ER action in a manner distinct from known antiestrogens. Current studies are focused on developing an approach to introduce these peptide antagonists into animals, which will permit a transient manipulation of receptor function *in vivo* and enable an evaluation of the relative contribution of ERα and ERβ to estrogen and xenoestrogen biology. In addition to their antagonist properties, the ER-interacting peptides have a broad range of applications including their use to predict the estrogenicity of environmental toxicants with potential endocrine disrupting function. Furthermore, the

use of the peptides as conformational probes has revealed that estrogen and xenoestrogens induce unique conformational changes in ER, demonstrating there are mechanistic differences between endogenous and exogenous ER ligands that correlate with their distinct biological activities. We anticipate that phage display technology can be used to identify specific peptide antagonists of other nuclear endocrine receptors, such as those for progestins, androgens, retinoids and thyroid hormone, and thus providing additional tools to evaluate the biological activities of endocrine disrupting toxicants through diverse hormone signaling pathways.

Q&A

Kanno: Questions and comments, please.

Q: Do you suspect that you have created a new protein, or that their might be an endogenous peptide that could also block ERβ?

Hall: I'm sorry, could you repeat that?

Q: In the LXXLL motifs that you have made, do you think you have created a new protein or do you think the cell has one just like that that it is using?

Hall: That is a really good question that we are actually asked a lot. Through all the peptides that I have screened through and sequenced, only one of them has correlated to a known protein, and that is the corepressor RIP140.

All of the other LXXLL containing peptides have been to nonsense proteins, or just theoretical proteins, so they actually are not to actual proteins, or they do not for the most part correspond to actual proteins within the cell, which could explain why one advantage of using these peptides over using pep to analyze structure and activity of the receptors is that they bind with much higher affinity than the actual known LXXLL motifs from real coactivators. They are almost like a super interacting protein for the receptors.

Q: A very fascinating presentation. I would like to know the distribution of this new protein, EBIT 56 and 92 and other binding proteins. Where they are located in the target tissues and so on?

Hall: I'm sorry, I did not understand you question.

Q: I understand that these proteins… are they located *in vivo* tube, or are they all human made proteins?

Hall: Oh, the EBIT 56 and 92? Yes, they are synthetic peptides. They do not correspond to real proteins.

Q: Are there very similar types of the proteins that could exist in the cells?

Hall: The LXXLL motifs are contained within real proteins, but most of the specific peptides that we identified do not correlate to real proteins.

Q: Do you think there is some possibility that similarly structured proteins are located in *in vivo* situation in several target tissues? As you know, the expression of ERβ and ERα in organism is different. So if you can find if these proteins are also located in cells *in vivo* situation, these proteins should have some action, i.e. the correlation between these receptors. Do you understand? [Japanese]

Hall: That is a good question: do ERα and ERβ overlap in their distributions, or whether they are, in fact, unique. They are expressed in many of the same tissues, although they also have their unique distributions, but what is important is that even when they are expressed in the same tissue, they seem in most cases to localize to different cells.

Q: How about the peptide which you have found?

Hall: Well, the work that we have done that I showed today has been done just in mammalian tissue culture. What we are hoping to do, what we are trying to do now and what we are doing, is developing viral delivery systems to introduce them into animals and we can then introduce them in a manner to target certain tissues as you are suggesting, so that we could, in fact, target maybe some effects of xenoestrogen action in some tissue and not others.

Q: Thank you.

Kavlock : Thank you.