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13. ABSTRACT ***(Maximum 200 Words)***

Estrogen is mitogenic in most estrogen receptor (ER) positive breast cancers and the use of anti-estrogen like tamoxifen has been quite successful in the treatment of this disease. Although patients initially respond well to anti-estrogens, resistant tumors often develop within 5-10 years of treatment. The purpose of this research is to develop mechanistically distinct therapeutics by directly blocking the interaction of ER with coactivator proteins required for its activity. In the previous funding period, we reported the identification of conformational peptide probes that detect the estrogen receptor conformations. In this granting period, we have extended our study in the following two directions. (1) Developing high affinity ER-specific peptides for targeting ER activity. (2) Identifying novel ER-interacting cofactor proteins that may facilitate the elucidation of ER pharmacology, and to validate these receptor:cofactor interaction surfaces as targets for therapeutic intervention.

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Table of Contents

Introduction:

The ultimate goal of this research project is to identify novel therapeutic strategies for breast cancer treatment. Since the successful use of anti-estrogen tamoxifen in breast cancer treatment, inhibiting the activity of estrogen receptor (ER) has become the major drug target for the treatment of this disease. The estrogen receptor is a transcription factor whose activity is modulated by the nature of the bound ligand. The classical estrogen receptor model holds that binding of an agonist leads to receptor activation and binding of an antagonist silences ER activity. The discovery of the selective estrogen receptor modulators (SERMs), which function as either receptor agonist or antagonist in a tissue selective manner, has challenged the classical model and highlighted the complexity of ER action. It is now recognized that the traditional on/off model to explain receptor pharmacology is over-simplified and a more complex system which includes the differential interaction of ER-ligand complexes with cellular cofactor proteins has emerged to become the main stream hypothesis. It has become clear in recent years that protein-protein interaction governs many biological processes, including transcriptional activation by the nuclear receptors, and that ligand induced structural alterations in the receptor can influence the interaction between cofactor proteins with receptor. We proposed to screen and obtain small peptides which can function to detect the conformations of various ER-ligand complexes. We expect that these peptides will serve as useful tools for dissecting the complex ER pharmacology. In addition, we believe these peptides can also detect important protein-protein interaction surfaces on ER, which highlight new targets for new drug development for modulating ER activity.

Body:

The major task accomplished in the previous granting period was the application of combinatorial peptide screening approach to dissect estrogen receptor signal transduction pathways and to identify conformational probes that can detect ligand-induced structural alterations within ER. Different ER-ligand complexes were used as targets in phage display random peptide screens and peptides that detect ER structural alterations as well as peptides which recognize important receptor:cofactor interaction surfaces were identified (1-3). We found several peptides that interact with the p160 coactivator binding pocket within ER, and demonstrated that overexpression of these peptides in cells can interfere with the association of ER with coactivators, leading to disruption of ER-mediated transcriptional activation. We have also demonstrated that the mechanisms by which tamoxifen and estradiol manifest agonist activity are not the same. We found that the ER-tamoxifen and ER-estradiol complexes may recruit distinct coactivator proteins, suggesting ER activity can be differentially regulated by targeting different receptor:cofactor interactions. The results of these studies were summarized in the previous progress report.

In this granting period, we have extended our study in the following two directions. (1) Developing high affinity ER-specific peptides for targeting ER activity. (2) Identifying novel ER-interacting cofactor proteins that may facilitate the elucidation of ER pharmacology, and to validate these receptor:cofactor interaction surfaces as targets for therapeutic intervention.

1. Identification of ER-specific, high affinity binding peptides using focused libraries.

We have demonstrated previously that expression of LxxLL-containing peptides in cells can efficiently disrupt ERα-mediated reporter gene transcription (3). These LxxLL-containing peptides bind to the coactivator docking site within ER ligand binding domain, and thus competitively block the interaction between coactivator and receptors. We have also found that different receptors have distinct preference for various LxxLL motifs and in the previous progress report, we reported the identified from a primary random peptide screen, an ERβ-selective peptide, #293, which binds to ERβ but not ERα. Although this #293 peptide discriminates between ERα and ERβ, it does cross-interact with a few other nuclear receptors such as TR and RAR. This result however, suggests that it is possible to develop receptor-specific peptide antagonists by targeting receptor:cofactor interaction surfaces. To follow up on this lead, we used estradiol-activated ERβ as target to select for ERβ-specific peptides from a focused library containing the core LxxLL motif (4). This was accomplished by panning the peptides against ERβ as target, and removing any peptides that cross-reacted with ERα in an ELISA assay. All the peptides which showed ERβ specific binding were brought forward and assayed in a mammalian two-hybrid test against a panel of ten other nuclear receptors. Using this approach, we identified two peptides EBIP92 and EBIP56 which interact specifically with only ERβ but not other receptors (Figure 1). Importantly, these two peptides when expressed in cells specifically blocked ERβ-mediated reporter gene transcription without interfering with ERα-dependent transcription (Figure 2). The outcome of this study is published in *Molecular Endocrinology* (see appendix).

2. Not all cofactors interact with ER in the same manner, highlighting the potential targets for new drug intervention.

In the previous report, we demonstrated using different ERα mutants that not all LxxLL motifs interact with ER-LBD in the same manner. When we compared the sequence of these peptides with the LxxLL motifs present in the known coactivators, we found that our three classes of LxxLL peptides matched three distinct classes of coactivators (see Table 1).

Table 1

Class	Consensus	Known coactivators
I	SRLxxLL	GRIP-1, SRC-1, ACTR (5-7)
II	PLLxxLL	TRAP220, DRIP205 (8, 9)
III	(S/T)ΦLxxLL	RIP140, PGC-1, SHP, DAX-1 (10-13)

* x: any amino acid, Φ: hydrophobic residue

It has been suggested before that different classes of coactivators may interact with nuclear receptors in a temporally distinct manner, and act at different steps in the nuclear receptor signal transduction pathway. In order to define the sequence of events which occur following activation of ER by different ligands, we in collaboration with F. Schaufele (UCSF) carried out a study to visualize the interactions between these peptides and ERα in living cells (14). Peptides which recognize different ER conformations were fused to the green fluorescent protein (GFP) and the ERα was labeled with the blue fluorescent protein (BFP), the spatial and temporal interactions between ERα and peptides were recorded using fluorescent microscopy. We found that different types of LxxLL motifs interacted with ERα with slightly different kinetics. The class I and III peptides interact with ERα with faster kinetics and with higher efficiency than with class II LxxLL peptides (Figure 3). It is possible that the interaction of class II type coactivators with ER may require certain modifications on ER following ligand activation, resulting in a delay of these peptides being recruited to the receptor. It is also possible that the class I and III peptide containing coactivators, such as GRIP1 and SRC1 may interact with ER immediately following agonist activation of the receptor and that the class II motif-containing coactivators (TRAP220) may interact with ER subsequent to the binding of p160 coactivators. These two possibilities are not mutually exclusive, although at this point, the significance of these findings remains unclear. The results from this study was published in *Molecular Endocrinology* (see appendix).

In addition to the differences in the kinetics of the interactions between these LxxLL peptides with ER, we also found that the class III LxxLL peptides interact somewhat differently when compared to other classes of peptides. The interactions between class I and II peptides as well as the p160 coactivators with ERα was disrupted when three charged residues in the helix-12 of ER were mutated to their corresponding amides (ER-3x mutant). These mutations, however, do not influence the ability of the class III LxxLL peptides to interact with ERα. This result was summarized in last year's report. Since the goal of the proposed research is to develop and validate modulators of ER:cofactor interactions as potential therapeutics for breast cancer treatment, we felt that it is necessary to identify as many cofactor proteins as possible to separate

out the ones that are targets for breast cancer treatments and the ones that produce favorable interactions. Since most of the p160 coactivators interact with ER and function in a similar manner, we wish to identify cofactor proteins that have a distinct way of interacting with ER. We have previously determined that the ER-3x functions in a cell type and promoter context specific manner (15), therefore, the cofactor(s) that allows this mutant ER to manifest its activity is of great interest to us. We performed a yeast two-hybrid screen using the ER-3x ligand binding domain as bait and identified several ER-interacting factors that interacted with ER-3x mutant in a manner similar to the class III peptides. The characterization of these clones are summarized as follows. We have isolated several cDNAs encoding the 140 kDa receptor-interacting protein (RIP140) (10) as well as the 1A isoform of the SRC-1 (steroid receptor coactivator-1) (5) as interactors of both wild-type ER and ER-3x mutant (Figure 4). As mentioned previously that the 3x mutation disrupted the interaction between ER and most other p160 coactivator proteins, the ability of RIP140 and SRC-1A to interact differently with ER-LBD may suggest a different function of these cofactor proteins. In addition, we also isolated the C-terminus of kinesin-2 which interacted in an estradiol-dependent manner with both wild-type and 3x mutant ER (16). However,we failed to show the full-length kinesin-2 to interact with ER. We are not certain at this point, the significance of this interaction. This interaction could be a false positive, but it is also likely that a special activation event may be required for the interaction between kinesin-2 and ER to occur. One novel sequence we called HB-199 was identified which interacts weakly with the ER-3x mutant, but shows a robust interaction with the wild-type receptor in an agonist dependent manner (Figure 5). We are still in the process of generating the full-length clone of this cDNA and characterizing the function of the protein it encodes. In addition, a clone we isolated called HB375, which interacted equally efficient with both the wild-type as well as the 3x mutant ER, has recently been characterized as 4E-T, a factor that mediates the nuclear import of eIF4E (Figure 6) (17). Whether this protein also has a function in intracellular trafficking of ER remains to be determined. If 4E-T does have a function in the localization of ER, we believe that protein-protein interaction of this sort can also be a target for new drug intervention. Although some of the factors that we identified in this screen have been described previously and some have not yet been assigned a function, the result of this screen highlight the versatility of ER to interact with multiple proteins. We hope when the functions of these proteins become clearer, we will be able to identify peptides or small molecules that can disrupt the interaction of only a selected subset of cofactor:ER interactions without affecting other favorable ER:cofactor associations.

3. Cancer cells use different mechanisms to escape anti-hormone treament.

In the previous report, we described the identification of one mechanism by which breast cancer cells develop tamoxifen resistance (2). Using peptide probes, we found that the tamoxifen-activated ERα presents a distinct conformation from that of the estradiol-activated ER and recruits different types of coactivator proteins to manifest its agonist activity. Since hormone resistance is also frequently found in prostate cancers, we wondered if the same mechanism we identified in breast cancer cells will also explain the anti-androgen resistance in prostate cancers. Although this part of study was not proposed in the original research plan, we felt that characterization of hormone resistance in prostate cancer may also provide insight into hormone resistance in breast cancer. In a mammalian two-hybrid assay, we used the peptides identified in our ER screen to probe the conformations of the androgen receptor (AR). Among

all the peptides we tested, only two interacted with AR (Figure 7). Since the interaction between these two peptides with AR is agonist dependent and this interaction correlates very well with transcriptional activation by AR, we believe that these two peptides detect an active conformation of the receptor. One of the frequently found genetic alterations in prostate cancer cells is the T877A mutation in the AR LBD (18-20), which allows the cells to switch from recognizing hydroxyflutamide (OHF) as an antagonist to an agonist (21, 22). When we probed the conformation of this mutant receptor using the same peptides, we found that in the presence of OHF, the AR/T877A does not gain the ability to interact with other coactivator-like peptides like tamoxifen-activated ER does. Rather, OHF-activated mutant AR appears to adopt conformation similar to the agonist-activated wild-type AR as judged by the fact that it interacted with the same peptides as the DHT-activated AR. Furthermore, we found that in addition to the OHF, progestins and estradiol, this mutant AR can also be activated by a number of endogenous as well as synthetic glucocorticoids (Figure 8), which may explain the failure of subsequent hormonal manipulations in patients who have failed flutamide treatment. We suspect that mutations in the AR may emerge under the androgen-deprived conditions, which would favor the receptor to be activated by either the anti-androgens used in the treatment, or other circulating hormones. Although ER mutations are less prevalent in breast cancers, a mutation in ER identified from human breast hyperplastic lesion, which allows it to interact more efficiently with the coactivator SRC-1 and is hypersensitive to estradiol, was described recently (23). This highlights a common mechanism by which both prostate and breast cancer cells may use to escape hormonal manipulation, and both involve the gain of function in recruiting coactivator proteins. We believe our approach, which targets directly the interaction between ER and coactivator proteins, may circumvent these resistance mechanisms tumor cells have developed.

Key Research Accomplishments:

July 1999 - June 2000:

1. Identified conformational-sensitive probes for ER.
2. Developed a cell-based assay system to probe ER conformations.
3. Identified different classes of LxxLL, coactivator:receptor interacting motifs.
4. Demonstrated that tamoxifen- and estradiol-induced transcriptional activities are mediated through different mechanisms.
5. Identified peptide antagonists for estradiol-induced ER transcriptional activity.
6. Identified peptide antagonists that can distinguish between ERα and ERβ.
7. Identified peptide antagonists which block tamoxifen partial agonist activity within intact cells.

July 2000 - June 2001:

1. Identified peptides that bind ERβ with high specificity and affinity using the LxxLL focused library.
2. Validated the ERβ-specific peptides as potent inhibitors of ERβ activity.
3. Confirmed the interactions of peptides identified in our screen with ER in living cells and discovered that different coactivator peptides interact with ER with different kinetics.
4. Identified proteins that interact with ERα in a manner distinct from p160 coactivators.
5. Identified one mechanism by which hormone resistance occurs in prostate cancer cells.

Reportable Outcomes:

Manuscripts:

- Julie M. Hall, Ching-yi Chang and Donald P. McDonnell
  Development of peptide antagonists that target estrogen receptor β-coactivator interactions. 2000. Molecular Endocrinology 14(12): 2010-2023.

- Fred Schaufele, Ching-yi Chang, Weiqun Liu, John D. Baxter, Steven K. Nordeen, Yihong Wan, Richard N. Day, and Donald P. McDonnell
  Temporally distinct and ligand-specific recruitment of nuclear receptor-interacting peptides and cofactors to subnuclear domains containing the estrogen receptor., Molecular Endocrinology 14(12): 2024-2039.
- Ching-Yi Chang, Philip J. Walther, and Donald P. McDonnell
  Glucocorticoids manifest androgenic activity in a cell line derived from a metastatic prostate cancer. Submitted.

Meeting Abstracts:

- American Society for Bone and Mineral Research 22nd Annual Meeting, Toronto, Ontario, Canada. September, 2000. J.of Bone Min. Res. (15): SA482, suppl.
  Mechanistic dissociation of the anti-apoptotic and classical transcriptional activities of the estrogen receptor using peptide antagonists.

- The Endocrine Society's 83rd Meeting, June 20-23, 2001. Denver, CO.
  Ching-yi Chang and Donald P. McDonnell
  Evaluation of ligand-dependent changes in androgen receptor structure by peptide probes provides insight into the mechanisms underlying anti-androgen resistance.

Conclusions:

Anti-estrogens play an important role in the treatment of breast cancers. Because estrogen receptor is required for the maintenance of several normal physiological functions, the ideal drug of choice should have anti-estrogenic activity in the breast but at the same time preserve the beneficial effects of estrogen in other tissues. Last year, we reported the development of conformational probes of ER and demonstrated that various protein-protein interaction surfaces on ER are exposed upon binding different ligands. We also showed ER activities can be differentially targeted by selectively blocking specific receptor:cofactor interactions using these conformational peptide probes. We demonstrated in this report that highly selective inhibitory peptides can be obtained using a combinatorial approach. Although the downstream events following receptor:cofactor interactions have not been fully characterized, we observed, using GFP-labeled peptides in living cells, that different coactivator peptides interact with ER with slightly different kinetics. This result highlights the complex regulations underlying the receptor activation process, and suggests that several targeting steps may exist, in which the receptor activity can be modulated by peptides or small molecule antagonists. We also performed a yeast two-hybrid screen to complement the peptide approach and identified several ER-interacting proteins, although detailed characterization of these interactions is still in progress. We wish to identify and characterize all the ER-interacting partners which participate in different ER-mediated functions. Then, by using highly selective peptide antagonists, we should be able to identify novel targets which can be used to modulate ER activity in a tissue-type specific manner and/or to circumvent the development of resistance associated with anti-estrogen therapy. Finally, since most of these peptide probes also interact with other nuclear receptors, we believe they will have great utility in the elucidation of other receptor-mediated diseases. For example, we have used the peptide probes identified in this study to examine the mechanism of anti-hormone resistance in prostate cancer cells.

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Appendices:

Figure 1

	ERα	ERβ	PR-A	PR-B	GR	AR	RARα	RXRα	TRβ	VDR	RORα	LXR	FXR
SRC-1	+	+	+	+	+	-	+	+	+	+	-	+	+
GRIP1	+	+	+	+	+	-	+	+	+	+	-	+	+
EBIP-37	-	+	+	+	+	-	+	+	+	+	+	+	+
EBIP-41	-	+	-	-	+	-	+	+	+	-	-	+	-
EBIP-44	-	+	+	+	+	-	+	+	+	-	-	+	+
EBIP-45	-	+	+	+	-	-	-	+	+	-	-	+	-
EBIP-49	-	+	-	-	+	-	-	-	-	-	-	-	-
EBIP-51	-	+	-	-	-	-	-	+	+	+	-	+	-
EBIP-53	-	+	+	+	+	-	-	+	+	-	-	+	-
EBIP-56	-	+	-	-	-	-	-	-	-	-	-	-	-
EBIP-60	-	+	+	+	+	-	-	+	-	-	-	+	-
EBIP-66	-	+	+	+	+	-	-	+	-	-	-	-	-
EBIP-70	-	+	-	-	-	-	+	+	+	+	-	+	-
EBIP-76	-	+	-	-	-	-	-	+	+	-	-	+	-
EBIP-87	-	+	+	+	+	-	+	+	+	-	-	+	-
EBIP-92	-	+	-	-	-	-	-	-	-	-	-	-	-
EBIP-96	-	+	+	+	+	-	-	-	+	+	+	+	-

+ denotes interaction
- denotes lack of interaction

Figure 1. The ability of the ERβ-selective LxxLL motifs to interact with several nuclear receptors was tested in the mammalian two-hybrid assay (+ denotes interaction, - denotes lack of interaction). ERβ-specific peptides are shaded. Shown here are interactions that form between the peptides and receptors in the presence of the receptor-specific agonists with the exception of RORα, which is constitutively active. An interaction (+) was defined by the observation of a statistically significant increase in activity when both the receptor and peptide were coexpressed compared to that present when either was cotransfected with the parent vector of the other. HepG2 cells were transiently transfected with each pVP16-receptor fusion expression vector in combination with each peptide-Gal4DBD fusion construct, the 5xGal4-tata-Luc reporter, and the pCMV-β-gal control plasmid. Each receptor expression construct includes the VP16 activation domain sequence fused 5' to the entire coding sequence for the human form of the respective receptor, with the exception of pVP16-FXR, which contains the rat FXR homolog. Following transfection, cells were treated with vehicle or the following hormones: 10^{-7} M 17β-estradiol for ERα and ERβ, 10^{-7} M progesterone for PR-A and PR-B, 10^{-7} M dexamethasone for GR, 10^{-7} M 5α-dihydrotestosterone for AR, 10^{-7} M 9-cis-retinoic acid for RARα and RXRα, 10^{-7} M T3 for TRβ, 10^{-7} M 1, 25-dihydroxyvitamin D3 for VDR, 10 μM 22R-hydroxycholesterol for LXR, and 50 μM chenodeoxycholic acid for FXR. After 24h luciferase assays were performed, and each value was normalized to the β-galactosidase activity.

Figure 2

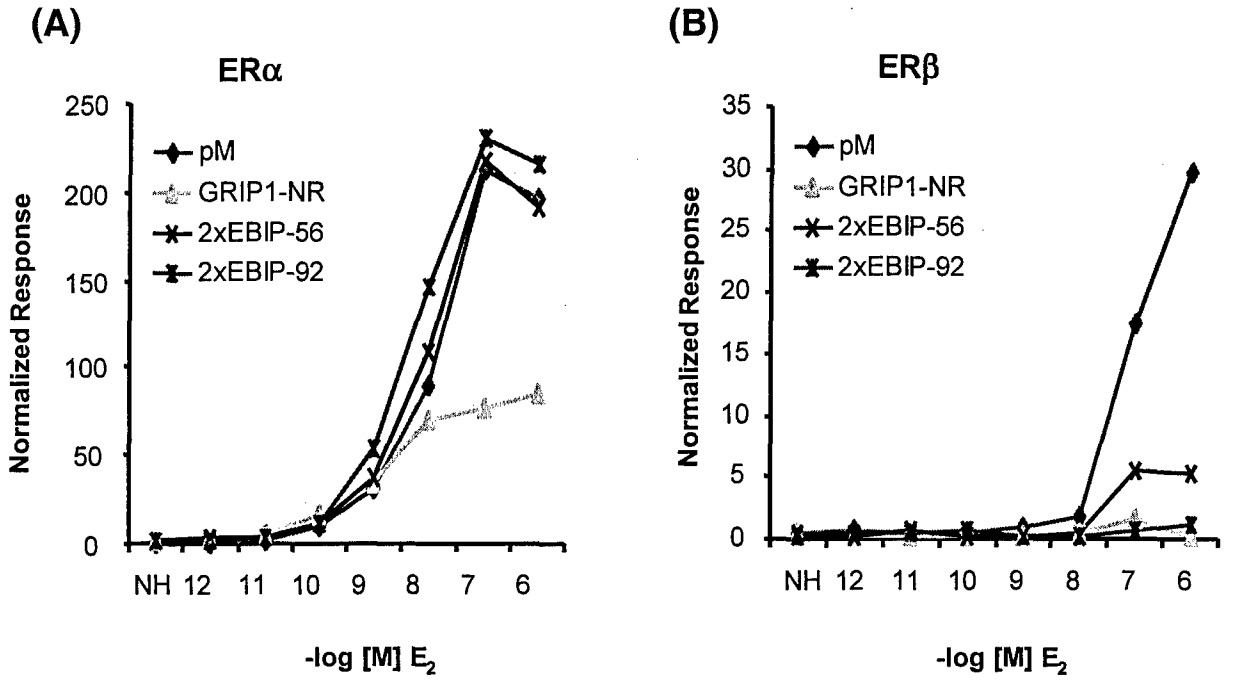

Figure 2. Evaluation of the antagonist properties of peptides that interact in a specific manner with ERβ. The ability of the ERβ-specific LxxLL peptides EBIP-56 and EBIP-92 to disrupt the transcriptional activities of ERα and ERβ was examined. For these experiments, constructs containing two copies of the peptides (2xEBIP-56 and 2xEBIP-92) were used. A peptide containing the three LxxLL motifs (NR-boxes) of the coactivator GRIP1 was used as a control. HepG2 cells were transiently transfected with ERα (A) or ERβ (B) expression vector together with the empty Gal4DBD vector (pM) or the Gal4DBD-peptide fusion constructs and the 3x-ERE-TATA-Luc and pCMV-β-gal plasmids. After transfection, cells were treated with vehicle (nh) or increasing concentrations (ranging from 10^{-12} M to 10^{-6} M) of 17β-estradiol (E2) for 24h, and luciferase assays were performed. Each value was normalized to the β-galactosidase activity. Each data point is the average of triplicate determinations, and the average coefficient of variance for each value is <10%.

Figure 3

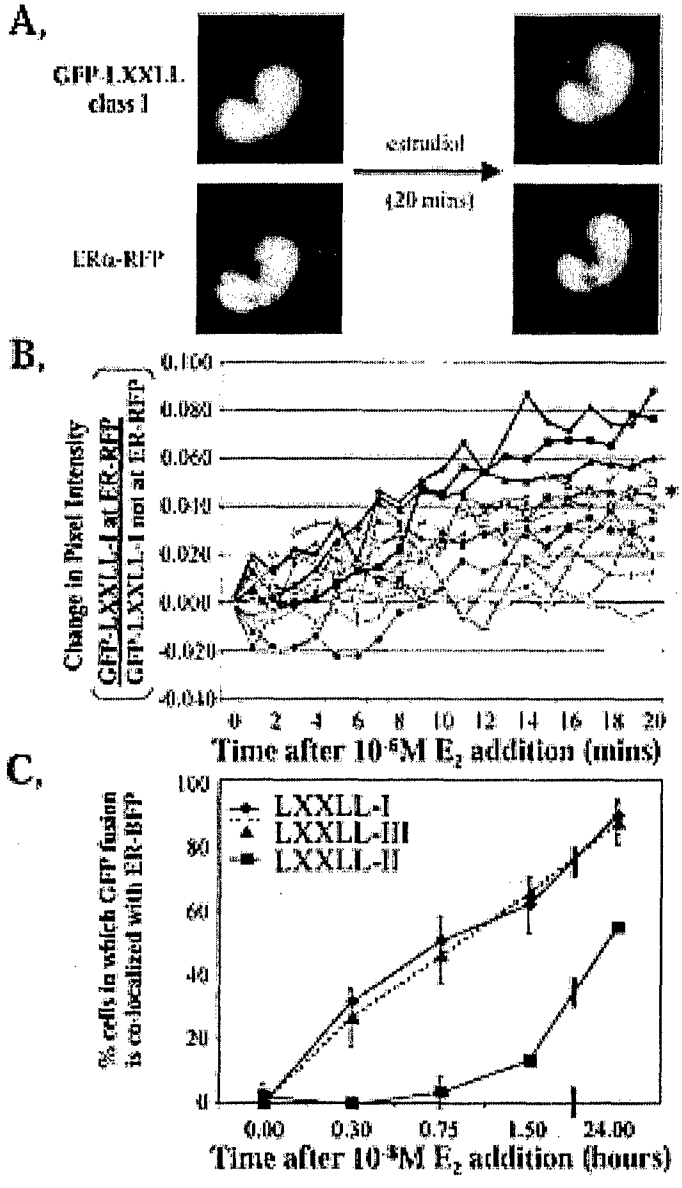

Figure 3. Different LxxLL-containing peptides are recruited to ERα with different temporal kinetics. (A) Sequential images of the same cell captured before and 20 min after the addition of 10^{-6} M E2 demonstrated partial colocalization of the class I LXXLL-GFP fusion protein with ERα fused to RFP. (B) Quantification of the level of GFP and RFP fluorescence at each pixel within the nucleus at 1-min time intervals after the addition of 10^{-6} M E2 shows variable concentration of class I LxxLL-GFP at the position of ERα-RFP in 15 different cells. (*): Readout of cell shown in Fig. 4A (open triangles). (C) The proportion of cells showing any colocalization of ERα-BFP with each LxxLL-GFP was scored with time after addition of 10^{-8} M E2. The slower time course and reduced binding of the class II peptide reflect the previously reported poorer interaction of this peptide with ERα. Each graph represents the mean ± SD in the percent of cells displaying colocalization from three independent experiments.

Figure 4

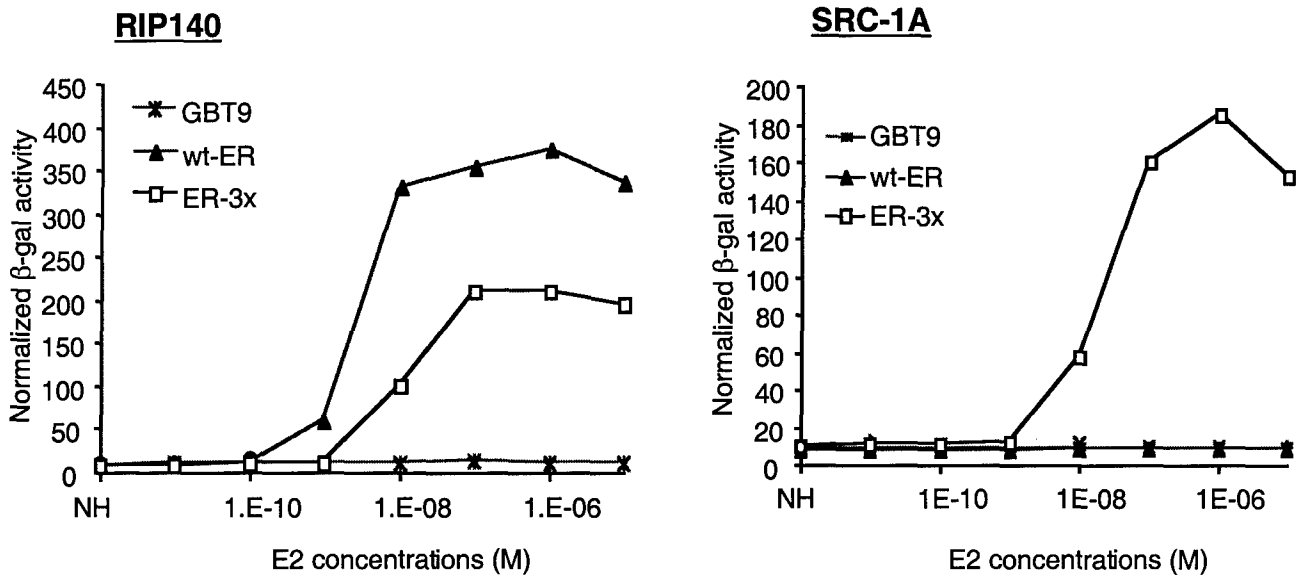

Figure 4. Identification of known ER-interacting proteins, SRC-1A and RIP140, validates the strategy of using ER-HBD3x as bait in the yeast two-hybrid screen. The ER-HBD3x was used as bait in yeast two-hybrid system to screen for ER-interacting proteins from human brain cDNA library. The C-terminal fragments of RIP140 and SRC-1A were identified as ER-HBD3 interacting proteins. Yeast strain HF-7c was transformed with either the pGBT9 empty vector, pGBT9-ER-HBDwt, or pGBT9-ER-HBD3x along with the library clones expressing RIP140 (A) or SRC-1A (B) fragments fused to yeast Gal4-activation domain. Cells were induced with different concentrations of 17β-estradiol for 4h and β-galactosidase activity was measured and normalized to cell density.

Figure 5

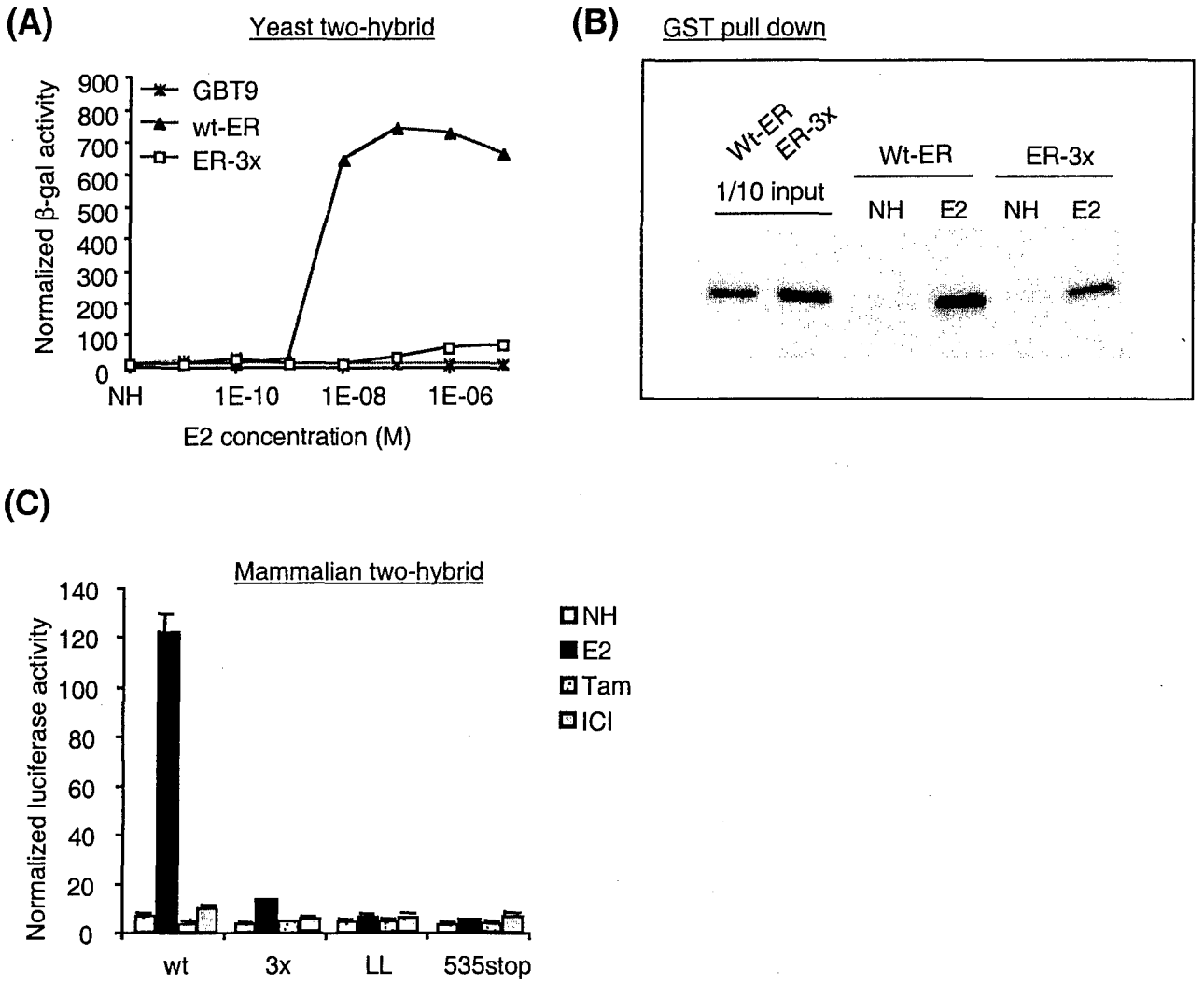

Figure 5. A novel cDNA HB199 was identified which shows strong interaction with wild-type ERα but weaker association with the ERα-3x mutant. (A) HB199 shows hormone-dependent interaction with ERα in yeast. Yeast strain HF-7c was transformed with either the pGBT9 empty vector, pGBT9-ER-HBDwt, or pGBT9-ER-HBD3x along with the library clone HB199. Cells were induced with different concentrations of 17β-estradiol for 4h and β-galactosidase activity was measured and normalized to cell density. (B). HB199 interacts with *in vitro* translated full-length wild-type ERα and ERα-3x in a GST-pull down assay. HB199 was expressed as GST fusion protein and incubated with S^{35}-labeled, *in vitro* translated ERα or ERα-3x, in the presence or absence of 10^{-6} M 17β-estradiol overnight. Non-specific binding was removed by washing four times with TBST and proteins remained bound to GST-HB199 were resolved by SDS-PAGE and detected by autoradiography. (C) Interactions between HB199 and ERα were analyzed in mammalian two-hybrid assay. HepG2 cells were transfected with pM-HB199 and VP16-ERα, VP16-ERα-3x, VP16-ERα-LL or VP16-ERα-535Stop, along with reporter constructs, 5xGal4Luc3 and pCMV-β-gal. PM-HB199 expresses HB199 fused to the Gal4DBD, and VP16-ERα constructs express the full-length ER with the VP16-activation domain fused at their amino termini. After transfection, cells were induced with vehicle control, 10^{-7} M 17β-estradiol, 10^{-7} M 4OH-tamoxifen or 10^{-7} M ICI-182,780 for 16h. Luciferase activity was determined and was normalized to the β-galactosidase activity.

Figure 6

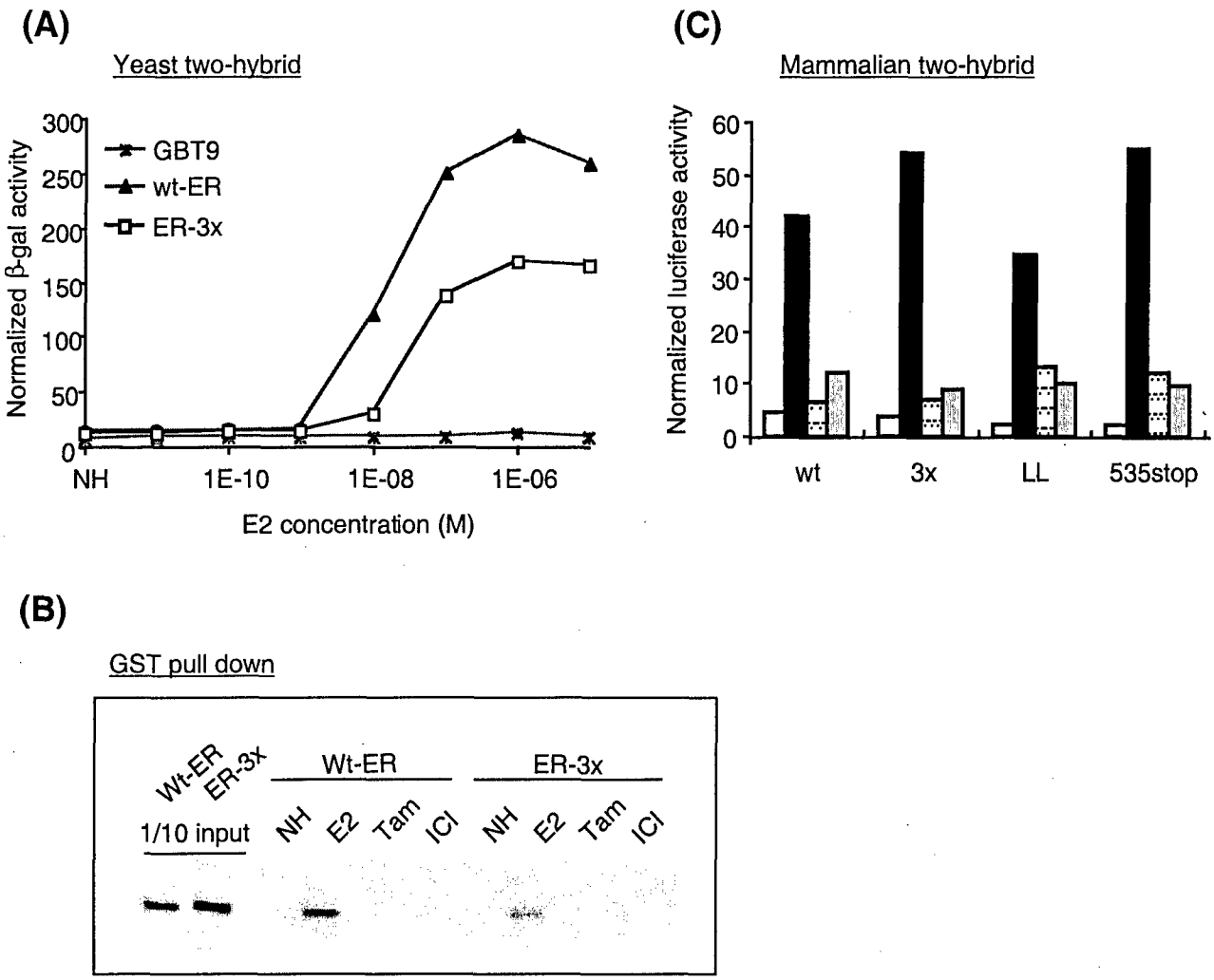

Figure 6. The interaction between HB375 and ERα *does not* require the AF-2 coactivator binding pocket (A) HB375 shows hormone-dependent interaction with ERα in yeast. Yeast strain HF-7c was transformed with either the pGBT9 empty vector, pGBT9-ER-HBDwt, or pGBT9-ER-HBD3x along with the library clone HB375. Cells were induced with different concentrations of 17β-estradiol for 4h and β-galactosidase activity was measured and normalized to cell density. Both wild-type and 3x mutant showed strong interaction with HB375. (B). HB375 interacts with *in vitro* translated full-length wild-type ERα and ERα-3x in a GST-pull down assay. HB375 was expressed as GST fusion protein and incubated with S^{35}-labeled, *in vitro* translated ERα or ERα-3x, in the presence of vehicle control, 10^{-6} M 17β-estradiol, 10^{-6} M 4OH-tamoxifen or 10^{-6} M ICI-182,780 overnight. Non-specific binding was removed by washing four times with TBST and proteins remained bound to GST-HB375 were resolved by SDS-PAGE and detected by autoradiography. (C) Interactions between HB375 and ERα were analyzed in mammalian two-hybrid assay. HepG2 cells were transfected with pM-HB375 and VP16-ERα, VP16-ERα-3x, VP16-ERα-LL or VP16-ERα-535Stop, along with reporter constructs, 5xGal4Luc3 and pCMV-β-gal. PM-HB375 expresses HB375 fused to the Gal4DBD, and VP16-ERα constructs express the full-length ER with the VP16-activation domain fused at their amino termini. After transfection, cells were induced with vehicle control, 10^{-7} M 17β-estradiol, 10^{-7} M 4OH-tamoxifen or 10^{-7} M ICI-182,780 for 16h. Luciferase activity was determined and was normalized to the β-galactosidase activity.

Figure 7

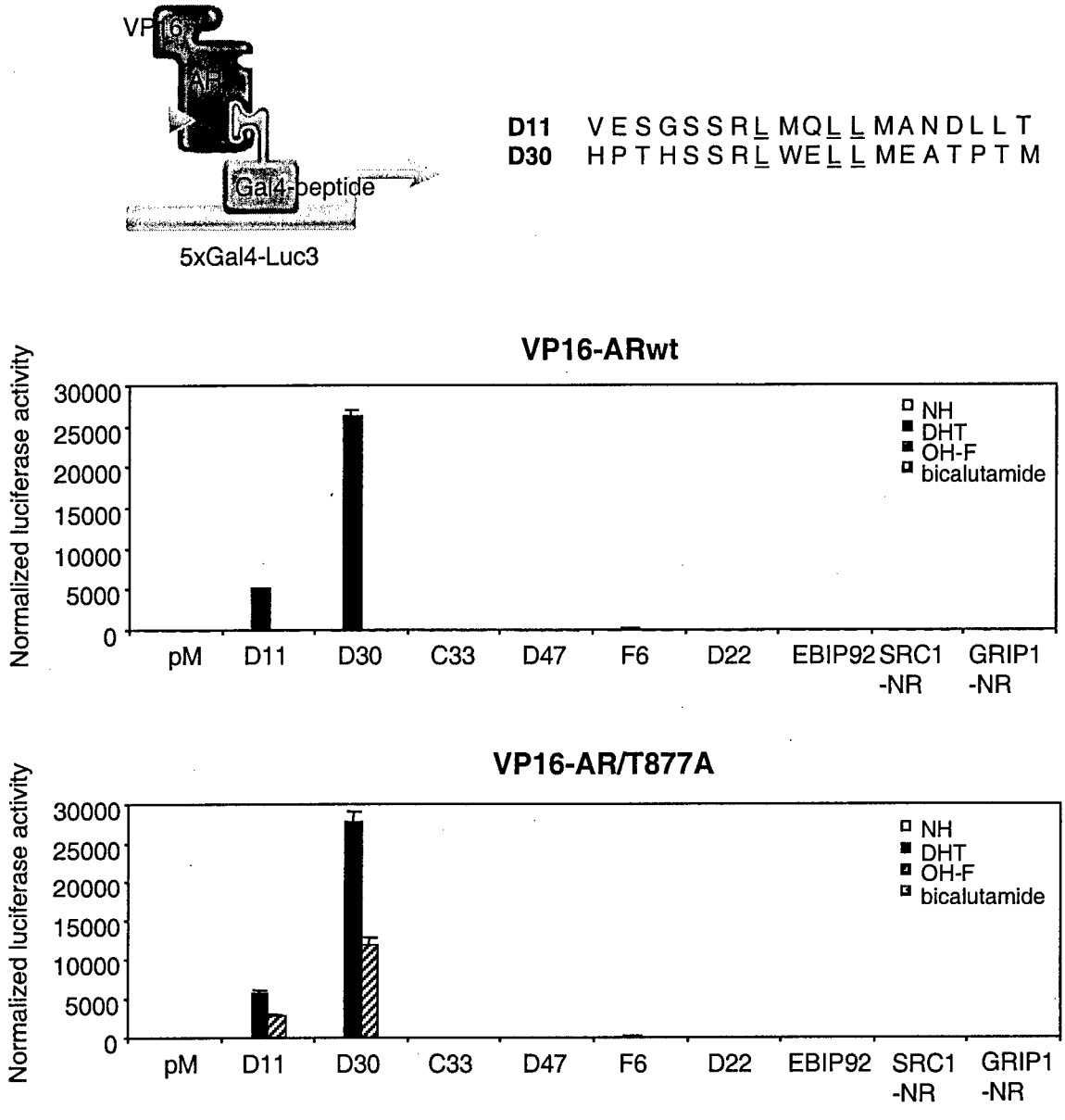

Figure 7. The T877A mutation permits anti-androgen bound AR to present an active conformation similar to that in the agonist-activated AR. Different LxxLL-motif containing peptides were fused to the Gal4-DBD, and the full length ARs (wild-type and the T877A mutant) were modified to include a VP16-activation domain at their amino termini. Interactions between peptides and AR were determined by measuring the expression of a reporter gene containing five copies of the Gal4-response elements. CV-1 cells were transfected with different peptide-Gal4DBD constructs together with either the VP16-ARwt or the VP16-AR/T877A expression plasmid, and reporter constructs 5xGal4Luc3 and pCMV-βgal. After transfection, cells were treated with either vehicle control (NH), 100 nM 5α-dihydrotestosterone (DHT), 100 nM hydroxyflutamide (OH-F) or 1 μM bicalutamide for 16 h. Luciferase activity was measured and normalized to the activity of the co-expressed β-galactosidase.

Figure 8

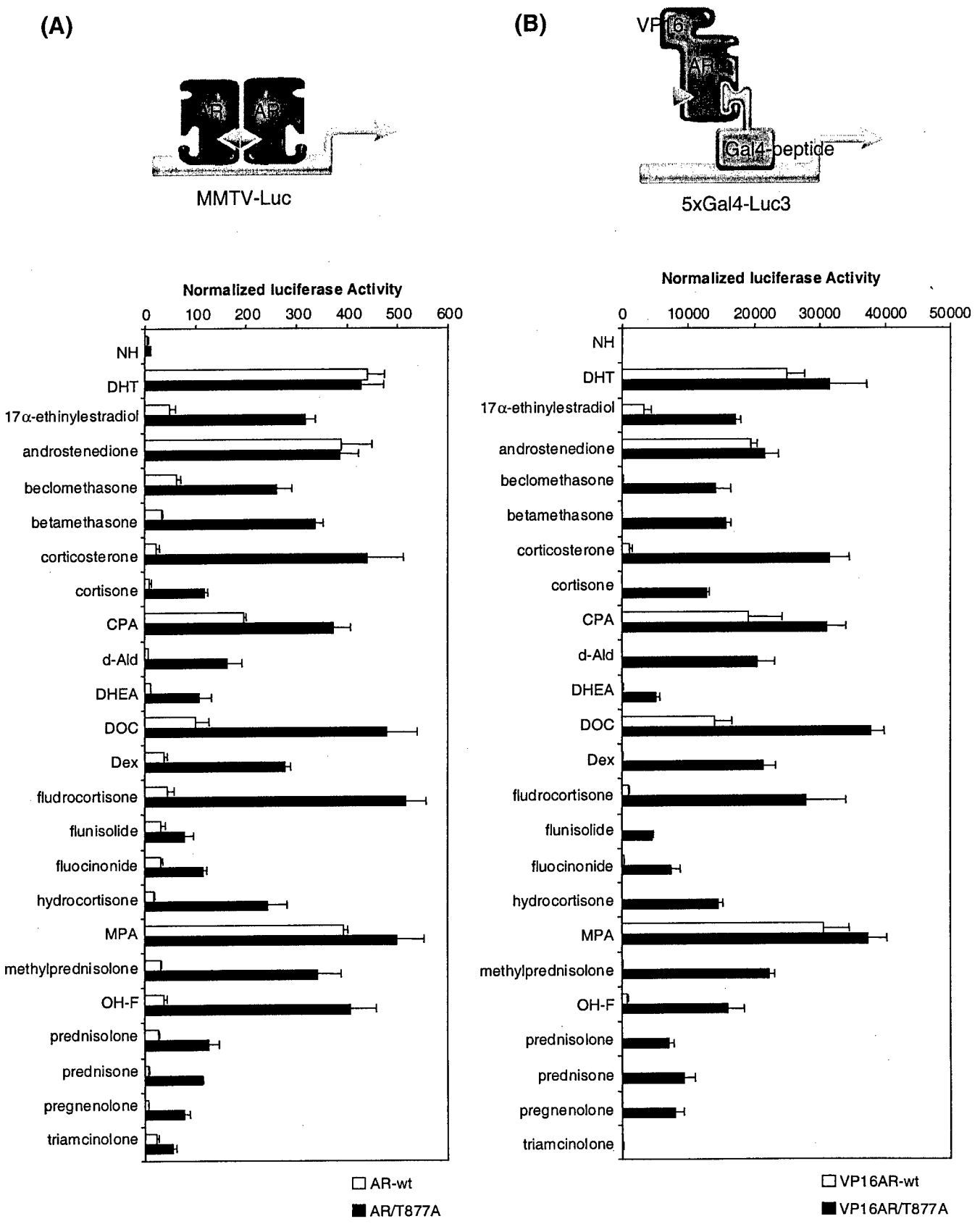
(A)
AR
AR
MMTV-Luc
(B)
VP16
AR
Gal4-peptide
5xGal4-Luc3
Normalized luciferase Activity
0
100
200
300
400
500
600
NH
DHT
17α-ethinylestradiol
androstenedione
beclomethasone
betamethasone
corticosterone
cortisone
CPA
d-Ald
DHEA
DOC
Dex
fludrocortisone
flunisolide
fluocinonide
hydrocortisone
MPA
methylprednisolone
OH-F
prednisolone
prednisone
pregnenolone
triamcinolone
AR-wt
AR/T877A
Normalized luciferase Activity
0
10000
20000
30000
40000
50000
NH
DHT
17α-ethinylestradiol
androstenedione
beclomethasone
betamethasone
corticosterone
cortisone
CPA
d-Ald
DHEA
DOC
Dex
fludrocortisone
flunisolide
fluocinonide
hydrocortisone
MPA
methylprednisolone
OH-F
prednisolone
prednisone
pregnenolone
triamcinolone
VP16AR-wt
VP16AR/T877A

Figure 8. A large number of structurally diverse compounds can activate the AR/T877A mutant. (A) CV-1 cells were transfected with either the wild-type AR or the AR/T877A mutant expression plasmids and the reporter gene MMTV-Luc together with a normalization vector pCMV-βgal. Transfected cells were induced with 1 μM of each compound for 16h, then the luciferase and β-galactosidase activities were measured. (B) CV-1 cells were transfected with the pM-D30 plasmid expressing the D30 peptide fused to the Gal4DBD, and either VP16AR-wt or VP16AR/T877A together with the reporter gene 5xGal4Luc3 and pCMV-βgal. Transfected cells were treated and assayed as in (A). NH: no hormone, DHT: 5α-dihydrotestosterone, CPA: cyproterone acetate, d-Ald.: d-aldosterone, DHEA: dehydroepiandrosterone, DOC: 11-deoxycorticosterone, Dex: dexamethasone, MPA: medroxyprogesterone, OH-F: hydroxyflutamide. Normalized luciferase activity was obtained by dividing the luciferase activity by the β-galactosidase activity. The values shown are the mean ± S.D. of three determinations. The results shown are representative of multiple experiments performed under the same conditions.

Meeting Abstract #1

Mechanistic Dissociation of the Anti-Apoptotic and Classical Transcriptional Activities of the Estrogen Receptor Using Peptide Antagonists

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Presentation Number: SA482

Estrogens exert anti-apoptotic effects on osteoblasts and osteocytes in vitro and in vivo. These effects are mediated by a rapid, presumably non-genotropic, yet estrogen receptor (ER)–dependent mechanism of action, which involves activation of extracellular signal regulated kinases. To determine whether the anti-apoptotic activity is dissociable from the classical transcriptional activity of the ER, we used a series of peptide antagonists that interact with ERalpha or ERbeta and modulate their functional activity. HeLa or 293 cells were co-transfected with plasmids carrying GAL4-peptide chimeras, ERE- or IL-6-luciferase constructs (representing paradigms of protein-DNA or protein-protein interactions, respectively), and ERalpha or ERbeta containing plasmids. Peptide alphaII, which binds to the ligand binding domain of the ERalpha, blocked transcription when mediated via protein-DNA or protein/protein interaction. Nonetheless, it had no effect on the anti-apoptotic effect of 17beta estradiol, in either cell. In contrast, an ERbeta selective peptide (293) or a peptide (F6) that binds both ERalpha and beta blocked transcription from both promoters in cells expressing the respective ER, and also blocked the anti-apoptotic activity through ERalpha or beta. Since the peptides bind and block different domains within the ligand binding region (E) of the ER, these results suggest that the anti-apoptotic activity of the ligand activated ERalpha or ERbeta must reside in a distinct region of the receptor protein, which we have termed anti-apoptotic domain, and have localized (as reported elsewhere in this meeting) within the carboxy terminus (E) domain of the protein, and requires only the AF-2 activity that resides within region E. The differential inhibitory activities of the two classes of peptide antagonists clearly distinguish the domains within ER (alpha and beta) required for transcriptional activity and prevention of apoptosis.

Meeting Abstract #2

EVALUATION OF LIGAND-DEPENDENT CHANGES IN ANDROGEN RECEPTOR STRUCTURE BY PEPTIDE PROBES PROVIDES INSIGHT INTO THE MECHANISMS UNDERLYING ANTI-ANDROGEN RESISTANCE .

C. Chang and D.P. McDonnell. Pharmacology and Cancer Biology, Duke University, Durham, NC, United States

Mutations in the androgen receptor (AR) are frequently found in relapsed prostate cancers, permitting anti-androgens as well as estrogens and progestins to function as androgens. However, the mechanism by which these mutations enable this shift in AR-pharmacology is still unknown. Resistance to anti-hormone therapy arises also in estrogen receptor (ER)-positive breast cancers, where it is believed that alterations in cofactor expression in the cells permit anti-estrogens like tamoxifen to function as agonists. In support of this hypothesis we have shown that tamoxifen binding to ER allows the presentation of novel protein-protein interaction surfaces on the receptor, enabling it to interact in an ectopic manner with transcriptional coactivators. In this study we wanted to see if the same mechanisms would also apply with respect to anti-androgen resistance. To explore this possibility, we used phage display to identify a series of LXXLL-containing peptides that interact with the AF2 domain of AR. We found that although the binding of peptides to wild-type AR was agonist dependent, these same peptides could also interact with the AR-T877A variant, a mutant frequently found in anti-hormone refractory prostate cancers, in the presence of either androgens or anti-androgens. This suggests that the agonist activity of anti-androgens and other physiologically relevant ligands occurs because they, in the background of the mutation, allow AR-AF2 to adopt an active conformation. Initially, this result seems to contradict other findings which suggest that coactivator binding to AR-AF2 is not required for AR activity. In probing this further, we have determined that the role of AR-AF2 appears to be to stabilize the overall structure of the receptor, allowing the amino terminus to interact with appropriate coactivators. This contention is supported by the finding that over-expression of the AF2-binding peptides does indeed block the interaction of the amino- and carboxyl- terminal of AR, but does not attenuate AR transcriptional activity. Thus we believe that mutations in AR which facilitate the formation of an AF2 pocket, have the potential to allow AR antagonists to manifest agonist activity.

Development of Peptide Antagonists That Target Estrogen Receptor β-Coactivator Interactions

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Department of Pharmacology and Cancer Biology
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The biological actions of estrogen are manifest through two genetically distinct estrogen receptors (ERα and ERβ) that display nonidentical expression patterns in target tissues. The phenotypic alterations in response to estrogens in mice disrupted for either or both of these receptors are not identical, suggesting that each subtype plays a unique role in ER-action. However, the lack of subtype-specific agonists and antagonists has made it difficult to define the processes that are regulated by ERα and/or ERβ. Previously, we have reported the identification and characterization of a series of LXXLL-containing peptide antagonists that block estrogen signaling by preventing the association of ERα with required coactivators. As expected, given the similarity of the coactivator binding pockets among nuclear receptors, most of the peptide antagonists identified inhibited the activity of multiple receptors. However, by altering sequences flanking the core LXXLL motif, some receptor selectivity was afforded. Building on this observation, we have screened combinatorial phage libraries, expressing peptides in the format X_7LXXLLX_7, for peptides that interact in a specific manner with ERβ. Using this approach, a series of highly specific, potent peptide antagonists have been identified that efficiently inhibit ERβ-mediated estrogen signaling when introduced into target cells. Interestingly, in cells where both ER subtypes were expressed, these ERβ antagonists were capable of attenuating ER action, suggesting that ERα and ERβ do indeed form functional heterodimeric complexes. We believe that suitably formulated versions of these peptides can be used to study ERβ action *in vitro* and *in vivo*. In addition, the unanticipated specificity of the peptides identified should serve as an impetus to investigate the use of this approach to develop peptide antagonists of other nuclear receptors and unrelated transcription factors. (Molecular Endocrinology 14: 2010–2023, 2000)

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Molecular Endocrinology 14(12): 2010–2023

INTRODUCTION

The human estrogen receptor (ER) belongs to the nuclear receptor superfamily of ligand-inducible transcription factors (1), 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 (2). In the absence of hormone, the receptor is sequestered within the nuclei of target cells in an inactive state. The binding of ligand induces an activating conformational change within ER, an event that permits the receptor to interact with transcriptional coactivators such as steroid receptor coactivator 1 (SRC-1) and glucocorticoid receptor interacting protein 1 (GRIP1) (3, 4), and which facilitates the association of the resulting complex with specific DNA response elements (EREs) located within the regulatory regions of target genes. Depending on the promoter context, the DNA-bound receptor can then exert either a positive or negative effect on target gene transcription (2, 5).

Until recently it was thought that all of the biological actions of estrogens and antiestrogens were manifest through a single receptor located within target cell nuclei. However, the identification of a second estrogen receptor, ERβ (6, 7), 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 (8) and interact with the same DNA response elements. 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 (7, 9–11). 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 (11). Thus, in cells where both receptors are expressed, overall estrogen responsiveness is reduced.

In parallel with studies performed *in vitro*, the creation and characterization of mice in which either ERα and/or ERβ have been disrupted (αERKO, βERKO and αβERKO, respectively) have demonstrated that the two receptors are not functionally equivalent and that each subtype plays a unique role in ER action *in vivo* (12, 13). However, it is clear that in addition to these mouse models, there is a need for subtype-selective agonists and antagonists that will permit the transient manipulation of receptor function in intact animals. Consequently, 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 (15), we have undertaken a novel approach to develop subtype-specific antagonists that inhibit ERβ action in a manner distinct from known antiestrogens.

All of the currently available ER antagonists function by 1) binding to the receptor ligand-binding domain, thereby blocking agonist access, and 2) inducing a conformational change within the receptor that prevents it from interacting efficiently with transcriptional coactivators such as SRC-1, GRIP1, and amplified in breast cancer 1 (AIB-1). Specifically, it has been shown that agonist binding to the receptor induces a conformational change that permits the formation of a hydrophobic pocket (16, 17), enabling the receptor to interact with the LXXLL motif contained within the receptor interaction domains of most of the validated coactivators (18, 19). The conformational changes induced in ER upon antagonist binding do not permit coactivator recruitment (19). 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, we have recently identified a series of LXXLL-containing peptides that interact very well with the coactivator binding pocket of ERβ, but which demonstrate distinct preferences in their ability to interact with other receptors (20). Thus, all LXXLL motifs are not functionally equivalent. Building on this observation, in the current study we have identified LXXLL-containing peptides that interact specifically with ERβ and inhibit its transcriptional activity. We believe that these novel peptide antagonists will serve as useful tools to evaluate the role of ERβ in estrogen signaling. In addition, we anticipate that the general approach used to obtain these ERβ antagonists can be applied to the development of peptide antagonists of other nuclear receptors and unrelated transcription factors.

RESULTS

Affinity Selection of ERβ-Binding LXXLL-Containing Peptides Using Phage Display

A critical step in ER action is the ligand-dependent recruitment of transcriptional coactivators to target gene promoters. Antiestrogens manifest their inhibitory activities by altering ER structure and independently blocking cofactor binding (19, 22). In this study, the feasibility of targeting ER-coactivator interactions directly as a mechanism of developing human ERβ-specific antagonists was evaluated. Using combinatorial phage display technology (21), we created and screened a phage library that expressed 19-mer peptides containing a central fixed Leu-X-X-Leu-Leu motif flanked by seven random amino acids on each side. Since we used an NNK nucleic acid format in the construction of this library it has a theoretical complexity of 1.2×10^{24}. However, since our library contained only 10^8 independent clones, we are only surveying a fraction of the potential LXXLL motifs that can interact with ERβ. This random LXXLL library was screened for phage that bound to ERβ either in the absence or presence of estradiol, and 70 of the most avid interactors identified were brought forward for further analysis. A secondary enzyme-linked immunosorbent assay (ELISA) was used to confirm the ERβ-binding characteristics of the plaque-purified phage. Cross-screening, using a similar approach, 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.

Characterization of the ERβ-Selective LXXLL-Containing Peptides in Intact Cells

We performed a mammalian two-hybrid assay to assess the ability of the peptide sequences identified by phage display to interact selectively with ERβ in intact cells. Each of the 33 ERβ-selective peptides identified *in vitro* were expressed as a yeast Gal 4 DNA-binding domain (Gal4DBD) fusion protein and tested for their ability to interact with ERα and/or ERβ expressed as fusions to the VP16 activation domain. Expression of the peptide-fusions was confirmed by Western immunoblotting (data not shown). A Gal4DBD-fusion of the NR-box of the ER coactivator SRC-1 (containing three LXXLL motifs), shown previously to interact with both ERα and ERβ, was used in this assay as a positive control. When analyzed in the two-hybrid assay, it was found that 15 of the 33 peptides studied interacted with ERβ, but not ERα. Representative examples are shown in Fig. 1 of those peptides that were isolated in the screen with the apo-ERβ (Fig. 1A), and those identified with the agonist-liganded receptor (Fig. 1B). Interestingly, several of the peptides were able to bind ERβ in the absence of ligand, suggesting either that a portion

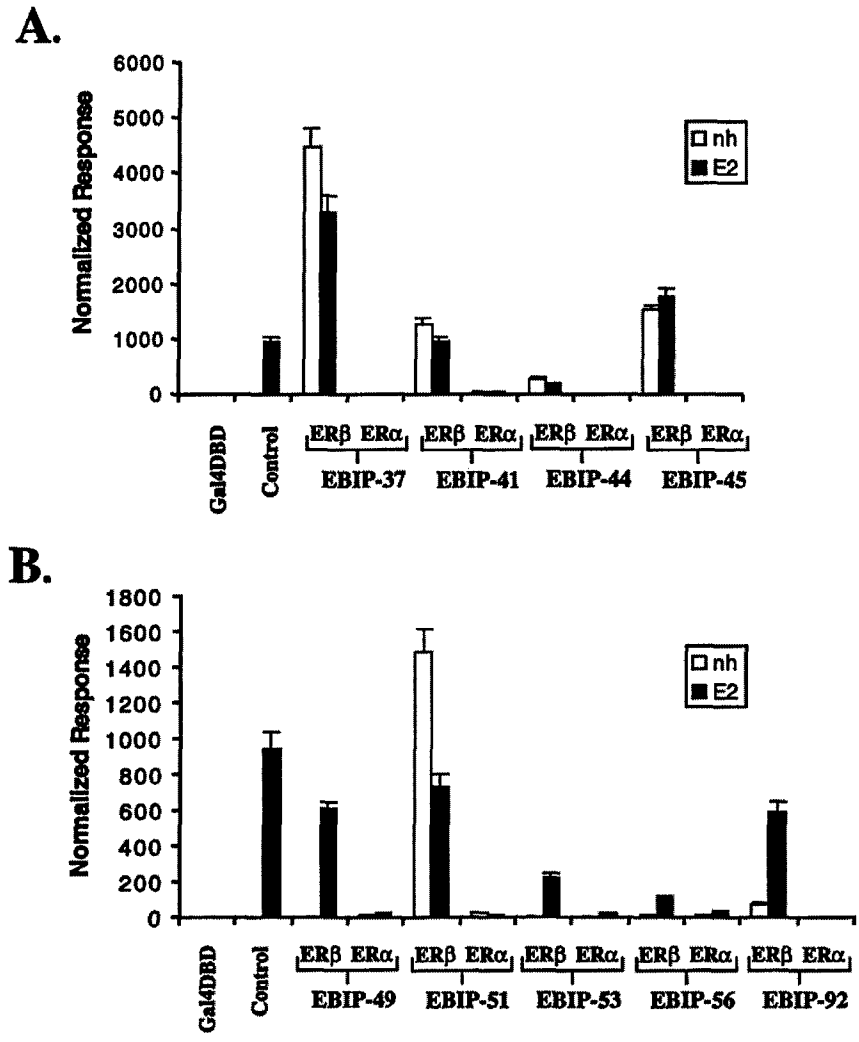

Fig. 1. Evaluation of ERβ-Selective LXXLL-Containing Peptides in Intact Cells

Mammalian two-hybrid assays were performed to examine the interactions between the peptides and ERα or ERβ in mammalian cells. Each ER expression construct includes the VP16 activation domain sequence fused 5′ to the complete coding sequence for the human ERα or ERβ. A, Analysis of the interaction of peptides that were isolated in the screen with apo-ERβ (four representative peptides are shown). B, Analysis of the interaction of peptides that were isolated in the screen with agonist-liganded ERβ (five representative peptides are shown). HepG2 cells were transiently transfected with the pVP16-ERα expression vector or the pVP16-ERβ expression vector together with each peptide-Gal4DBD fusion construct, the 5x-GAL4-TATA-Luc reporter, and the pCMV-β-gal control plasmid. The SRC-1 (NR-box)-Gal4DBD fusion was used as a control. Cells were induced with vehicle (nh) or 10^{-7} M 17β-estradiol (E_2) for 24 h, and luciferase assays were performed. Each value was normalized to the β-galactosidase activity. Each data point is the average of triplicate determinations, and the average coefficient of variance for each value is <10%.

of the ERβ aporeceptor resides in an active conformation, or that the binding of LXXLL-containing motifs to the receptor may facilitate activation. Using the mammalian-two hybrid assay, it was also shown that the introduction of inactivating point mutations into the AF-2 region of ERβ (11) completely abolished the interaction of each peptide with the receptor (see Fig. 7A, below). These results suggested that the peptides were targeting the coactivator binding pocket, and thus would be able to antagonize ERβ transcriptional activity.

ERβ-Selective LXXLL Peptides Display Hormone Specificity

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 (3–5). The surprising finding that some LXXLL-containing peptides can interact with apo-ER begged a re-evaluation of the role of ligand in regulating ERβ-LXXLL interactions. This was accomplished using the mammalian two-hybrid assay to examine the effect of

different ER ligands on peptide binding. As expected, the interactions between the Gal4DBD-SRC-1, and Gal4DBD-GRIP1 NR-box fusions and ERβ were enhanced by the addition of the agonists 17β-estradiol and genistein (Fig. 2A). However, administration of antiestrogens alone antagonized the basal receptor-peptide interactions. Interestingly, both of the control peptide fusions showed significant levels of hormone-independent interaction with ERβ. A similar observation was made *in vitro*, where glutathione-S-transferase (GST)-pull down assays revealed that both SRC-1 and GRIP1 interacted with ERβ in the absence of hormone (data not shown).

When the ERβ-selective peptides were analyzed in this assay, we were able to divide them into two classes: those that interacted with the receptor in the absence of hormone (Fig. 2B) and those whose interaction was strictly agonist dependent (Fig. 2C). The profiles of three representative peptides from each group are shown. Several of the peptide fusions (ERβ-interacting peptides EBIP-37 and EBIP-44) interacted equally well with ERβ in the absence of hormone and

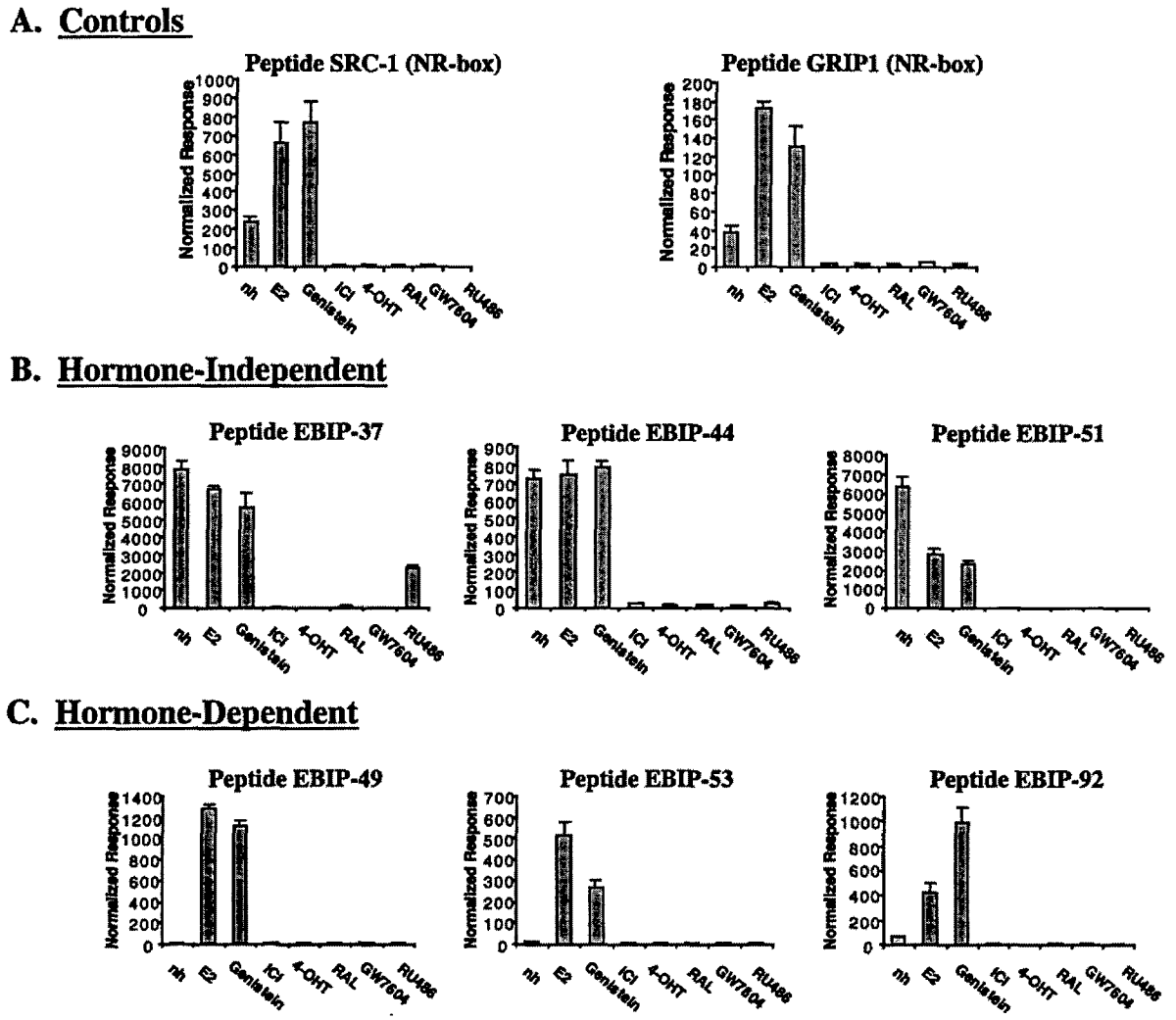

Fig. 2. ERβ-Selective LXXLL Peptides Display Hormone Specificity

Mammalian two-hybrid assays were performed to examine the interactions between the peptides and ERβ in the presence of different ER ligands. HepG2 cells were transiently transfected with the pVP16-ERβ expression vector together with each peptide-Gal4DBD fusion construct, the 5x-GAL4-TATA-Luc reporter, and the pCMV-β-gal control plasmid. After transfection, cells were treated with vehicle (nh) or either 10^{-7} M 17β-estradiol (E_2), ICI182,780 (ICI), 4-hydroxytamoxifen (4OH-T), raloxifene (RAL), GW7604, RU486, or 10^{-6} M genistein. After 24 h cells were harvested and assayed for luciferase activity, and all transfections were normalized for efficiency using the internal pCMV-β-gal control plasmid. Each data point is the average of triplicate determinations, and the average coefficient of variance for each value is <10%. A, The SRC-1 NR-box and GRIP1 NR-box were used as controls. Based upon the pattern of interactions, the peptides were divided into two classes: B, hormone-independent (six peptides) and C, hormone-dependent (nine peptides). The activities of three representative peptides are shown in both parts B and C, and these illustrate the three different patterns of interactions observed in each case.

in the presence of agonist (Fig. 2B). However, these interactions were antagonized by antiestrogen administration. Interestingly, peptide EBIP-51 interacted most efficiently with apo-ERβ, suggesting the existence of cofactors that prefer to associate with ERβ in a constitutive manner (see *Discussion*). The binding of LXXLL motifs to ERβ in the absence of hormone was puzzling, in view of the fact that it was previously observed that the human ERβ does not demonstrate ligand-independent transcriptional activity (11). This result suggests that the aporeceptor may recruit cofactors in some contexts, but that this interaction is not transcriptionally productive. In these studies, we also observed that the antiprogestin RU486 was able to decrease interactions between the peptides and receptor in these assays, a result that agrees with recent reports that RU486 can function as an ERβ antagonist (23).

Nine of the peptide fusions studied were found to interact only with agonist-activated ERβ. Interestingly, within this class, three distinct binding patterns were observed (Fig. 2C). Specifically, peptide EBIP-92 appeared to interact more efficiently with genistein-activated ERβ than that activated by 17β-estradiol, whereas the estradiol-activated receptor interacted equally as well with EPIP-49, and more efficiently with EBIP-53. 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 functional properties that have recently been ascribed to genistein (8, 24). Similar differences in efficacy were noted when the experiments were repeated over a full range of ligand concentrations (data not shown). These results suggest that estradiol and genistein induce unique conformational changes within ERβ. This hypothesis is supported by recent crystallography studies that showed that ERβ helix 12 (AF-2) assumes a distinct position when occupied by genistein compared with the distinctive agonist position observed for helix 12 of the estradiol-bound ERα (16, 25). If the estradiol-ERα crystallographic data are extrapolated to ERβ, it is possible that genistein-liganded ERβ interacts with cofactors in a different manner than the estradiol-activated receptor or that the former complex recruits a unique coactivator in some settings.

Affinity Does Not Explain the Hormone-Independent Interaction of LXXLL Peptides with ERβ

One of the most interesting classes of peptides identified in this study were those that interacted with ERβ in a constitutive manner but that were unable to interact with the receptor when occupied by antagonists. A trivial explanation for these observed binding characteristics was that these peptides had a higher affinity for ERβ than the peptides that required ligand. Alternatively, these peptides may interact in a unique manner with the coactivator binding pocket within ERβ. These different possibilities were tested using a quantitative phage ELISA. Specifically, the phage stocks of each peptide-expressing clone were titered, and then the binding of different concentrations of phage expressing peptides to ERβ was tested in the presence and absence of estradiol. The results of this analysis, shown in Fig. 3, indicated that even at the lowest input phage concentrations, the hormone-independent peptides maintained their ligand-independent interactions with ERβ; two representative peptides are shown. This was in contrast to the hormone-dependent peptides, which maintained their estradiol-dependent interaction with ERβ throughout the range of phage concentrations (representative example shown in Fig. 3C). Neither the absolute numbers of phage binding

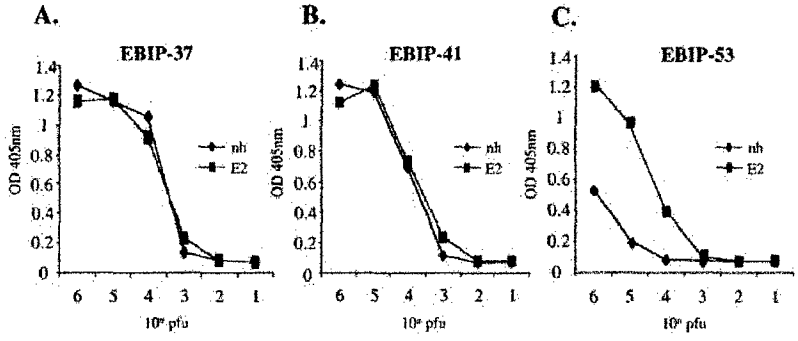

Fig. 3. Affinity Does Not Explain the Hormone-Independent Interaction of LXXLL Peptides with ERβ

The binding of each titered phage clone to ERβ was measured by ELISA. Purified ERβ protein was added to 96-well plates, and 50 μl of each phage stock or serial dilutions were added to individual wells and incubated with the ER target for 1 h at room temperature. The assays were performed in the absence and presence of 10^{-6} M 17β-estradiol. The wells were washed with PBST to remove nonbinding phage and incubated with a horseradish peroxidase-conjugated anti-M13 antibody. Immunocomplexes were detected with ABTS (2′, 2′-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid) supplemented with 0.05% H_2O_2. The colorimetric change was quantitated by measuring the absorbance at 405 nm. The binding patterns of two representative peptides from the hormone-independent class (A and B), and the binding of one representative peptide from the hormone-dependent class (C) are shown.

to each target nor the apparent binding affinity were significantly different. These results indicate that receptor-binding affinity is not what distinguishes peptides that interact with ERβ in a ligand-dependent manner from those that bind constitutively, but rather these classes of LXXLL-containing peptides interact in different ways with the ERβ-coactivator binding pocket.

Disruption of ERβ Transcriptional Activity by LXXLL-Containing Peptides

The ability of the ERβ-interacting peptides to function as ER subtype-selective antagonists in target cells was next assessed. This was accomplished using transcriptional interference assays in mammalian cells transfected with either ERα or ERβ, together with an empty Gal4DBD vector (pM) or Gal4DBD fusions of the peptides. The EBIP-37, EBIP-41, and EBIP-45 peptides were used in these initial experiments, because they appeared to interact most efficiently with ERβ in the mammalian two-hybrid system (Fig. 1A). Under the conditions of this assay, we noted that the transcriptional activity of ERα was unaffected by overexpression of the ERβ-selective peptides (Fig. 4A). Coexpression of the GRIP1 NR-box sequence (three LXXLL motifs), however, inhibited ERα transcriptional activity by 77%, thus validating the use of LXXLL peptides as antagonists of ER transcriptional responses. When the effect of our peptides on ERβ activity was assessed, we observed that all of the LXXLL sequences functioned as effective antagonists (Fig. 4B). Significantly, coexpression of peptide EBIP-37 with ERβ resulted in 100% inhibition of the transcriptional response, whereas the EBIP-41, EBIP-45, and GRIP-1 (NR-box) peptide fusions produced 85, 70, and 90% inhibition, respectively. Western immunoblotting revealed that expression of the peptides did not alter the cellular levels of ERβ (data not shown). These results indicate that it is possible to target ERβ-coactivator interactions in a selective manner and validate this general approach to develop antagonists of additional members of the nuclear receptor superfamily.

ERβ-Selective LXXLL-Containing Peptides Show Distinct Preferences for Other Nuclear Receptors

One of the major objectives of this study 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 ERα, ERβ, and 11 different nuclear receptors. Table 1 summarizes the interactions observed between each of the peptides and receptors in the presence of their cognate ligands. No clear pattern emerged from these studies, as each receptor appeared to exhibit distinct peptide binding preferences. Based on these results, it is possible that most of the receptors will eventually be found to display different cofactor preferences. The observation that the androgen receptor (AR) did not interact with any of the peptides tested and that RORα interacted with only two of the 15 peptides suggest that these receptors might have very specific coactivator requirements.

Interestingly, the peptides identified in our screen using unliganded ERβ as bait (EBIP-37, EBIP-41, EBIP-44, EBIP-45), and which interacted with the receptor in an agonist-independent manner in mammalian cells (Fig. 3), were found to interact with most of

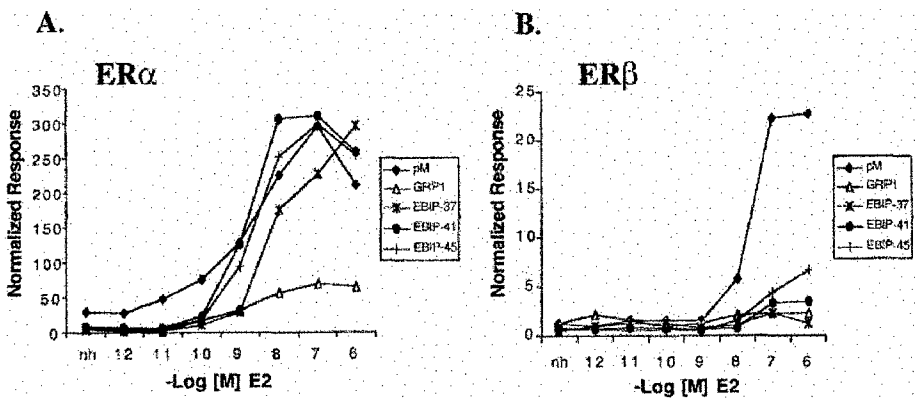

Fig. 4. Disruption of ERβ Transcriptional Activity by LXXLL-Containing Peptides

The effects of three LXXLL-containing peptides (EBIP-37, EBIP-41, and EBIP-45) on ERα and ERβ transcriptional activity were examined. A peptide containing the LXXLL motifs (NR box) of the coactivator GRIP1 was used as a control. HepG2 cells were transiently transfected with either the ERα (A) or ERβ (B) expression vector, together with the empty Gal4DBD vector (pM) or the Gal4DBD-peptide fusion constructs and the 3x-ERE-TATA-Luc reporter and pCMV-β-gal control plasmid. Cells were induced with vehicle (nh) or increasing concentrations (ranging from 10^{-12} M to 10^{-6} M) of 17β-estradiol (E_2) for 24 h, and luciferase assays were performed. Each value was normalized to the β-galactosidase activity. Each data point is the average of triplicate determinations, and the average coefficient of variance for each value is <10%.

Table 1. ERβ-Selective LXXLL-Containing Peptides Show Distinct Preferences for Other Nuclear Receptors

	ERα	ERβ	PR-A	PR-B	GR	AR	RARα	RXRα	TRβ	VDR	RORα	LXR	FXR
SRC-1	+	+	+	+	+	−	+	+	+	+	−	+	+
GRIP1	+	+	+	+	+	−	+	+	+	+	−	+	+
EBIP-37	−	+	+	+	+	−	+	+	+	+	+	+	+
EBIP-41	−	+	−	−	+	−	+	+	+	−	−	+	−
EBIP-44	−	+	+	+	+	−	+	+	+	−	−	+	+
EBIP-45	−	+	+	+	−	−	−	+	+	−	−	+	−
EBIP-49	−	+	−	−	+	−	−	−	−	−	−	−	−
EBIP-51	−	+	−	−	−	−	−	+	+	+	−	+	−
EBIP-53	−	+	+	+	+	−	−	+	+	−	−	+	−
EBIP-56	**−**	**+**	**−**	**−**	**−**	**−**	**−**	**−**	**−**	**−**	**−**	**−**	**−**
EBIP-60	−	+	+	+	+	−	−	+	−	−	−	+	−
EBIP-66	−	+	+	+	+	−	−	+	−	−	−	−	−
EBIP-70	−	+	−	−	−	−	+	+	+	+	−	+	−
EBIP-76	−	+	−	−	−	−	−	+	+	−	−	+	−
EBIP-87	−	+	+	+	+	−	+	+	+	−	−	+	−
EBIP-92	**−**	**+**	**−**	**−**	**−**	**−**	**−**	**−**	**−**	**−**	**−**	**−**	**−**
EBIP-96	−	+	+	+	+	−	−	−	+	+	+	+	−

The ability of the ERβ-selective LXXLL motifs to interact with several nuclear receptors was tested in the mammalian-two-hybrid assay (+ denotes interaction; − denotes lack of interaction). ERβ-specific peptides are indicated in *bold*. Shown here are the interactions that form between the peptides and receptors in the presence of the receptor-specific agonists, with the exception of RORα, which is constitutively active. An interaction (+) was defined by the observation of a statistically significant increase in activity when both the receptor and peptide were coexpressed compared to that present when either was cotransfected with the parent vector of the other. HepG2 cells were transiently transfected with each pVP16-receptor fusion expression vector in combination with each peptide-Gal4DBD fusion construct, the 5x-GAL4-TATA-Luc reporter, and the pCMV-β-gal control plasmid. Each receptor expression construct includes the VP16 activation domain sequence fused 5′ to the entire coding sequence for the human form of the respective receptor, with the exception of pVP16-FXR, which contains the rat FXR homolog. Following transfection, cells were treated with vehicle or the following hormones: 10^{-7} M 17β-estradiol for ERα and ERβ, 10^{-7} M progesterone for PR-A and PR-B, 10^{-7} M dexamethasone for GR, 10^{-6} M 5α-dihydrotestosterone for AR, 10^{-7} M 9-*cis*-retinoic acid for RARα and RXRα, 10^{-7} M T_3 for TRβ, 10^{-7} M 1,25-dihydroxyvitamin D_3 for VDR, 10 μM 22R-hydroxycholesterol for LXR, and 50 μM chenodeoxycholic acid for FXR. After 24 h luciferase assays were performed, and each value was normalized to the β-galactosidase activity; − indicates that no significant increase in activity was observed when the receptor and peptide were coexpressed.

the nuclear receptors tested. The most important result, however, was that EBIP-56 and EBIP-92 interacted exclusively with ERβ and did not interact with any other receptor under the conditions tested. Other peptides, such as EBIP-87 and EBIP-96, were found to interact with multiple receptors. Cumulatively, the results of these studies indicate that it is possible to identify LXXLL-containing peptides that interact in a highly specific manner with ERβ.

Evaluation of the Antagonist Properties of Peptides That Interact in a Specific Manner with ERβ

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 (18). 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 NR-box 2 and NR-box 3 of 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 (Fig. 5A). 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 (Fig. 5B). Similar results were obtained using 1xEBIP-56 and 1xEBIP-92, although higher levels of expression of these peptides were required to attain the same degree of antagonism as their dimeric counterparts. Western immunoblotting was used to demonstrate that expression of these peptides did not alter cellular levels of ERβ (data not shown). Thus, ERβ-specific LXXLL-containing peptides can function as potent inhibitors of ERβ transcriptional activity.

ERβ-Specific LXXLL-Containing Peptides Disrupt the Transcriptional Activity of ERα/ERβ Heterodimers

We and others have demonstrated that the transcriptional activity of agonist-activated ERα is significantly greater than ERβ in most cell and promoter contexts

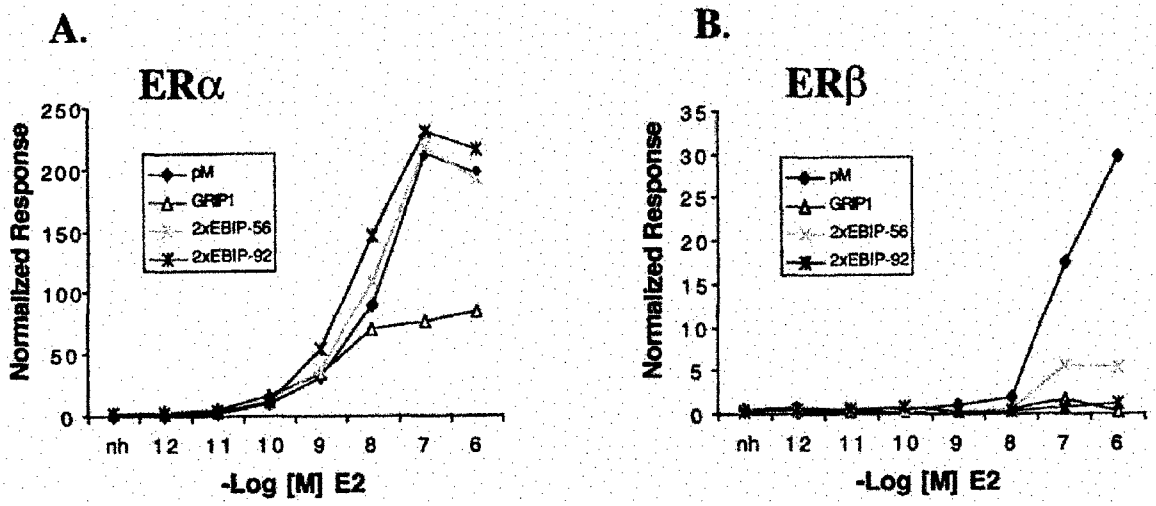

Fig. 5. Evaluation of the Antagonist Properties of Peptides That Interact in a Specific Manner with ERβ

The ability of the ERβ-specific LXXLL peptides EBIP-56 and EBIP-92 to disrupt the transcriptional activities of ERα and ERβ was examined. For these experiments, constructs containing two copies of the peptides (2x-EBIP-56 and 2x-EBIP-92) were used. A peptide containing the three LXXLL motifs (NR-boxes) of the coactivator GRIP1 was used as a control. HepG2 cells were transiently transfected with the ERα (A) or ERβ (B) expression vector together with the empty Gal4DBD vector (pM) or the Gal4DBD-peptide fusion constructs and the 3x-ERE-TATA-Luc and pCMV-β-gal plasmids. After transfection, cells were treated with vehicle (nh) or increasing concentrations (ranging from 10^{-12} M to 10^{-6} M) of 17β-estradiol (E_2) for 24 h, and luciferase assays were performed. Each value was normalized to the β-galactosidase activity. Each data point is the average of triplicate determinations, and the average coefficient of variance for each value is <10%.

(7, 9–11). Not surprisingly, therefore, in cells engineered to produce both ER subtypes, the overall response to estradiol is reduced. This suggests that one of the functions of ERβ is to modulate ERα transcriptional activity in target cells. At subsaturating concentrations of agonist, ERβ completely suppresses ERα transcriptional activity, whereas no inhibition is observed when the assay is performed in the presence of saturating concentrations of 17β-estradiol (11). These findings suggest that the role of ERβ in estrogen signaling is complex. Previously, we have shown that ERβ is bound constitutively to DNA in the absence of hormone (11). Consequently, at subsaturating concentrations of hormone, the receptor is capable of competitively inhibiting the activity of ERα homodimers and ERα/ERβ heterodimers by blocking their ability to interact with target gene promoters. Under saturating concentrations of hormone, we have proposed that ERα/ERβ heterodimers form, and that the functional properties of this complex are similar to that of an ERα homodimer. However, without a specific ERβ antagonist, it has not been possible to prove that the heterodimeric ERα/ERβ complex was functionally active in cells.

The identification of specific peptide antagonists of ERβ has enabled us to evaluate the functional significance of ERα/ERβ heterodimers. This was accomplished by determining the impact of expressing the 2xEBIP-92 antagonist in cells and examining its impact on ERα and ERβ-mediated transcriptional activity. The results of this analysis are shown in Fig. 6. As observed previously, expression of 2x-EBIP-92 in cells had no effect on ERα transcriptional activity, whereas it completely suppressed ERβ activity. Importantly, however, in cells expressing both receptor subtypes it was demonstrated that the ERβ-specific antagonist, 2xEBIP-92, was capable of significantly reducing the transcriptional activity of the ERα/ERβ heterodimer. To rule out the possibility that, in the context of the heterodimer, the ERβ-specific peptides may interact directly with ERα we used mammalian two-hybrid assays to assess the interaction of the heterodimeric complexes with a subset of the EBIPs. For this purpose, we created an ERβ mutant that disrupts AF-2 but that has no effect on the receptor's ligand binding characteristics. As shown in Fig. 7A, an ERβ-VP16, but not an ERβ-3x-VP16, chimera was able to interact with the LXXLL peptides when tested in the two-hybrid assay. Since ERβ-3x heterodimerizes with ERα in a manner that was indistinguishable from ERβ it was possible to use this mutant to test the interaction of the ERβ-interacting peptides with the ER α/β heterodimer (11). First, we demonstrated that expression of ERα in target cells had no effect on the ability of ERβ to interact with EBIPs 37, 41, 56, or 92 (Fig. 7B). Although it would be difficult to quantitate the amount of heterodimer formed under the conditions of this assay, the results suggest that ERβ specificity is maintained in this context. The most important result, however, was that in cells expressing ERα, the ERβ-3x-VP16 chimera was inactive in the two-hybrid assay (Fig. 7B). Given the characteristics of the ERβ-3x chimera, a

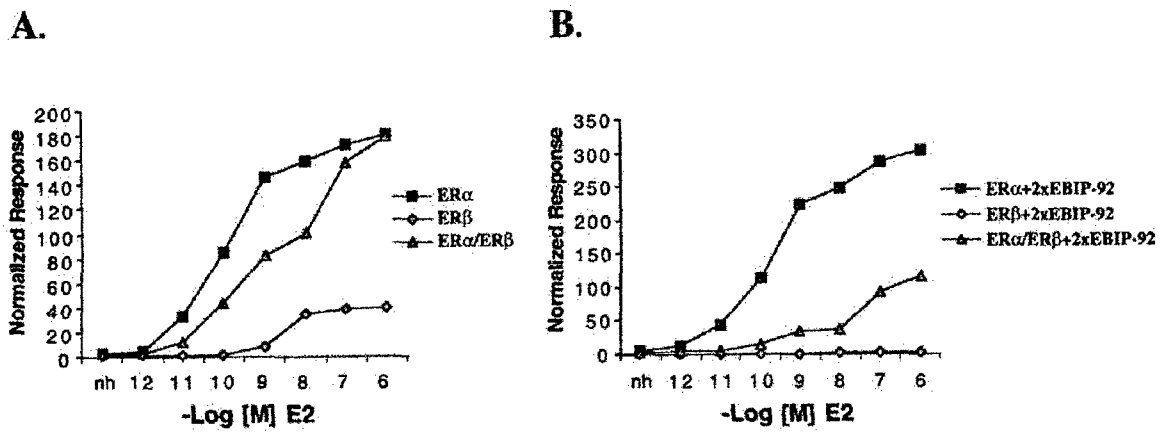

Fig. 6. ERβ-Specific LXXLL Containing Peptides Disrupt the Transcriptional Activity of ERα/ERβ Heterodimers

HepG2 cells were transiently transfected with either the ERα or ERβ expression vectors, or equal quantities of both vectors together with the pM (Gal4DBD) empty vector (A) or the 2XEBIP-92 peptide-Gal4DBD fusion construct (B) and the 3x-ERE-TATA-Luc and pCMV-β-gal plasmids. Cells were induced with vehicle (nh) or increasing concentrations (ranging from 10^{-12} M to 10^{-6} M) of 17β-estradiol (E_2) for 24 h, and luciferase assays were performed. Each value was normalized to the β-galactosidase activity. Each data point is the average of triplicate determinations, and the average coefficient of variance for each value is <10%.

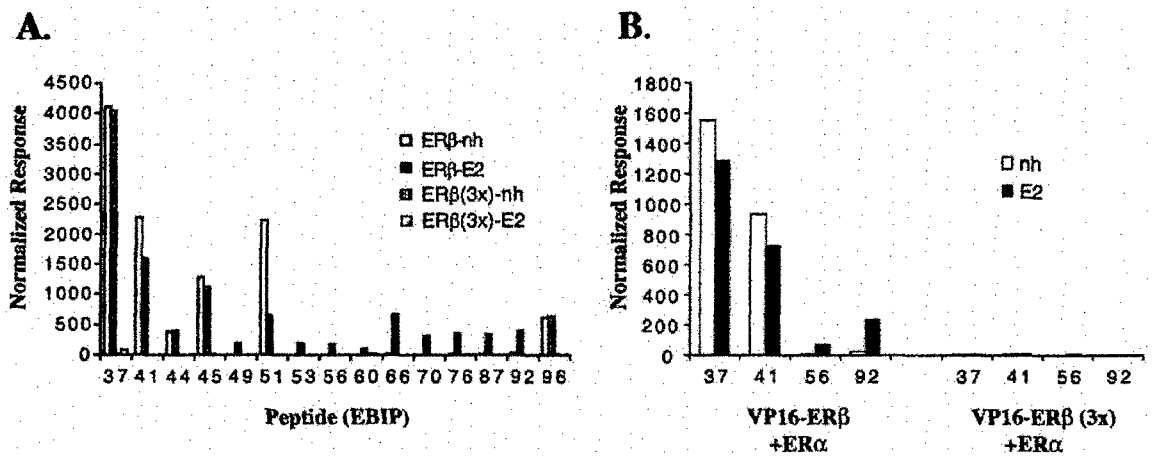

Fig. 7. The Specificity of the ERβ-Interacting Peptides Is Maintained within the Context of an ERα/ERβ Heterodimer

A, Mammalian two-hybrid assays were performed to examine the interactions between the peptides and ERβ or ERβ(3x). ERβ(3x) contains three point mutations introduced into AF-2, which nullify the AF-2 activity of the receptor. HepG2 cells were transiently transfected with the pVP16-ERβ or pVP16-ERβ(3x) expression vector together with each peptide-Gal4DBD fusion construct, the 5x-GAL4-TATA-Luc reporter, and the pCMV-β-gal control plasmid. B, Mammalian two-hybrid assays were performed to examine the interactions between the peptides and ERα/ERβ heterodimers or ERα/ERβ(3x) heterodimers. The interaction of four representative peptides are shown. HepG2 cells were transiently transfected with the pVP16-ERβ or pVP16-ERβ(3x) expression vector and an ERα expression vector together with each peptide-Gal4DBD fusion construct, the 5x-GAL4-TATA-Luc reporter, and the pCMV-β-gal control plasmid. After transfection, cells were treated with vehicle (nh) or 10^{-7} M 17β-estradiol (E_2). After 24 h, cells were harvested and assayed for luciferase activity, and all transfections were normalized for efficiency using the internal pCMV-β-gal control plasmid. Each data point is the average of triplicate determinations, and the average coefficient of variance for each value is <10%.

positive interaction in the two-hybrid assay would only have been possible if the peptides were able to interact with the coactivator binding pocket of ERα. We conclude from this experiment that even within the context of an ERα/ERβ heterodimer that the ER-interacting characteristics of the EBIPs are maintained. Therefore, the inhibitory effects of 2xEBIP-92 on the ERα/β heterodimer is mediated by blocking ERβ function. Thus, although it has been shown previously that ERα and ERβ preferentially form heterodimers when coexpressed (26, 27), we now demonstrate ERβ contributes in a positive

manner to the overall activity of the complex under agonist saturating conditions.

Comparison of the Amino Acid Sequences of the ERβ-Interacting Peptides

Alignment of the three classes of ERβ-interacting peptides identified (hormone-independent, hormone-dependent, and ERβ-specific) was next performed (Table 2). Surprisingly, minimal homology was observed outside of the conserved LXXLL motif between members within the same class. However, we observed that the two ERβ specific peptides, EBIP-56 and EBIP-92, contain a tryptophan residue at position −5 that was not found in any 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 (data not shown). 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 2. Comparison of the Amino Acid Sequences of the ERβ-Interacting Peptides

	−3−2−1	LXXLL	+1+2+3
ERβ-selective peptides			
Hormone-independent			
Peptide EBIP-37	TGGGVSL	**LLHLL**	NTEQGES
Peptide EBIP-41	RRDDFPL	**LISLL**	KDGALSQ
Peptide EBIP-44	YGLKMSL	**LESLL**	REDISTV
Peptide EBIP-45	MSYDMLS	**LYPLL**	TNSLLEV
Peptide EBIP-51	FPAEFPL	**LTYLL**	ERQGMDE
Peptide EBIP-96	VESEFPY	**LLSLL**	GEVSPQP
Hormone-dependent			
Peptide EBIP-49	VSSEGRL	**LIDLL**	VDGQQSE
Peptide EBIP-53	DTPQSPL	**LWGLL**	SSDRVEG
Peptide EBIP-60	GGTQDGY	**LWSLL**	TGMPEVS
Peptide EBIP-66	SLPEEGF	**LMKLL**	TLEGDAE
Peptide EBIP-70	VMGNNPI	**LVSLL**	EEPSEEP
Peptide EBIP-76	VLVEHPI	**LGGLL**	STRVDSS
Peptide EBIP-87	QTPL	**LEQLL**	TEHIQQG
ERβ-specific peptides			
Peptide EBIP-56	GSWQDSL	**LLQLL**	NRTELMA
Peptide EBIP-92	SVWPGPE	**LLKLL**	SGTSVAE

The ERβ-selective peptides were divided into two classes: hormone-independent and hormone-dependent. Also shown are the sequences of the ERβ-specific peptides EBIP-56 and EBIP-92, which constitute a third class of ERβ-interacting peptides. The conserved tryptophan at position −5 relative to the LXXLL motif in these two sequences is shown in *bold*.

DISCUSSION

Development of ER Peptide Antagonists

The most important outcome of this series of studies was the identification of highly potent, specific ERβ antagonists. Using these peptides, it is possible to efficiently inhibit ERβ transcriptional activity by disrupting interactions between the receptor and cellular coactivators. These reagents are important tools that will facilitate an evaluation of the role of this ER subtype in estrogen signaling. For instance, we have used these peptides to demonstrate that ERα/ERβ heterodimeric complexes can form within cells, and that ERβ contributes in a positive manner to the overall activity of the estrogen-activated complex.

Recently, it has been suggested that the two ER subtypes may oppose the actions of each other in target organs. Although controversial, this hypothesis is supported by the observation that the βERKO mouse displays epithelial hyperplasia in the prostate and bladder (14), an increase in bone mineral content (28), and an increased responsiveness to estrogen in the uterus (29), reflecting possibly an enhancement of ERα-mediated transcriptional activity. Using an appropriate delivery system, it may be possible to antagonize ERβ action using the receptor-specific peptides and test directly the hypothesis that ERβ functions as an ERα modulator in some tissues.

It has recently been shown that both ER subtypes are expressed in breast tumors (30–32) and that ERβ expression is up-regulated in tumors that have developed tamoxifen resistance (32). Thus, there is an unmet medical need to develop novel ER antagonists as 1) potential breast cancer therapeutics and 2) tools to specifically define the role of ERβ in breast cancer cell biology. The finding that none of the LXXLL-containing sequences in this study interact with antiestrogen-liganded receptor suggests that suitably formulated ER peptide antagonists could be coadministered with tamoxifen to completely block estrogen-stimulated proliferative pathways in the breast, using two mechanistically distinct modes of antagonism. Recent studies provide evidence that tamoxifen resistance in breast tumors may arise from the up-regulation of coactivator proteins, which may permit cells to recognize tamoxifen as an agonist and growth stimulant (22). The identification of peptides that disrupt receptor-coactivator interactions provides a novel mechanism by which the mitogenic actions of activated ER can be blocked in both antiestrogen-responsive and -resistant breast cancer cells. Theoretically, the peptide antagonists that we have identified could be developed as second line pharmaceutical treatments for ER-positive, tamoxifen-refractory tumors.

Previous studies in our laboratory (20) reported the identification of the ERβ selective peptide 293. However, while the peptide displays selectivity for ERβ over ERα, 293 was found to interact with many of the other nuclear receptors. In this study our goal was to develop peptides that interacted in a completely specific manner with ERβ, which was accomplished in the discovery of peptides EBIP-56 and EBIP-92. A similar study was recently reported (33) in which a panel of LXXLL-containing peptides were identified that demonstrated selectivity for ERβ over thyroid hormone receptor (TR). However, the authors of that study indicated that most of their ERβ-interacting peptides cross-react with ERα. Thus, EBIP-56 and EBIP-92 represent the only reagents available that can be used to specifically inhibit ERβ transcriptional activity.

Ligand-Independent Recruitment of LXXLL Motifs

One of the most important findings of this study was that the unliganded ERβ is capable of recruiting many of the LXXLL peptides. Interestingly, studies with ERα showed that LXXLL-containing sequences were capable of a low but significant basal level of interaction in the absence of hormone (20). These results suggested that a fraction of the ERα molecules in a cell might reside in an active conformation, thus permitting recruitment of LXXLL motifs in the absence of receptor agonists. This may explain why ERα can activate transcription in some contexts in the absence of hormone. Surprisingly, although apo-ERβ is capable of binding several different LXXLL-containing peptides, this form of the receptor does not activate transcription in the absence of agonist (11). Consistent with this observation, we have shown using *in vitro* protein-protein interaction studies that ERβ, but not ERα, can bind to GRIP1 in the absence of ligand (our unpublished results). One possibility is that the ERβ aporeceptor is present in an inhibitory complex containing both coactivators and corepressors, and that the binding of hormone enhances the functionality of associated activators and promotes the dissociation of repressor proteins. Alternatively, unliganded ERβ may bind to some cofactors in a manner that is not transcriptionally productive. An activity of this nature has not yet been demonstrated for ERβ; however, it has been shown that unliganded peroxisome proliferator activated receptor-γ (PPARγ) interacts with the coactivator PGC-1 (PPARγ coactivator 1, a protein that has no apparent coactivator activity), and this protein is responsible for recruiting SRC-1 when agonist is added (34).

The ability of nuclear receptors to interact with LXXLL motifs in their apo- state raises the possibility that ligand regulation of coactivator recruitment may have evolved to enable receptor activity to respond to changes in cellular homeostasis. Consistent with this hypothesis is the observation that several orphan receptors [estrogen-receptor-related proteins (ERR1, ERR2, and ERR3)] that have not yet been shown to require ligands bind SRC-1, GRIP1, or activator of thyroid receptor (ACTR) in a ligand-independent manner (35, 36). Similarly, the orphan receptor 1/ retinoid X receptor (OR1/RXR) heterodimer is capable of ligand-independent cofactor recruitment (37). Recent studies have also illustrated that ligand-independent signaling pathways can result in activation of ERβ by promoting agonist-independent coactivator binding (38). These observations suggest that the general mechanisms of hormone-dependent and independent transcriptional activation by nuclear receptors may be similar, and that in some cases the role of ligand may be as a catalyst, but not as a required part of receptor activation. Thus, ERβ may be a receptor whose state of evolution is intermediate between the orphan receptors and the more classical steroid receptors.

Nuclear Hormone Receptors Have Distinct Preferences for LXXLLs

A recurring theme in these studies is that nuclear receptors have distinct preferences for LXXLL motifs. Previous work in our laboratory using peptide display has demonstrated that the sequences flanking the core LXXLL domain are important determinants of receptor selectivity (20). Mutagenesis studies have also been used to identify residues important for both receptor binding affinity and specificity (18, 39). McInerney *et al.* demonstrated that of the three helical LXXLL-containing regions of SRC-1, a single helical domain was sufficient for ER activation, whereas a combination of two distinct helical regions were required for PR, TR, retinoic acid receptor (RAR), and PPARγ actions (40). These studies indicate that different receptors can interact with the same cofactor in different ways.

To complement these previous studies, we observed that peptide EBIP-37 (identical to an LXXLL motif in RIP140) interacted selectively with ERβ, but not ERα. Therefore, since RIP140 can bind to and repress the transcriptional activities of both ERα and ERβ (Ref. 41 and our unpublished data), it is likely that each receptor subtype utilizes distinct LXXLL motifs within this factor, enabling them to bind. The observation that each of the receptors examined in our study displayed a unique pattern of interaction with LXXLL peptides also provides evidence that the receptors may bind different coactivators, or alternatively, recruit the same factors by utilizing distinct binding regions. It is likely therefore, that it will be possible to develop LXXLL-containing antagonists for many of the nuclear receptors. It was surprising, given the structural conservation among the nuclear receptors and associated cofactors, that peptides could be identified which block these interactions in a highly specific manner. However, given that it has been possible to develop specific ERβ antagonists

using this approach, we believe that it will be feasible to identify inhibitors of a wide variety of transcription factors by interfering with specific protein-protein interactions.

MATERIALS AND METHODS

Biochemicals

DNA restriction and modification enzymes were obtained from Roche Molecular Biochemicals (Indianapolis, IN), New England Biolabs, Inc. (Beverly, MA), or Promega Corp. (Madison, WI). PCR reagents were obtained from Perkin Elmer Corp. (Norwalk, CT) or Promega Corp. 17β-Estradiol, genistein, 4-hydroxytamoxifen, 9-*cis*-retinoic acid, dexamethasone, 5α-dihydrotestosterone, T_3, progesterone, 22R-hydroxycholesterol, and chenodeoxycholic acid were purchased from Sigma (St. Louis, MO). RU486 was a gift from Ligand Pharmaceuticals, Inc. (San Diego, CA). The estrogen receptor antagonist ICI 182,780 was a gift from Dr. Alan Wakeling (Zeneca Pharmaceuticals, Macclesfield, UK). Raloxifene was a gift from Dr. Eric Larsen (Pfizer, Inc., Groton, CT). GW7604 was a gift from Dr. Tim Willson (Glaxo-Wellcome, Research Triangle Park, NC); 1,25-dihydroxyvitamin D_3 was purchased from Duphar Pharmaceuticals (Daweesp, The Netherlands). The mouse monoclonal anti-Gal4DBD antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The rabbit polyclonal ERβ antibody was a gift from Dr. Geoffrey Greene (University of Chicago, Chicago, IL). Secondary antibodies, Hybond-C extra transfer membranes, and ECL reagents were purchased from Amersham Pharmacia Biotech (Arlington Heights, IL).

Affinity Selection of ERβ-Binding Peptides

Baculovirus-expressed human ERβ (amino acids 1–477) was purchased from PanVera Corp. (Madison, WI). ERβ (4 pmol) was added to 100 μl of $NaHCO_3$, pH 8.5, in single wells of a 96-well Immulon 4 plate (Dynex Technologies, Inc.). The protein was then incubated in the absence or presence of 10^{-6} M 17β-estradiol overnight at 4 C. A duplicate well containing BSA alone was used as a control. The wells were blocked with 150 μl of 0.1% BSA in $NaHCO_3$ for 1 h at room temperature and then washed five times with PBST (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.3, 0.1% Tween 20). Twenty five microliters of the phage library ($>10^{10}$ phage) were preincubated on ice for 1 h in 125 μl PBST, 0.1% BSA, and 10^{-6} M 17β-estradiol or vehicle. The phage library was added to the wells, and the plate was sealed and incubated at room temperature for 8 h with gentle agitation. The wells were washed five times with PBST to remove nonbinding phage. The binding phage were eluted with 100 μl of 50 mM glycine-HCl, pH 2.0 (prewarmed to 50 C), and subsequently eluted with 100 μl of 100 mM ethanolamine, pH 11.0. The first eluant was neutralized with 200 μl of 200 mM Na_2HPO_4, pH 8.5, before being combined with the second eluant. The bound phage were amplified in DH5αF′ cells for 6 h and recovered by centrifugation. The amplified phage were used for subsequent rounds of panning. Three rounds of panning were performed. The enrichment of ERβ binding phage in each round of panning was confirmed by ELISA. Individual phage clones were purified after the third round of panning. The single-stranded phage DNA was isolated from each clone, and the peptide sequences were determined by DNA sequencing.

ELISA

Purified ERβ protein (0.4 pmol) was added to 96-well Immulon 4 plates as detailed above. Fifty microliters of each purified phage were added to an individual well and incubated with the ER target for 1 h at room temperature. The assays were performed in the absence and presence of 10^{-6} M of various ER ligands. The wells were washed five times with PBST to remove nonbinding phage. The binding of each peptide to full-length ERα (provided by Panvera Corp.) in the presence of various ER ligands was also tested in this assay. A horseradish peroxidase-conjugated anti-M13 antibody (Amersham Pharmacia Biotech) was diluted 1:5000 in PBST, 100 μl of the mixture was added to each well, and the solutions were incubated for 1 h at room temperature. The wells were washed five times with PBST, and immunocomplexes were detected with ABTS (2′,2′-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid) supplemented with 0.05% H_2O_2. The colorimetric change was quantitated by measuring the absorbance at 405 nm on a plate reader (Multiskan MS; Labsystems, Marlboro, MA).

Plasmids

The Gal4DBD-peptide fusions were constructed as follows. The peptides were excised from the mBAX phage vectors with *Xba*I and *Xho*I. The parent pMsx vector (20) (containing the Gal4DBD) was digested with *Sal*I and *Xba*I. The peptides were then ligated in frame to the pMsx vector, creating Gal4DBD-peptide fusion constructs. The constructs containing two copies of the LXXLL-containing peptides (2x-EBIP-56 and 2x-EBIP-92) were created as follows: pM-EBIP-56 and pM-EBIP-92 were digested with *Xba*I. The linker region between the second and third LXXLL motifs within the GRIP1 cDNA was amplified by PCR, digested with *Nhe*I and *Xba*I, and ligated into pM-EBIP-56 and pM-EBIP-92, at the 3′ of the peptide coding region. These vectors were then digested with *Sal*I and *Xba*I and an oligonucleotide encoding a second copy of the peptide was inserted into these sites 3′ to the GRIP1 linker. The construction of pM-SRC-1 (NR-box) and pM-GRIP1 (NR-box) has been described previously (20).

The mammalian expression plasmid for the peptide EBIP-92 mutant was constructed by site-directed mutagenesis as follows. The pM-EBIP-92 vector was used as the template, and a point mutation in the conserved tryptophan residue was created using PCR-based oligonucleotide-directed mutagenesis, according to the manufacturer's protocol (Stratagene, La Jolla, CA). The sequences of the oligonucleotides used for PCR were 5′-CTCGAGAAGTGT-TGAGCCGGGTCCGGAGCTGCTTAAGCTGCTGTCGGGGA-CGAGTGTGGCGGAG (forward) and 3′-CTCCGCCACAC-TCGTCCCCGACAGCAGCTTAAGCAGCTCCGGACCCGGC-TCAACACTTCTCGAG (reverse).

pVP16ERα, pVP16ERβ, pVP16RARα, and pVP16RXRα have been described previously (20). VP16GR, VP16PR-A, VP16PR-B, and VP16AR expression plasmids were gifts from J. Miner (VP16GR), D. X. Wen (VP16PR-A and VP16PR-B), and K. Marschke (VP16AR) (Ligand Pharmaceuticals, Inc., San Diego, CA). VP16VDR was a gift of J. W. Pike (University of Cincinnati, Cincinnati, OH), and the VP16TRβ expression plasmid (pCMX-VP-F-hTRβ) was provided by D. D. Moore (Baylor College of Medicine, Houston, TX). pVP16RORα-LBD was a gift from A. R. Means (Duke University Medical Center, Durham, NC). The cDNAs for the human liver X receptor (LXR) and rat farnesoid X receptor (FXR) were provided by D. J. Mangelsdorf (University of Texas, Dallas, TX). pVP16LXR, and pVP16FXR were created as described previously for the other nuclear receptor VP16 fusions (20).

The mammalian expression plasmids for ERα (pRST7ER) and ERβ (pRST7ERβ) have been described previously (11, 42). The reporter 5x-GAL4-TATA-Luc (a gift from Dr. Xiao-Fan Wang, Duke University Medical Center) contains five palin-

dromic copies of the GAL4 transcription factor response element cloned into pGL2-TATA-Inr (Stratagene). The 3x-ERE-TATA-Luc reporter contains three copies of the vitellogenin ERE (43).

All of the PCR-based constructs were sequenced to verify the accuracy of the amplified sequences.

Cell Culture and Transient Transfection Assays

HepG2 cells were maintained in MEM (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% FCS (Life Technologies, Inc.), 0.1 mM nonessential amino acids, and 1 mM sodium pyruvate. Cells were plated in 24-well plates (coated with gelatin for transfections of HepG2 cells) 24 h before transfection. DNA was introduced into the cells using lipofectin (Life Technologies, Inc.). Triplicate transfections were performed using 3 μg of total DNA. In standard mammalian two-hybrid assays, 1,500 ng of reporter (5x-GAL4-TATA-Luc), 500 ng of receptor-VP16 fusion, 500 ng of pM (Gal4DBD)-peptide fusion constructs, 100 ng of the pCMV-βgal normalization vector (44), and 400 ng of the control vector pBSII-KS (Stratagene) were used. For receptor disruption studies, 1,500 ng of reporter (3x-ERE-TATA-Luc), 250 ng of receptor (either pRST7ERα or pRST7ERβ), 1000 ng of pM-peptide fusion constructs or the parent pM vector, 100 ng of pCMV-βgal, and 150 ng of pBSII-KS were used. Cells were incubated with the DNA/lipofectin mix for 3 to 6 h and then washed with PBS and the transfection mix was replaced with phenol red-free MEM containing 10% charcoal-stripped FCS (HyClone Laboratories, Inc., Logan, UT). The receptor ligands were added to the cells 20–24 h before the assays. Luciferase and β-galactosidase assays were performed as described previously (45). All experiments were repeated a minimum of three times.

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Temporally Distinct and Ligand-Specific Recruitment of Nuclear Receptor-Interacting Peptides and Cofactors to Subnuclear Domains Containing the Estrogen Receptor

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Ligand binding to estrogen receptor (ER) is presumed to regulate the type and timing of ER interactions with different cofactors. Using fluorescence microscopy in living cells, we characterized the recruitment of five different green fluorescent protein (GFP)-labeled ER-interacting peptides to the distinct subnuclear compartment occupied by blue fluorescent protein (BFP)-labeled ERα. Different ligands promoted the recruitment of different peptides. One peptide was recruited in response to estradiol (E_2), tamoxifen, raloxifene, or ICI 182,780 incubation whereas other peptides were recruited specifically by E_2 or tamoxifen. Peptides containing different sequences surrounding the ER-interacting motif LXXLL were recruited with different time courses after E_2 addition. Complex temporal kinetics also were observed for recruitment of the full-length, ER cofactor glucocorticoid receptor-interacting protein 1 (GRIP1); rapid, E_2-dependent recruitment of GRIP1 was blocked by mutation of the GRIP1 LXXLL motifs to LXXAA whereas slower E_2 recruitment persisted for the GRIP1 LXXAA mutant. This suggested the presence of multiple, temporally distinct GRIP 1 recruitment mechanisms. E_2 recruitment of GRIP1 and LXXLL peptides was blocked by coincubation with excess ICI 182,780. In contrast, preformed E_2/ER/GRIP1 and E_2/ER/LXXLL complexes were resistant to subsequent ICI 182,780 addition whereas ICI 182,780 dispersed preformed complexes containing the GRIP1 LXXAA mutant. This suggested that E_2-induced LXXLL binding altered subsequent ligand/ER interactions. Thus, alternative, ligand-selective recruitment and dissociation mechanisms with distinct temporal sequences are available for ERα action *in vivo*. (Molecular Endocrinology 14: 2024–2039, 2000)

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INTRODUCTION

Ligand binding to ER alters ER conformation and thereby affects its interactions with cofactors regulating gene expression (1–3). Many cofactors that interact specifically with the estradiol (E_2)-bound ER or other ligand-bound nuclear receptors contain one or more copies of the consensus sequence LXXLL (4). Mutation of the LXXLL motifs abrogates ligand-dependent cofactor binding to the ligand-binding domain of many nuclear receptors (4, 5). Peptides containing the LXXLL motifs are themselves sufficient to bind to nuclear receptors (6, 7). Structural studies showed that the LXXLL peptides form an amphipathic α-helix, of which the hydrophobic surface fits into a hydrophobic cleft that forms on the surface of the ER ligand-binding domain in response to E_2 binding (6, 7). The ligand-induced hydrophobic cleft is conserved in the ligand

binding domain of most nuclear receptors (8) and is required for ligand-activated transcription via activation function-2 (AF-2) (9).

The hydrophobic cleft does not form properly upon ER binding to tamoxifen or raloxifene (7, 10), which may account for the antiestrogenic action of these ligands in some tissues. Conversely, tamoxifen and raloxifene have estrogenic effects on other tissues. Like estrogens, tamoxifen and raloxifene promote interaction of some cofactors or peptides with ER structures outside of the hydrophobic cleft (11–14). These interactions probably contribute to the AF-2-independent estrogenic actions of tamoxifen and raloxifene. Novel ER ligands that possess estrogenic activities in most tissues and antiestrogenic activities in the breast and uterus will be clinically useful for reducing the estrogen-mediated increase in breast and endometrial tumors that accompanies otherwise beneficial postmenopausal hormone replacement therapies (15–19). Identification of such improved selective estrogen receptor modulators (SERMs) will be aided by the development of techniques that discern the effects of each putative SERM on the types and timing of ER interactions with ligand-selective ER-interacting targets.

Previously we used phage display to isolate a large number of peptides that bound to different sites on nuclear receptors including ERα (12–14). Each peptide differed in their interactions with specific nuclear receptors or in response to different ligands. Some of the nuclear receptor-interacting peptides contained the LXXLL motif and could be grouped into three classes based upon sequence conservation of the two amino acids immediately amino terminal to LXXLL (12). All three classes of LXXLL are naturally found in cofactors that interact with AF-2. Some cofactors contain multiple LXXLL motifs predominantly of a single class. Others contain LXXLL motifs of varying classes and even LXXLL motifs that are distinct from these three classes. It is thought that such divergence in LXXLL sequence (5, 6, 20), combined with nuclear receptor- or ligand-specific divergences in the structure of the hydrophobic activation function-2 cleft (21), and variations in the interactions of cofactors to other nuclear receptor surfaces, contributes to the divergent actions of different ligands and nuclear receptors.

Although the molecular alterations that accompany ligand binding to nuclear receptors have been intensely characterized (1–3), very little is known of the specificity and order of those events within living cells. Recent studies of fluorophore-labeled nuclear receptors and their interacting cofactors (22–27) demonstrated that the temporal and spatial characteristics of nuclear receptors could be directly examined within cells by fluorescence microscopy. Here, we used fluorescence microscopy to measure in intact cells the ligand-specific interactions of ER with the nuclear receptor cofactor GRIP1 and five peptides that we recently selected from combinatorial libraries for their binding to ligand-bound ER (12–14). Human ERα expressed as a fusion with blue fluorescent protein (28) (BFP) localized to discrete subdomains of the nucleus. GRIP1 (glucocorticoid receptor-interacting protein 1) and the peptides expressed in cells as fusions with the spectrally distinct green fluorescent protein (28) (GFP) were more evenly distributed throughout the nucleus; the GFP-peptide fusions were also present in the cytoplasm. When coexpressed with ERα-BFP in cells not treated with ER ligand, the GFP-peptides and GFP-GRIP1 exhibited the same distributions as when expressed alone. When incubated with E_2, three peptides containing variants of LXXLL relocalized to assume the intranuclear position of ER. A fourth, unrelated peptide was selectively recruited in response to tamoxifen whereas recruitment of a fifth peptide was promoted by any of E_2, tamoxifen, raloxifene, or the antiestrogen ICI 182,780. GRIP1 was selectively recruited by E_2 or tamoxifen incubation. Simultaneous incubation with an excess of ICI 182,780 blocked recruitment of GRIP1, each LXXLL peptide, and the tamoxifen-specific peptide.

Recruitment of the peptides and GRIP1 to the intranuclear location of ERα in living cells mimicked their previously reported ligand dependence and efficacy of ERα interaction. In addition to confirming in living cells the ligand specificities of these interactions, the intranuclear recruitment assay uniquely enabled us to determine that each peptide and GRIP1 varied in the timing of recruitment after ligand addition. Surprisingly, temporal studies of dissociation showed that preformed complexes involving LXXLL interactions with ER uniquely were not disrupted even after 4 h of incubation with a 1,000-fold molar excess of ICI 182,780. Thus, we report a novel procedure for investigating the ligand-specific recruitment of labeled factors or peptides to nuclear receptors in living cells. This allowed us to determine the unique timing of different ligand-specific complexes formed with ER and to discover that LXXLL-dependent interactions alter the availability of the receptor for subsequent ligand binding in living cells.

RESULTS

E_2-Dependent Relocalization of Class I, II, and III LXXLL Peptides to the Intranuclear Subcompartment Containing ERα

We previously isolated three different classes of LXXLL-containing, ERα-interacting peptides by phage display (12–14). All three classes are represented in known ERα-interacting cofactors, including a receptor-interacting protein of 140 kDa (RIP140) (29), a thyroid hormone receptor accessory protein of 220 kDa ($TRAP_{220}$) (30), a vitamin D receptor-interacting protein of 205 kDa ($DRIP_{205}$)(31), and the homologous coac-

tivators glucocorticoid receptor-interacting protein (GRIP1) (32, 33), and steroid receptor coactivator (SRC-1a)(34). For instance, RIP140 contains 11 LXXLL motifs, eight of which are of the class III type (S/T,Φ,LXXLL where Φ is any hydrophobic amino acid) whereas $TRAP_{220}$ and $DRIP_{205}$ each contain two LXXLL motifs, both of the class II type (P,Φ,LXXLL). GRIP1 and SRC-1a have, in common, three divergent LXXLL motifs, the most amino terminal of which is of the class I type (S/T, K/R, LXXLL), and two more carboxy-terminal LXXLL motifs that do not readily fit into any of the three classes.

Oligonucleotides encoding peptide sequences representative of each of the class I, II, and III peptides were fused in frame to the carboxy terminus of GFP (see Fig. 1) and expressed in mouse GHFT1–5 cells. The intracellular locations of GFP and each GFP-labeled LXXLL peptide were identified by fluorescence microscopy after their expression. GFP (not shown) and the three GFP-LXXLL fusions were distributed throughout the cytoplasm and nucleus (Fig. 2, A–C, *left panels*). The proportion of GFP-LXXLL fluorescence in the nucleus and cytoplasm varied from evenly distributed between nucleus and cytoplasm to some nuclear preference. The variation in nuclear/cytoplasmic partitioning was independent of expression level and was globally similar for GFP and each GFP-LXXLL fusion.

To determine whether ERα expression altered the distribution of GFP-LXXLL, ERα was coexpressed as an in-frame fusion with BFP. This allowed us to separately track the locations of ERα-BFP and GFP-LXXLL in the same cell by selectively exciting and detecting their corresponding blue and green emissions (35). The ERα-BFP fusion was functional as, like native ERα (36), it cooperated with the transcription factor Pit-1 to activate the PRL promoter in GHFT1–5 cells (not shown). The ERα-BFP fusion also activated the transcription of a minimal promoter under the control of an isolated ER binding site in HeLa and DU145 cells (not shown). In contrast to the cytoplasmic and uniform intranuclear distributions of GFP-LXXLL, ERα-BFP was exclusively nuclear and assumed a reticular pattern of distribution within the nucleus (Fig. 2, ERα-BFP). This reticular intranuclear distribution has been previously reported for ER (22, 27) as well as other nuclear receptors (23–26) and is more pronounced

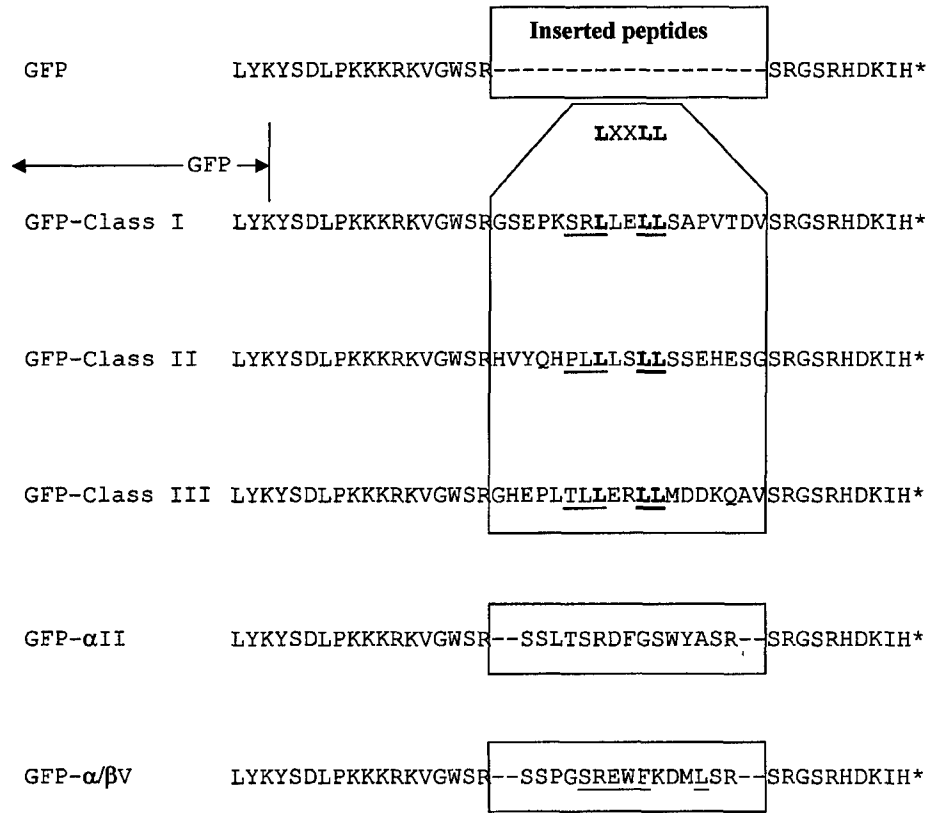

Fig. 1. ER-Interacting Peptides Fused to the Carboxy Terminus of GFP

Box represents sequence of ER-interacting peptides isolated in Chang *et al.* (12) and Norris *et al.* (13). *Underlined* amino acids in peptide sequence are those conserved in the class I, class II, and class III LXXLL peptides (12). *Underlined* in the α/βV peptide are those amino acids conserved in other isolated peptides and in receptor potentiating factor 1 (13). *, Carboxy terminus of fusion proteins. The spacer between GFP and the ER-interacting peptide sequence contains the Simian Virus 40 NLS that, because of the small size of GFP, did not have much effect on nuclear localization.

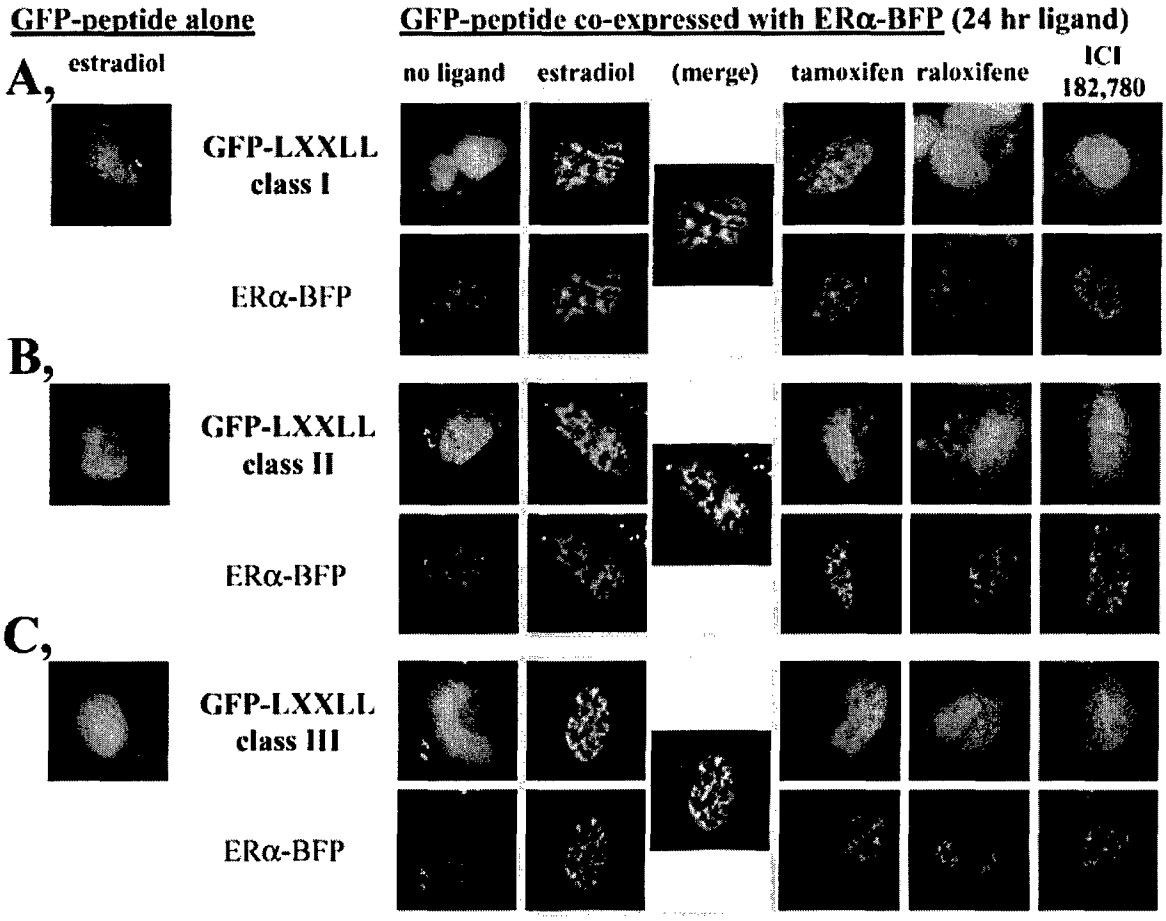

Fig. 2. E_2-Dependent Recruitment of GFP Fused to Class I, Class II, and Class III LXXLL Peptides to the Intranuclear Location of ERα-BFP

Fluorescence microscopic detection of the intracellular localization of the three GFP-LXXLL fusions (A–C) coexpressed with ERα-BFP in cells. One day after transfection, cells were treated either with ethanol vehicle (no ligand), 10^{-8} M estradiol, 10^{-6} M tamoxifen, 10^{-7} M raloxifene or 10^{-7} M ICI 182,780. One day after ligand addition, green and blue fluorescence was selectively detected by fluorescence microscopy as described in *Materials and Methods*. *Merge*, Merged blue and green images of the cells incubated with E_2 indicate overlap in the intranuclear location of ERα-BFP and each GFP peptide as a cyan coloration. Green fluorescence from each GFP-peptide fusion in the absence of ERα-BFP expression is shown from cells incubated with 10^{-8} M estradiol (*left panels*).

when the cells are incubated with E_2 or SERMs (22, 27).

In cells grown in E_2-free media, the dispersed cellular distribution of each GFP-LXXLL fusion was unchanged upon coexpression of ERα-BFP (Fig. 2, A–C, no ligand). In contrast, incubation of cells coexpressing ERα-BFP and any of the GFP-LXXLL fusions with 10^{-8} M E_2 caused the GFP-LXXLL to assume the reticular pattern characteristic of ERα-BFP in the nucleus (Fig. 2, A–C, estradiol). Complete overlap of GFP-LXXLL with ERα-BFP in the identical subnuclear compartment after E_2 addition is indicated by the exclusively cyan-colored image obtained when the separate blue and green images are merged (Fig. 2, A–C, merge). This was observed in cells that express GFP-LXXLL in low stoichiometry relative to ERα-BFP. In cells expressing more GFP-LXXLL than ERα-BFP, colocalization of GFP-LXXLL and ERα-BFP was observed as a concentration of green fluorescence at the site of blue fluorescence (not shown). When ERα-BFP was not coexpressed, there was no intranuclear redistribution of GFP-LXXLL in the presence of E_2 (Fig. 2, A–C, *left panels*) or any other ER ligand (not shown). Similarly, GFP itself did not redistribute to ERα-BFP upon incubation with E_2 or any other ER ligand (not shown). Thus, relocalization of GFP-LXXLL was specifically dependent upon the LXXLL peptide, coexpression of ERα-BFP, and addition of E_2.

Intracellular Relocalization of Different LXXLLs to ERα Parallels Their Interaction Profiles

To further characterize the ligand dependence of GFP-LXXLL colocalization with ERα-BFP, we determined

the E_2-induced relocalization kinetics of each of the class I, class II, and class III GFP-LXXLLs to ERα. Each GFP-LXXLL was coexpressed with ERα-BFP in cells grown in E_2-free media. One day after transfection, parallel coverslips were incubated with no hormone, or with 10^{-10}, 10^{-9}, 10^{-8} or 10^{-7} M E_2 for 24 h. We then determined the fraction of cells in which GFP-LXXLL colocalized with ERα-BFP for each E_2 concentration.

By fluorescence microscopy, we scanned the coverglass using blue fluorescence excitation and emission filters to first identify cells expressing ERα-BFP. We then rapidly switched to the green filter set to determine whether the cell contained visible GFP-linked target. If the GFP-linked target was also present, it was then scored as colocalized if there was any concentration of green fluorescence at the site of the ER. By scoring GFP-peptide or cofactor-expressing cells only after determining which cells obviously contained ERα-BFP, we avoided the bias in which a bright, reticular GFP fluorescence pattern would inflate our detection of colocalized cells containing otherwise undetectable levels of the generally less fluorescent ERα-BFP. By setting the colocalization criterion as "any" colocalization, we also removed any biases that would have resulted if we had attempted to subjectively score cells for the variable extent of colocalization. Since the proportion of non-colocalized cells decreases with increasing colocalization, the recruitment of specific factors or peptides is measured as the change in the proportion of cells that responded after the addition of different concentrations of E_2. The validity of this approach was confirmed by the high reproducibility of the data obtained from multiple independent experiments, which are plotted in Fig. 3A as the mean ± SD in the percent of cells showing colocalization at each ligand concentration. Half-maximal binding to ERα-BFP with each class of GFP-LXXLL was reached at 3–7 × 10^{-10} M E_2, approximately the concentration of E_2 needed for activation of ER-regulated promoters in cell transfection studies (37).

Essentially complete colocalization with ERα-BFP was achieved with 10^{-8} M E_2 for both the class I and the class III GFP-LXXLL fusions. In contrast, colocalization of the class II GFP-LXXLL did not increase beyond a maximum of 57 ± 6% of the cells. This limit did not appear to be a function of the level of peptide expressed in the cell as the proportion of cells showing colocalization remained constant over a wide range of GFP-class II LXXLL expression (Fig. 3B). In these studies, expression of GFP-LXXLL was modulated from a tetracycline-inducible promoter by varying the levels of the inducer, doxycycline. Note that all images in Fig. 3B were taken with the same short exposure times that were insufficient to detect the basal expression level of the GFP-LXXLL peptide in the absence of doxycycline. Thus, the observed deficiency in the *in vivo* ERα-BFP interaction of the class II LXXLL relative to the class I and III LXXLLs was not related to differences in the expression of these peptides. The reduced efficiency of colocalization of the GFP-class II LXXLL with ERα-BFP accurately mimicked the poorer interaction of the class II peptide with ERα that we had previously observed (12).

Delayed Temporal Kinetics of Class II LXXLL Recruitment to ERα

Sequence-specific differences in colocalization of the three LXXLL peptides with ERα were also evident in time course studies. We conducted single cell recordings of the E_2-induced intracellular recruitment of LXXLL to ERα. First, we identified cells, grown in the absence of E_2, that expressed both the class I GFP-LXXLL and ERα fused to red fluorescent protein (RFP). The ERα-RFP fusion protein was functionally active in the ligand-induced activation of estrogen-responsive promoters (data not shown). ERα-RFP and GFP-LXXLL digital images of the same cell were captured using red and green fluorescent filter sets before the addition of ligand and at 1-min intervals after the addition of 10^{-6} M E_2. An example of one cell before E_2 addition and 20 min after E_2 addition is provided in Fig. 4A. Appropriate controls, using cells expressing only ERα-RFP or GFP-LXXLL of intensities equivalent to those in the coexpressing cells, showed that there was no fluorescence bleedthrough between the red and green images (not shown).

Only partial colocalization of GFP-LXXLL with ERα-RFP (or ERα-BFP) was obtained after these short incubation periods (see Fig. 4A), which contrasts with the complete overlap of GFP-LXXLL and ERα-RFP (or ERα-BFP) after 24-h incubations (Fig. 2). To quantify partial colocalization at the short time frames, we measured the intensity of green GFP-LXXLL and red ERα-RFP fluorescence for each pixel within the nucleus of each cell image. The intensity of green fluorescence within the nucleus that colocalized with the red fluorescence of ERα-RFP was divided by the intensity of green fluorescence in the regions of the nucleus from which ERα-RFP was less concentrated (defined at those regions of the nucleus in which ERα-RFP intensity was less than 75% of the maximal ERα-RFP intensity). This ratio was calculated before the addition of hormone and at 1-min time intervals after E_2 addition for 15 different single cell recordings. The change in this ratio is plotted over 20 min after E_2 addition in Fig. 4B; a positive change in the pixel intensity ratio indicates that more GFP-LXXLL was concentrating at the intranuclear location of ERα-RFP. Notably, the response for each cell varied from cells displaying relatively random fluctuations of green/red pixel intensities of −0.02 to +0.02 to cells in which there was an obvious concentration of the GFP-LXXLL at the intranuclear location of ERα-RFP over the 20-min time course. For comparison, the read-out obtained from a cell shown in Fig. 4A, in which con-

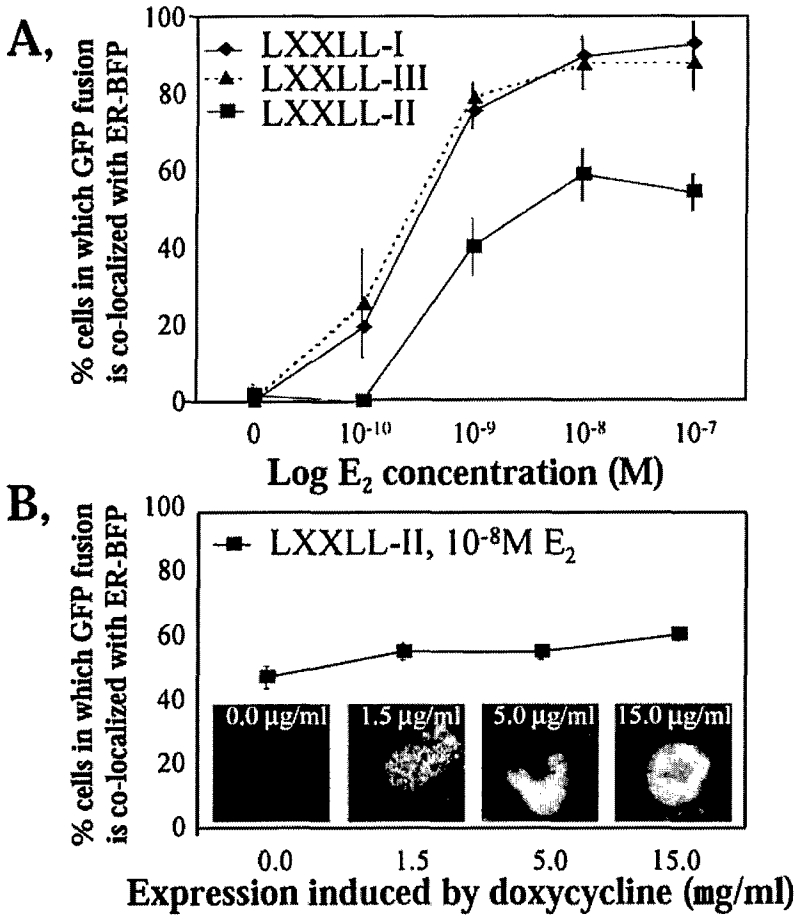

Fig. 3. Different LXXLL-Containing Peptides Are Recruited to ERα with Different Kinetics

A, The proportion of cells in which class I, class II, or class III GFP-LXXLL colocalizes with ERα-BFP increases with increasing E_2 dose. Half-maximal colocalization is achieved at E_2 concentrations required for promoter activation (37, 50). The *graph* represents the mean ± SD in the percent of cells displaying colocalization from three independent experiments. B, The poorer colocalization of the class II GFP-LXXLL with ERα-BFP is independent of the expression level of class II GFP-LXXLL. Representative green fluorescence from individual cells shows the different expression levels of class II GFP-LXXLL induced by the indicated concentrations of doxycycline used to drive the linked tetracycline-inducible promoter. In the absence of doxycycline, green fluorescence was visible but not captured with the short exposure times used to collect the images shown. The *graph* represents the mean ± SD in the percent of cells displaying colocalization from a single transfection collected three separate times over 1 day.

centration of GFP-LXXLL at the site of ERα-RFP is at the threshold of being visible by the naked eye, is indicated by the *open triangles* (see Fig. 4B, *). Thus, over short time periods, recruitment of GFP-LXXLL to ERα-RFP, measured quantitatively, is highly variable with time after E_2 addition.

The cell-to-cell variability in recruitment over short time periods required that we score large numbers of cells at each time point to obtain data in which we have confidence. This could not be accomplished by recording individual cells for prolonged time periods. However, our prior experience demonstrated that we could readily and reproducibly score by visual inspection 50–150 cells within a 10-min window (Fig. 3). We coexpressed each GFP-LXXLL together with ERα-BFP in cells grown in the absence of ligand and then scored those cells, exactly as for Fig. 3, for colocalization in 10-min windows between 15–25 min, 40–50 min, and 85–95 min at 24 h after the addition of 10^{-8} M E_2 (Fig. 4C).

Colocalization of the class I and class III GFP-LXXLLs with ERα-BFP was detected within 20 min after the addition of 10^{-8} M E_2 and increased thereafter. This represented the time required for E_2 to enter the cell, bind to ERα-BFP, and have detectable amounts of freely diffusing GFP-LXXLL concentrate at the intranuclear location of the liganded ERα-BFP. Whereas the colocalization of GFP-class I LXXLL and GFP-class III LXXLL fusions with ERα-BFP showed identical time courses and reached similar levels in response to saturating levels of E_2, the redistribution of the class II peptide to ERα-BFP was much less rapid (Fig. 4C).

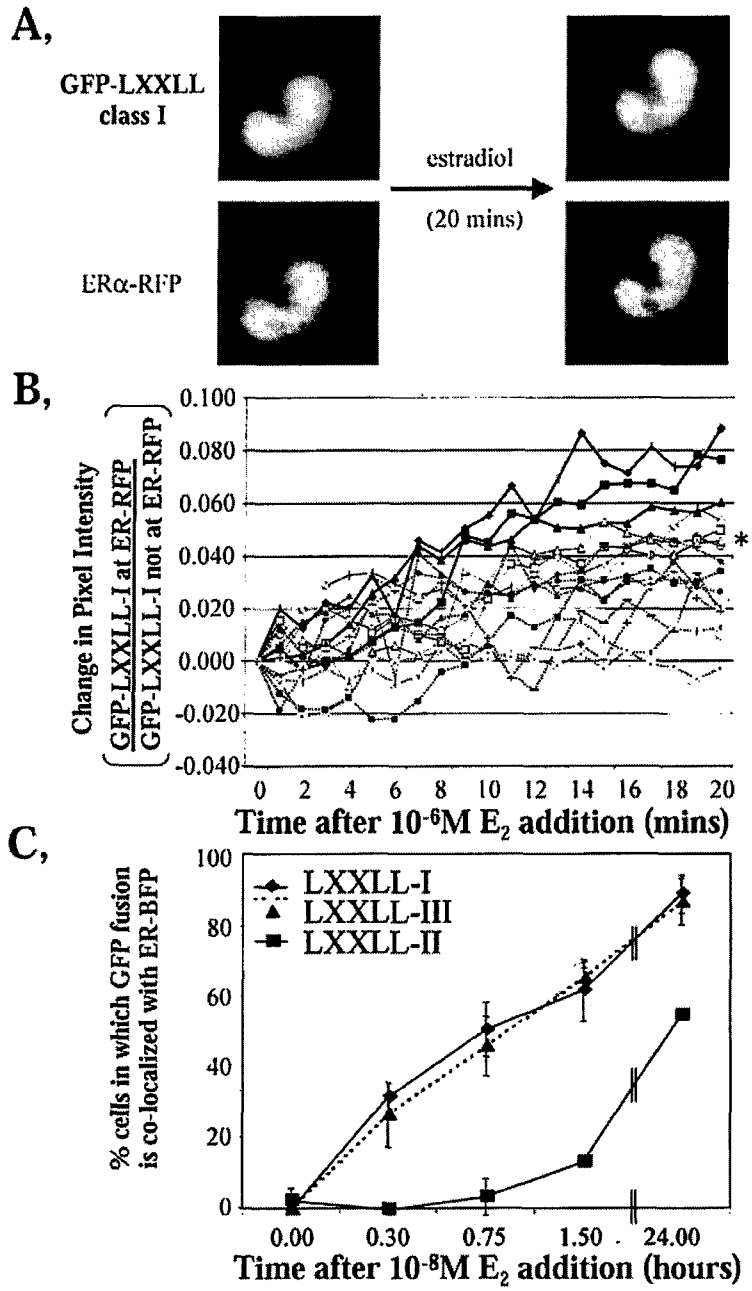

Fig. 4. Different LXXLL-Containing Peptides Are Recruited to ERα with Different Temporal Kinetics

A, Sequential images of the same cell captured before and 20 min after the addition of 10^{-6} M E_2 demonstrate partial colocalization of the class I LXXLL-GFP fusion protein with ERα fused to RFP. B, Quantification of the level of GFP and RFP fluorescence at each pixel within the nucleus at 1-min time intervals after the addition of 10^{-6} M E_2 shows variable concentration of class I LXXLL-GFP at the position of ERα-RFP in 15 different cells. *, Readout of cell shown in Fig. 4A (*open triangles*). C, The proportion of cells showing any colocalization of ERα-BFP with each GFP-LXXLL was scored with time after addition of 10^{-8} M E_2. The slower time course and reduced binding of the class II peptide reflect the previously reported poorer interaction of this peptide with ERα (12). Each *graph* represents the mean ± SD deviation in the percent of cells displaying colocalization from three independent experiments.

The delayed time course of class II LXXLL intracellular colocalization with ERα-BFP, which would not have been detected in other assays, demonstrated that ER association with different LXXLL sequences follows different temporal kinetics. An intriguing possibility is that the different LXXLL temporal kinetics underlies a previously proposed (38) sequential recruitment of co-factors to ER after E_2 addition.

Table 1. Ligand-Specific Complexes Formed by ERα and Indicated Peptides or GRIP1

Ligand : (24 hour incubation) / GFP -	no ligand	ICI 182,780 5×10^{-7}M	Raloxifene 5×10^{-7}M	Tamoxifen 5×10^{-7}M	Estradiol 5×10^{-8}M	ICI inhibition: E_2, 10^{-9}M or Tam, 10^{-7}M to α/βV	ICI inhibition: + ICI, 10^{-6}M
LXXLL -I	2 +/- 2%	0 +/- 0%	0 +/- 0%	21 +/- 9%	93 +/- 6%	79 +/- 7%	0 +/- 0%
LXXLL -II	2 +/- 2%	1 +/- 1%	1 +/- 1%	3 +/- 2%	64 +/- 17%	47 +/- 4%	0 +/- 0%
LXXLL -III	0 +/- 0%	*	*	*	90 +/- 8%	76 +/- 4%	3 +/- 1%
αII	0 +/- 0%	85 +/- 8%	46 +/- 5%	76 +/- 1%	56 +/- 2%	32 +/- 11%	84
α/βV	0 +/- 0%	0 +/- 0%	2 +/- 2%	81 +/- 14%	2 +/- 2%	52 +/- 10%	1 +/- 0%
GRIP1 wt	9 +/- 5%	3 +/- 3%	4 +/- 3%	21 +/- 12%	81 +/- 17%	63 +/- 17%	6 +/- 3%
GRIP1 ΔLXXLL	6 +/- 3%	3 +/- 3%	0 +/- 0%	5 +/- 4%	48 +/- 6%	43 +/- 5%	3 +/- 1%

*, no co-localization in some LXXLL-III data not quantified by cell counting

shading represents indicated % co-localization with ERα

X%	X%	X%	X%	X%
0-9%	10-34%	35-59%	60-89%	90-100%

Ligand-Specific Differences in Class I, II, and III LXXLL Colocalization with ERα

Incubation of cells coexpressing GFP-LXXLL and ERα-BFP overnight with 10^{-6} M tamoxifen resulted in a slight concentration of green fluorescence emitted from the class I GFP-LXXLL over the reticular pattern of ERα-BFP fluorescence (Fig. 2A). This weak colocalization was reproducible and was quantified in Table 1 as the percentage of cells in which the indicated GFP-linked peptide showed any visible colocalization with ERα-BFP in response to the indicated ER ligand. Tamoxifen promoted class I GFP-LXXLL colocalization with ERα-BFP but not class III GFP-LXXLL colocalization with ERα-BFP (Table 1). Thus, the class I and class III peptides, which behaved identically in response to E_2 (Figs. 3A and 4C), differed in their response to tamoxifen. The class II GFP-LXXLL also did not appreciably respond to tamoxifen (Table 1).

In contrast to tamoxifen, incubating the cells overnight with two other SERMs, raloxifene or ICI 182,780, did not promote overlap in the intracellular distributions of coexpressed ERα-BFP and any of the class I, II, or III GFP-LXXLLs (Table 1). Raloxifene and ICI 182,780 were effective in promoting the colocalization of another, unrelated peptide with ERα-BFP (Fig. 5A). This peptide, αII, was previously selected for the ability to interact with ERα bound by either E_2 or tamoxifen (13). In the absence of ligand, the intracellular distribution of GFP-αII was diffuse (Fig. 5A). However, E_2, tamoxifen, raloxifene, and ICI 182,780 each induced GFP-αII to redistribute to the intranuclear location occupied by ERα-BFP (Fig. 5A and Table 1). Relocalization was specifically dependent upon the αII peptide, ERα-BFP coexpression, and ligand addition. Thus, E_2, tamoxifen, raloxifene, and ICI 182,780 were all capable of entering the cell and promoting specific ERα-peptide interactions. The different ligand specificities of

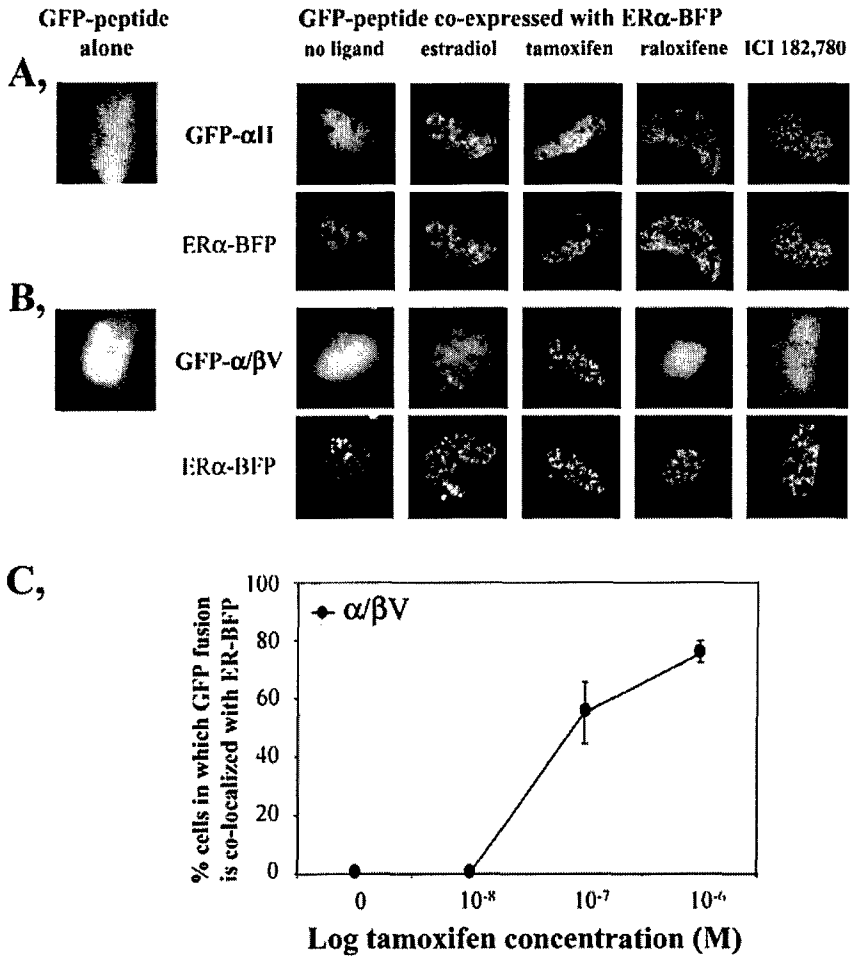

Fig. 5. Different Ligand Dependence of the Recruitment of non-LXXLL-Containing, ER-Interacting Peptides to the Intranuclear Location of ERα

A, Intracellular recruitment of GFP fused to the αII peptide (13) to ERα-BFP is induced by incubating the cells with any of E_2, tamoxifen, raloxifene, or ICI 182,780. B, Tamoxifen-dependent, intranuclear colocalization of ERα-BFP and GFP fused to the α/βV peptide (13). Ligand incubation and fluorescence detection are as described in the legend to Fig. 2. Green fluorescence from each GFP-peptide fusion in the absence of ERα-BFP expression (*left panels*) is shown from cells incubated with 10^{-8} M E_2 (A) or 10^{-6} M tamoxifen (B). C, Half-maximal recruitment of GFP-α/βV to ERα-BFP is achieved at tamoxifen concentrations that parallel tamoxifen induction of ERα promoter activation (13). The *graph* represents the mean ± SD in the percent of cells displaying colocalization from three independent experiments.

the LXXLL and αII peptide interactions demonstrate the subtle differences in receptor conformation promoted by each ligand.

Tamoxifen-Specific Colocalization of an ER-Interacting Peptide with ERα-BFP

SERM-selective *in vivo* recruitment also was shown by the tamoxifen-specific recruitment of another peptide, α/βV, selected previously from a combinatorial library for interaction only with ERα bound to tamoxifen (13). We fused the α/βV peptide in frame to GFP (Fig. 1) and determined that, like GFP, GFP-LXXLL, and GFP-αII, the GFP-α/βV fusion distributed throughout the nucleus and was present to varying degrees in the cytoplasm (Fig. 5B). In cells not expressing ERα-BFP, GFP-α/βV remained distributed after treatment with tamoxifen (Fig. 5B, *left panel*) or any other ligand. Similarly, when coexpressed with ERα-BFP in cells grown in the absence of ligand or after incubation with 10^{-8} M E_2, 10^{-7} M raloxifene, or 10^{-7} M ICI 182,780, the subcellular localization of GFP-α/βV was not altered. In contrast, incubation with 10^{-6} M tamoxifen resulted in a concentration of GFP-α/βV at the intranuclear reticular pattern characteristic of ERα-BFP. Tamoxifen dose-response curves (Fig. 5C) showed that GFP-α/βV relocalization to ERα-BFP corresponded with the promoter activation profile of tamoxifen bound to ERα (37). Tamoxifen-selective GFP-α/βV colocalization with ERα-BFP demonstrated that ERα

adopts a conformation *in vivo* that is different than that adopted by the E_2-, raloxifene-, or ICI 182,780-bound receptors.

Ligand-Dependent Recruitment of the Full-Length ER-Interacting Cofactor GRIP1 to the Intranuclear Compartment Containing ERα

The ligand-specific and temporally distinct associations of different ER-interacting peptides with ERα suggested that a similar approach could be employed to demonstrate the ligand specificity and pattern of recruitment of full-length ER-interacting cofactors to ERα *in vivo*. Indeed, one ER coactivator SRC-1a fused to GFP recently was shown to be recruited to the intranuclear location of ERα fused to the cyan fluorescent protein upon E_2, but not tamoxifen or ICI 182,780, incubation (27). We determined that the related ER-interacting cofactor GRIP1, fused to GFP, was also recruited upon ligand addition to the intracellular subcompartment containing ERα-BFP (Fig. 6) and then detailed the ligand specificity and kinetics of that recruitment (Fig. 7).

GFP-GRIP1, when expressed by itself, was exclusively nuclear and dispersed throughout the intranuclear compartment, although absent from nucleoli (Fig. 6A). In cells coexpressing GFP-GRIP1 and ERα-BFP and incubated with ICI 182,780 or raloxifene, GFP-GRIP1 retained its characteristic dispersed distribution (Fig. 6B). In contrast, both E_2 and tamoxifen were very effective in recruiting GFP-GRIP1 to ERα-BFP (Fig. 6B and Table 1). This again illustrated that E_2 and each SERM promote very distinct ER interactions with specific ER-interacting factors and motifs.

Temporal Variation in LXXLL Requirements for E_2 and Tamoxifen Recruitment of GRIP1 to ERα

E_2 and tamoxifen-specific recruitment of GFP-GRIP1 to ERα-BFP was distinguished by their different time courses and dependencies upon the LXXLL motifs in GRIP1 (Fig. 7). E_2 recruitment was characterized by a rapid LXXLL-dependent phase followed by a slow LXXLL-independent phase. Rapid recruitment was detected as an initial plateau of 30–40% of the cells displaying colocalization of GFP-GRIP1 with ERα-BFP within 20 min after E_2 addition (Fig. 7A, GRIP1-wt). This early phase plateau was blocked (Fig. 7A, GRIP1-ΔLXXLL) by mutation to LXXAA of the two LXXLL motifs of GRIP1 required for interaction with the ER

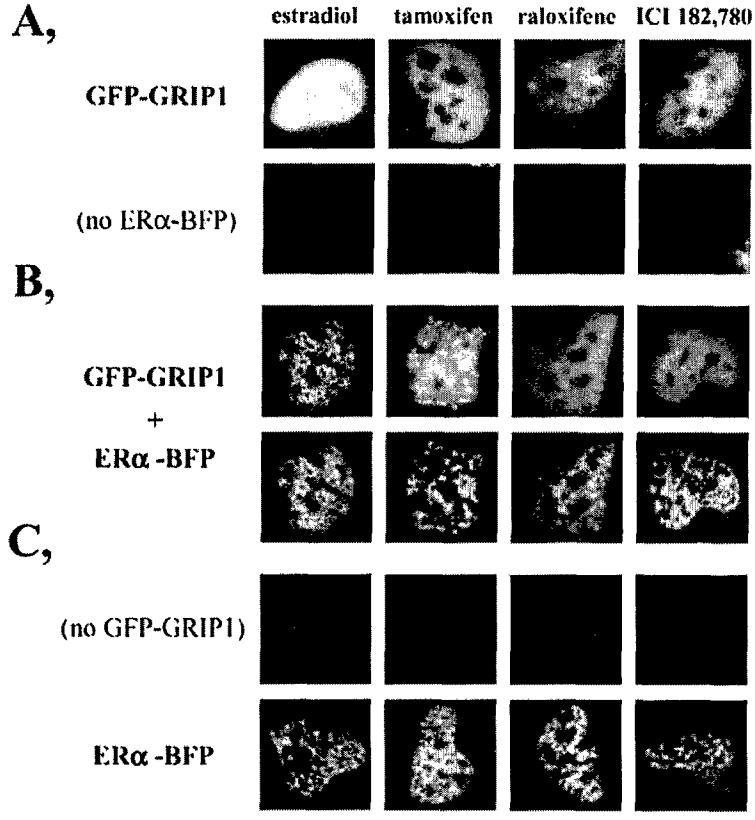

Fig. 6. Recruitment of GRIP1 to the Intranuclear Location of ERα Is Ligand-Selective

A, In the absence of coexpressed ERα-BFP, GRIP1 is evenly distributed throughout the nucleoplasm but is absent from nucleoli. B, When coexpressed with ERα-BFP, GFP-GRIP1 assumes the intranuclear location of ERα-BFP only if incubated with E_2 or tamoxifen. C, Cells expressing ERα-BFP only. Ligand incubation and fluorescence detection are as described in the legend to Fig. 2.

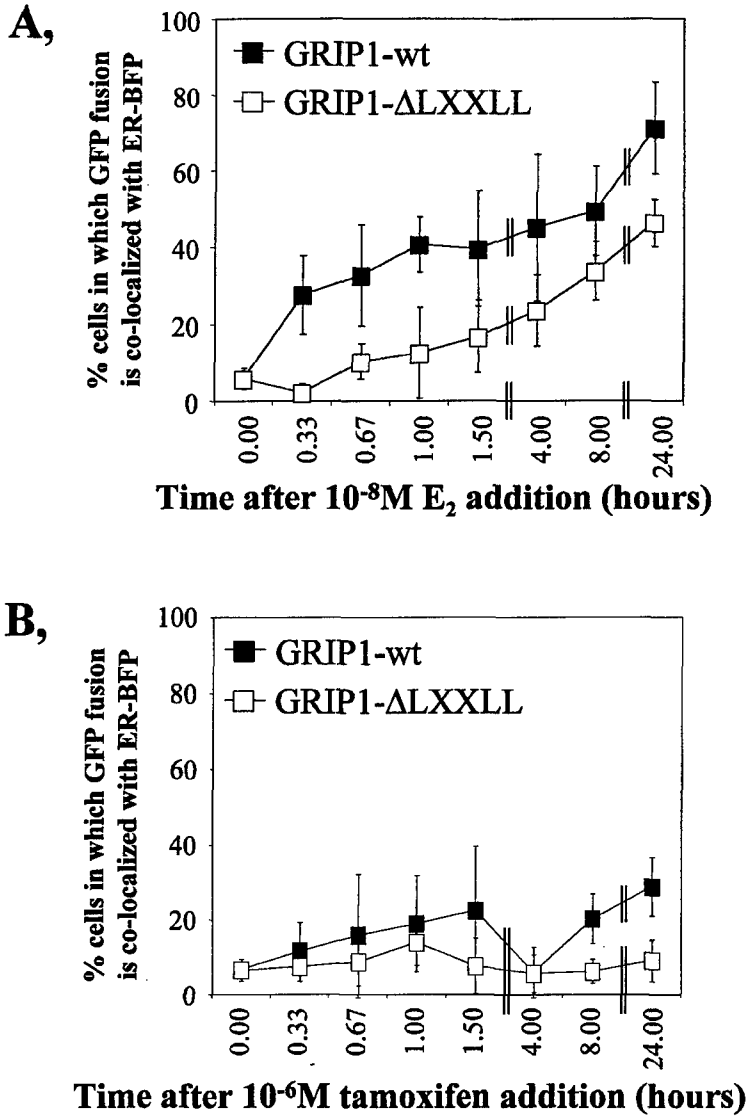

Fig. 7. Different Temporal Kinetics for LXXLL-Dependent and LXXLL-Independent Recruitment of GFP-GRIP to ERα-BFP

A, The proportion of cells in which GRIP1 colocalizes with ERα-BFP increases rapidly with time after E_2 addition. Rapid colocalization is lost if the second and third LXXLL motifs of GRIP1 are mutated to LXXAA (GRIP1-ΔLXXLL). GRIP1-ΔLXXLL is still induced by E_2 to colocalize with ERα but with much reduced temporal kinetics indicating that two separate, and temporally distinct mechanisms govern E_2-dependent, intranuclear recruitment of GRIP1 to ERα. B, Recruitment of GRIP1 to the intranuclear location of ERα is LXXLL-dependent at 8 h or more after tamoxifen addition. Recruitment at earlier time points is variable, but not statistically significant. Each *graph* represents the mean ± SD in the percent of cells displaying colocalization from three independent experiments.

ligand binding domain (5). A more gradual increase in GRIP1/ERα colocalization that followed 1.5 h after E_2 addition was not abrogated by the LXXAA mutations. These complex temporal kinetics and LXXLL dependencies suggest time-dependent variations in the available types of E_2/ER/GRIP1 associations with some lagging associations possibly dependent upon interim interactions and/or enzymatic processes.

The weaker tamoxifen-dependent recruitment of GFP-GRIP1 to ERα-BFP also displayed a complex time course (Fig. 7B) that was mechanistically distinct from that induced by E_2. Tamoxifen-induced colocalization of GFP-GRIP1 and ERα-BFP was statistically significant at 8 and 24 h after tamoxifen addition. Before that, a slow gradual recruitment of GRIP1 was not statistically significant. A precipitous drop in colocalization at 4 h after tamoxifen addition may indicate some tendency toward a temporally biphasic response, but this interpretation is questionable given that the change in colocalization at the early time points was not statistically significant. The statistically significant tamoxifen-dependent colocalization at 8

and 24 h was disrupted by the mutation of the GRIP1 LXXLL motifs to LXXAA (Fig. 7B). Because it is unlikely that the LXXLL motifs of GRIP1 interact directly with the tamoxifen-bound ER, the LXXLL dependence of the slow, tamoxifen-dependent GRIP1 recruitment may reflect a more indirect recruitment of GRIP1 to the tamoxifen-bound ER or a dependence on additional motifs present in GRIP1.

E_2/ERα/LXXLL Complexes Become Resistant to Subsequent Challenge with Antiestrogen

Previous reports showed that ligand-induced binding of LXXLL to ER *in vitro* caused an alteration in the rate by which the ligand dissociates from the ER (39). To determine whether LXXLL interaction with ER *in vivo* might similarly slow ligand access to ER, we examined whether ERα/cofactor or ERα/peptide complex formation altered access to the antiestrogen ICI 182,780. ICI 182,780 did not promote ERα-BFP colocalization with GFP-GRIP1, GFP-α/βV, or any of the three GFP-LXXLL fusions (Table 1). Consistent with its role as an antiestrogen, simultaneous addition of 10^{-6} M ICI 182,780 with 10^{-9} M E_2 abrogated colocalization of ERα-BFP with each of the three GFP-LXXLL fusions and GFP-GRIP1 (Table 1, ICI inhibition) whereas colocalization of the ICI 182,780-responsive, αII peptide was unaffected. ICI 182,780 (10^{-6} M) also blocked colocalization of ERα-BFP and GFP-α/βV in response to 10^{-7} M tamoxifen (Table 1).

Having showed that 10^{-6} M ICI 182,780 effectively blocked recruitment of GRIP1 and the α/βV, LXXLL-I, LXXLL-II and LXXLL-III peptides to ERα, we next determined the temporal kinetics of complex dissociation. To do so, we challenged preformed complexes with an excess of ICI 182,780 to block the reformation of transiently dissociated complexes. Initially, the proportion of cells containing ERα colocalized with GFP-α/βV after 24 h incubation with 10^{-7} M tamoxifen was determined, 10^{-6} M ICI 182,780 was added to the media, and the cells subsequently were scored for any colocalization at the indicated time points after ICI 182,780 addition (Fig. 8A). Complete disruption of colocalization of GFP-α/βV and ERα-BFP was observed within 2 h of ICI 182,780 addition. This established that 2 h was sufficient time for ICI 182,780 to enter the cells, disrupt all preformed complexes, and completely release all GFP-α/βV concentrated at the ERα subcompartment.

For complexes of ERα with GRIP1, the GRIP1 LXXAA mutant, and the LXXLL peptides, dissociation by 10^{-6} M ICI 182,780 was assessed after a 24-h treatment with 10^{-9} M E_2. In contrast to the α/βV peptide, GRIP1 remained as tightly associated with ERα 4 h after ICI 182,780 addition as it was before ICI 182,780 addition (Fig. 8B). Complete resistance to ICI 182,780 challenge also was observed for E_2/ER/GRIP1 complexes formed after only 1 h of E_2 preincubation (not shown). The relative stability of the E_2/ER/GRIP1 complexes depended upon the integrity of the LXXLL sites as GRIP1 containing the LXXAA mutations was dispersed by ICI 182,780 addition in a time-dependent fashion (Fig. 8B). The E_2/ER/GRIP1 complexes similarly displayed an LXXLL-dependent resistance to challenge with 10^{-6} M raloxifene (not shown). These data suggested that interaction of the LXXLL motif with ERα might regulate the subsequent ligand access to ERα *in vivo* as suggested by *in vitro* studies (39). Indeed, ERα complexes formed with the isolated LXXLL peptides were also resistant to dispersal by ICI 182,780 (Fig. 8C), demonstrating that interaction with LXXLL alone was sufficient to alter the subsequent response of the preformed complexes to challenge with ligand.

DISCUSSION

We found that different ER ligands caused the recruitment of specific panels of nuclear receptor-interacting peptides and proteins to the intranuclear location occupied by ERα (Figs. 2, 5, and 6). The correlation of the ligand specificities of intranuclear recruitment (Figs. 2–7 and Table 1) with the previously reported ligand specificities of the direct interactions of GRIP1 and each peptide with ERα (6, 7, 12–14, 20) suggests that some colocalization involves direct interactions between ERα-BFP and the GFP-linked GRIP1 and target peptides. Consistent with direct interaction, simultaneous addition of an excess of antiestrogen also blocked intranuclear recruitment. Thus, recruitment in living cells faithfully reflected the known biochemical and molecular properties of well characterized ER-interacting factors.

In addition to confirming in living cells the ligand specificities of these previously known interactions, the analysis of intranuclear recruitment allowed us to follow complex formation with time after ligand addition. Temporal variations in both recruitment and dissociation were observed (Figs. 4, 5, 7, and 8). Temporally delayed recruitments may represent a secondary association of some complexes via intermediary factors that are initially recruited in response to ligand binding. Such an indirect interaction may be responsible for the delayed (Fig. 7) interaction of ERα with GRIP deleted of the two LXXLL motifs previously described (5) to be necessary for direct GRIP1 interaction with ERα. Alternatively, GRIP1 is known to interact with other regions of ERα (11), and the temporal delay of intranuclear recruitment of GRIP1ΔLXXLL may result from time-dependent ERα interactions or modifications that may be required for the proper folding of these alternative GRIP1 interaction sites.

The molecular basis for the delayed recruitment to ERα of the isolated class II LXXLL (Fig. 4C) similarly remains to be defined. However, the direct interaction of the class II peptide with AF-2 in ERα is weak compared with the class I and class III LXXLL interactions (12), and the delayed intracellular recruitment may

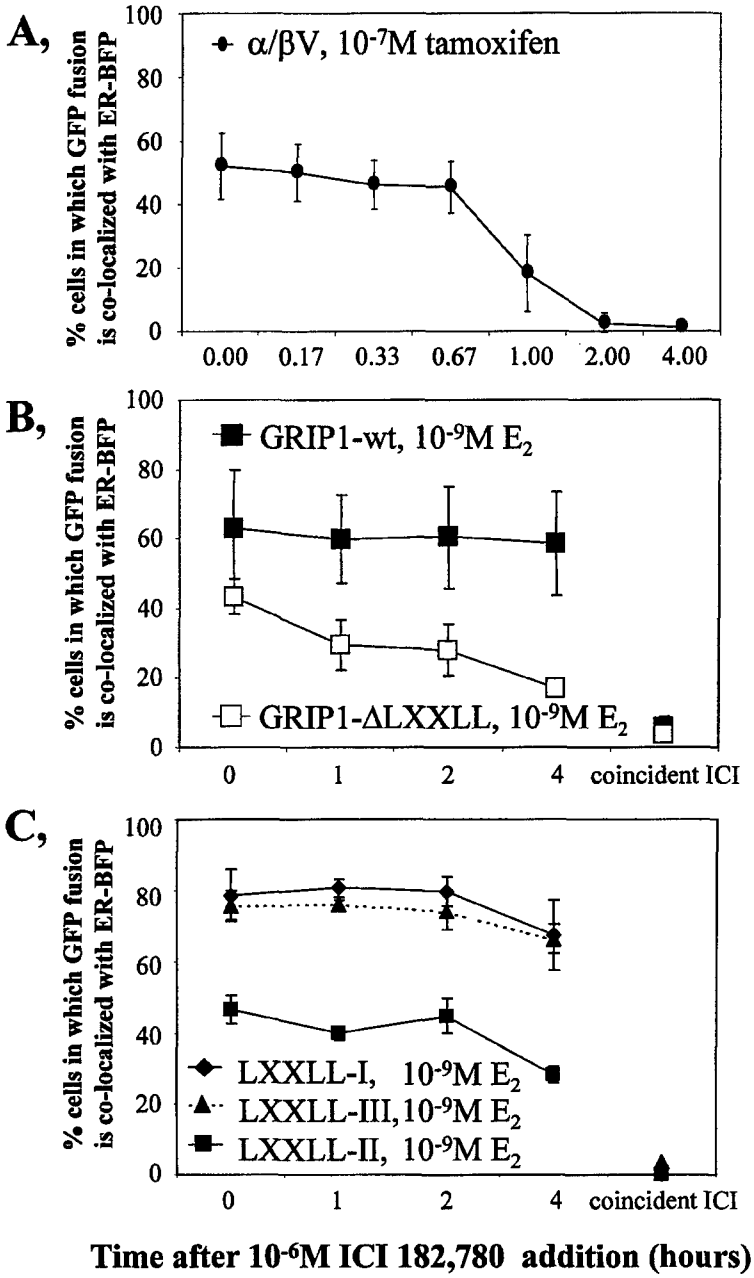

Fig. 8. Colocalized E_2/ERα/LXXLL Complexes Are Resistant to Dispersal by Antiestrogen

A, Colocalization of the non-LXXLL-containing GFP-α/βV peptide with ERα-BFP induced by 10^{-7} M tamoxifen is reversed by subsequent incubation with 10^{-6} M ICI 182,780. Complexes of ERα with GRIP1 (panel B) and each of the LXXLL peptides (panel C) induced by 10^{-9} M E_2 are not disrupted even 4 h after the addition of 10^{-6} M ICI 182,780. In contrast, colocalized GRIP1-ΔLXXLL is readily dispersed from the intranuclear location of ERα upon ICI 182,780 addition. Coincident ICI, percent cells showing colocalization 24 h after simultaneous addition of 10^{-6} M ICI 182,780 and 10^{-9} M E_2 in parallel experiments. Each *graph* represents the mean ± SD in the percent of cells displaying colocalization from four independent experiments.

arise from class II LXXLL E_2-induced associations with other regions of ERα, other ER-interacting factors, or even with ERα covalently modified by cofactors recruited to the E_2-bound ER. Although the mechanisms for these temporal variations in intranuclear recruitment remain unknown, the previously unrecognized variations in the timing and sequence of complexes recruited to ER after ligand addition are likely to be a key determinant of ligand response and adaptation and are uniquely detected by the intranuclear recruitment assay.

Challenge of the preformed complexes with competitive antagonists demonstrated that each peptide or cofactor was displaced from the complexes with

unique dissociation kinetics (Fig. 8). Complexes of ERα with LXXLL-containing peptides or cofactors were considerably more resistant to disruption by ICI 182,780 or raloxifene than complexes that did not involve LXXLL interactions, demonstrating that specific ligand-dependent interactions uniquely changed the nature of the complexes. The LXXLL-dependent reduction in the dissociation kinetics of the preformed complexes may be a consequence of the decreased off rate of ligand upon LXXLL binding to ER as recently reported *in vitro* (39). Alternatively, translocation of the LXXLL-containing peptide or GRIP1 from ER to another protein that resides in the same intranuclear position as ER could explain why the LXXLL-containing peptides and GRIP1 remain localized for prolonged periods of time after subsequent antagonist challenge.

The ligand specificity and temporal characteristics of intranuclear recruitment of ERα with each ligand indicated that, in the cellular environment, distinct conformations of ER are formed in response to E_2 and each SERM (7, 10). Recruitment was measured as a function of the percentage of cells responding to each ligand. The underlying basis for the cell-to-cell variability in recruitment remains to be described but may be responsible for the previous observation that dose-dependent transcriptional activation by a nuclear receptor ligand arises through an increase in the proportion of cells responding to the ligand rather than an equivalent, incremental increase in all cells (40).

The different abilities of E_2, tamoxifen, and raloxifene to promote ERα colocalization of the α/βV peptide, the class I LXXLL peptide, and GRIP1 provided dramatic evidence for the differing cellular and molecular properties of these clinically useful ligands. The E_2- and tamoxifen-induced recruitment of GRIP1 to the intranuclear location of ERα (Figs. 6 and 7) also contrasted with recruitment of related cofactor SRC-1a, which responded only to E_2, and not tamoxifen (27). This difference may be attributable to cell type or other experimental differences between laboratories, or to different ligand specificities for related cofactors in the context of the living cell. Nevertheless, the detection of these differences in living cells may prove useful in dissecting the differing clinical properties of E_2, tamoxifen, and raloxifene in different tissues (16, 18, 41, 42). The ability to quantify these changes on a pixel level (Fig. 4B) provide a first indicator that automated equipment can be developed for the high throughput measurement of ligand-specific effects on intranuclear recruitment and dissociation in living cells.

Recently, it was shown that the reticular intranuclear distribution of estrogen, SERM, and antiestrogen-bound ERα paralleled the tight binding of ERα to the nuclear matrix and that one ER-interacting factor, SRC-1a, was corecruited to the nuclear matrix via the ligand-bound ER (27). The results presented here suggest that other ER-interacting complexes may be similarly recruited to the nuclear matrix compartment upon ER binding to different ligands and that each ligand promotes the recruitment of specific nuclear receptor-interacting peptides and proteins (Table 1) with unique temporal kinetics (Figs. 4, 5, and 7). The ligand-regulated association of ER and ER-interacting complexes with the nuclear matrix is intriguing given the historical association of transcription markers and enhancer/promoter sequences with the nuclear matrix (43, 44). The nuclear matrix may aid the organization of transcriptionally competent chromosomal domains (45) but a decisive correlation of nuclear matrix association with transcriptional activation or repression remains to be established (46).

Thus, we demonstrated that recruitment of ER-interacting factors to the intranuclear position of ERα is differentially regulated by the nature of the interacting sequence and the type of ligand. The complex temporal kinetics and ligand specificities of the association of ERα and its cofactors illustrated a variety of possible responses of ERα to ligand addition for which the intranuclear colocalization assay provided a direct read-out *in vivo*. Other methods currently used to detect ER-peptide or cofactor interactions rely on various *in vitro* binding assays or on two-hybrid assays in cells. The advantage of the intranuclear colocalization assay is that it is an *in vivo* assay in which direct and indirect interactions of ER with specific peptide of cofactor targets are readily measured in real time. Therefore, the intranuclear colocalization assay allows the intracellular actions of each ligand to be dissected in unprecedented detail. The availability of many more ER-interacting peptides and cofactors (6, 7, 12–14, 20, 47) will permit the detection of an even more expanded series of ER activities and may also facilitate the identification of novel ligands that induce specific subsets of cofactor interactions with ER or other nuclear receptors. Such novel ligands could be used to probe for the specific molecular events involved in nuclear receptor regulation of different genes and may even provide a rapid means for the identification of compounds with improved specificity for hormone replacement therapies.

MATERIALS AND METHODS

Expression Vectors

The cDNA encoding the BFP Y66H, Y145F variant of GFP (28) or the cDNA encoding RFP (CLONTECH Laboratories, Inc. Palo Alto, CA) were fused in frame to the carboxy terminus of human ER and placed under the control of the cytomegalovirus promoter in the previously described BFP expression vector (35). The EGFP cDNA (CLONTECH Laboratories, Inc.), modified to include the SV40 nuclear localization signal (NLS) at its carboxy terminus, was inserted into the pTRE "Tet-On" expression plasmid (CLONTECH Laboratories, Inc.). Because of its small size, the modified EGFP-NLS remained distributed throughout the cytoplasm and nucleus when expressed. LXXLL (12) and α/βV (13) ER-interacting peptides were fused in frame to the carboxy terminus of EGFP-NLS in the pTRE expression plasmid. The αII (13) peptide was fused to the carboxy terminus of EGFP-NLS in the pEGFP-C3 vec-

tor (CLONTECH Laboratories, Inc.). The representative class I, class II, and class III LXXLL peptide sequences are from the D2, D47, and F6 peptides isolated in Ref. 12. Native GRIP1 and GRIP1 containing mutations in which ER-interacting, LXXLL boxes II and III (5) were mutated to LXXAA were appended to the carboxy terminus of GFP in the EGFP-C2 vector (CLONTECH Laboratories, Inc.).

Transfection

GHFT1–5 cells were grown in a 1:1 mixture of phenol red-free Ham's F12-DMEM containing estrogen-free 10% newborn calf serum. The cells were harvested and transfected by electroporation as described previously (35, 48) with 10 μg of the cytomegalovirus (CMV)-ERα-BFP or CMV-ERα-RFP expression vector, 10 μg of pEGFP-GRIP1 or pEGFP-GRIP1-ΔLXXLL, and 5 μg of pEGFP-αII or 3 μg of the pTRE-GFP-LXXLL or pTRE-GFP-α/βV expression vectors. pUHG17–1 (1.2 μg), which expresses the tetracycline repressor/VP16 activator (49) used to regulate expression of the pTRE plasmid, was cotransfected with the pTRE-GFP-LXXLL or pTRE-GFP-α/βV expression vectors. The transfected cells were plated onto coverslips and grown in estrogen-free media. Doxycycline (5 μg/ml) was added to the media to induce the Tet-On promoter except in Fig. 3B in which concentrations of doxycycline were varied from 0 to 15 μg/ml. One day after transfection, ER ligands were added at the indicated concentrations and imaged 24 h later (Figs. 2–6). For the E_2 time course experiments (Figs. 4C and 7), 10^{-8} M E_2 was added at the indicated time before imaging on the second day after transfection. For the ICI 182,780 antagonism time courses, cells were treated with 10^{-7} M tamoxifen (Fig. 8A) or 10^{-9} M E_2 (Fig. 8, B and C) for 24 h followed by addition of 10^{-6} M ICI 182,780 at the indicated times before imaging.

Microscopy and Image Analysis

After addition of ligand or ethanol control vehicle, fluorescence images from the transfected cells were acquired with a Axioplan microscope equipped with a 63×-oil immersion objective lens (Carl Zeiss, Thornwood, NY). Single-cell recordings of cells grown in chamber slides were obtained on an IX-70 inverted microscope (Olympus Corp., Lake Success, NY) and analyzed with Metamorph (Universal Imaging Corp., West Chester, PA) colocalization software. Dual color imaging using Hoechst and fluorescein isothiocyanate filter sets or GFP and rhodamine filter sets (Chroma Technology Corp., Brattleboro, VT) selectively distinguished blue from green fluorescence and green from red fluorescence, respectively. Appropriate controls in which ERα-BFP, ERα-RFP, or each GFP-peptide or GFP-GRIP were expressed individually ensured a lack of fluorescence bleedthrough between the channels. Grayscale images of the cells were obtained using a Xillix microscope (Carl Zeiss) or Hamamatsu ORCA microscope (Olympus Corp.) cooled CCD cameras. The digital images were background-subtracted and then converted to red-green-blue (RGB) images by assigning the GFP signal to the green channel, BFP signals to the blue channel, and RFP signals to the red channel of RGB digital images. Integration times and image processing were kept constant within each set of experiments.

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