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Cells

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13. ABSTRACT (Maximum 200 Words) Unlike most nuclear receptors, the Estrogen Receptor-Related Receptors (ERRs) activate transcription constitutively, interacting with coactivators and target gene promoters in the absence of ligand. Structurally, this subfamily of receptors is related to the classical estrogen receptors and has been shown to positively regulate the transcription of several estrogen responsive genes. Interestingly, the transcriptional activity of ERR α is not inhibited by classical anti-estrogens suggesting that its ability to regulate ER-responsive genes may contribute to the development of tamoxifen resistant breast cancer. Without pharmacological agents to regulate ERR α activity it has been difficult to define the specific roles of this orphan receptor in the pathogenesis of breast cancer and thus its potential as a therapeutic target is unknown. To address this issue we have developed approaches to both positively and negatively regulate ERR α activity in target cells. Specifically, we have developed peptide antagonists to inhibit ERR α activity by blocking cofactor binding and have developed activating "protein ligands" by creating modified coactivators that selectively regulate ERR α transcriptional activity. With these tools, we have characterized the critical regions of the receptor important for coactivator binding and defined differential binding requirements between coactivator families. In addition, we are identifying the target genes and processes regulated by ERR α .				
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Introduction

Estrogens and estrogen receptors (ERs) serve a critical function in the development and progression of breast cancer. Indeed, hormone receptor status is an important prognostic indicator of survival and hormone therapy is the first line approach to the treatment of ER positive breast cancers. However, within five years of treatment, most tumors will develop resistance to hormonal therapies such as tamoxifen. Thus, an understanding of the regulation of estrogen production and ER activity is essential for the development of better drug treatments for breast cancer. The estrogen receptor-related receptors (ERRs) are orphan members of the nuclear receptor (NR) superfamily of ligand-dependent transcription factors (Giguere 2002). Based on their similarity to the estrogen receptor (ER), it has been hypothesized that ERRs may have a role in the development and or progression of breast cancer. Indeed, one member of this subfamily, ERR α , can regulate the transcription of several estrogen responsive genes and ERR α immunoreactivity in breast tumors correlates with an unfavorable prognosis (Ariazi 2002; Lu 2001; Suzuki 2004; Vanacker 1999). The transcriptional activity of ERR α is not inhibited by clinically important anti-estrogens suggesting that expression levels may be an important consideration in the treatment of breast tumors (Coward 2001). In addition, its ability to regulate ER-responsive genes may contribute to the development of anti-hormonal resistance in patients. Thus ERR α has emerged as a potentially useful target for breast cancer chemotherapeutics.

Despite significant homology with the ERs, ERR α does not bind estrogen and it is unclear whether receptor activity can be enhanced by small lipophilic molecules (Giguere 1988). While ERR α transcriptional activity can be inhibited by diethylstilbesterol, toxaphene, and chlordane, these drugs lack the specificity necessary to adequately study ERR α physiological function (Lu 2001; Yang and Chen 1999). We have developed two novel approaches to both positively and negatively regulate ERR α activity in target cells. In the first, peptide antagonists were developed that inhibit ERR α activity in a highly selective manner by blocking cofactor binding. The second approach involves the development of activating "protein ligands" by creating modified coactivators that selectively enhance ERR α transcriptional activity. These selective ERR α reagents have been used to explore regulatory surfaces of the protein, the molecular determinants of cofactor binding, and the regulatory processes ERR α controls.

The goal of our proposal was to determine the role of estrogen receptor-related receptor alpha (ERR α) in cellular signaling, particularly how they modulate estrogen receptor (ER) transcriptional activity. Because of the absence of a highly selective ligand to modulate ERR α we developed peptide antagonists that can specifically interact with and inhibit the transcriptional activity of ERRs by competing for cofactor binding sites. The ERR α antagonists were developed using M13 phage display technology to identify high affinity peptides that interfere with coactivator binding of ERRs but not with other nuclear receptor (NRs). These peptides inhibit ERR activity in cells without disrupting the transcriptional activity of other highly related receptors. We also developed selective "protein ligands" that are customized coactivators which specifically activate ERR α in target cells. They were generated by replacing the receptor interaction domains in peroxisome proliferator activated receptor gamma coactivator-1alpha (PGC-1 α), a known ERR α coactivator, with the specific peptides described above. Although wildtype PGC-1 α coactivates many receptors, the modified coactivator selectively activates ERR α in cells when delivered via an adenovirus. We validated this approach to study ERR α (and potentially other orphan receptors) by demonstrating that mitochondrial biogenesis and fatty acid β -oxidation, processes ERR α is known to regulate, are robustly activated in cells expressing the customized PGC-1 α . The success to date of this new technology indicates that these "customized coactivators" will have broad applicability to the study of orphan NRs and will allow a specific determination of the biological consequences of individual receptor:coactivator pairs.

In last year's annual report (April 2004), we outlined the identification and development of peptide antagonists to ERR in fulfillment of the first two specific objectives of the statement of work. Outlined here are the significant findings from that portion of the study.

Task 1 Identification of peptides that interact with ERR using phage display technology.

Peptides that interact *in vitro* with full-length purified ERR α were identified by using phage display technology. Peptides were selected from seven peptide libraries, which included libraries with constrained motifs such as a X₆GX₆ or X₇LXXLLX₇ motif (where X denotes any amino acid, G represents glycine, and L represents leucine). A total of 175 peptides were selected from the seven libraries and of these 126 (72%) interacted with ERR α . These peptides were brought forward for analysis in cell based assays. The peptides were initially screened for their ability to bind to the three subtypes of ERR (collectively referred to as ERR unless the specific subtype is specified), ER α , and ER β . All peptides were able to interact with each subtype of ERR though there were minor differences in relative abilities to bind. Whether these variances actually reflect differences in receptor conformation and ability to recruit coactivators is not known. Of all the peptides that interacted with ERR α , 37 peptides (29%) did not also interact with either ER α or ER β . These peptides were then tested for their ability to interact with a panel of 12 additional NRs. Six peptides appeared to bind specifically to ERR with minimal binding (<10%) to the other receptors tested (Table).

The sequences of the peptides that interacted to the highest degree with ERR α were deduced by DNA sequencing. It was surprising to find that all peptides contained an LxxLL motif, even those that were selected from non-LxxLL constrained libraries. This motif has

previously been shown to be important for nuclear receptor-coactivator interactions (Darimont 1998; Heery 1997). Since this motif interacts primarily with the hydrophobic cleft of the LBD of NRs, the data suggests that this is the primary binding site of the ERR α peptides. Sequences surrounding the LxxLL motif can dictate receptor binding and selectivity (Chang 1999; Darimont 1998; Hall 2000; Heery 2001; Needham 2000). A sequence analysis of the ERR selective peptides suggests that ERRs have a preference for a glutamic acid in the -1 position relative to the LxxLL. This is in contrast to most other nuclear receptors that prefer to bind to sequences with a hydrophobic residue in the -1 position (Chang 1999; Heery 2001; Savkur and Burris 2004). Interestingly, the third LxxLL motif of PGC-1 α , which is apparently *only* involved in interactions with ERR (Huss 2002; Schreiber 2003), also has a glutamic acid in the -1 position suggesting that this residue may confer specificity between ERRs and coactivator interaction domains. In sum, we identified peptides using phage display technology that selectively interact with the ERR subfamily of receptors and this selectivity may be a result of specific sequence preferences of the ERRs.

	ERR α	ERR β	ERR γ	ER α	ER β	PR-A	GR	AR	RAR	RXR	ROR	TR β	VDR	LXR	FXR	LRH	PPAR γ
SRC-1	+	+	+	+++	-	+	++	-	++	++	-	++	++	++	+	nd	nd
L3-2	+++	+++	+++	-	-	+	++	-	+	-	-	-	-	+	-	nd	nd
L3-7	+++	+++	++	-	-	-	-	-	-	-	-	-	-	+	-	nd	nd
L3-12	+++	+++	+++	-	-	+++	++	-	-	++	-	-	-	+	-	nd	nd
L3-20	++++	++++	++++	-	-	-	-	-	+	-	-	-	-	+	+	nd	nd
L3-33	++++	++++	++++	-	-	-	-	-	+	-	+	-	-	+	+	nd	nd
L3-37	++++	+++	+++	-	-	++	-	-	++	+++	-	-	-	-	-	nd	nd
L3-39	+++	+++	++	-	-	-	-	-	-	-	-	-	-	+	-	nd	nd
L3-49	+++	+++	+++	-	-	++	-	-	++	++++	-	-	-	-	-	nd	nd
L3-53	+++	+++	+++	-	-	+	++	-	-	-	-	-	-	+	-	nd	nd
L3-57	+++	+++	+++	-	-	+	++	-	+	-	-	-	-	+	-	nd	nd
L3-62	+++	+++	++	-	-	-	-/+	-	-	-	-	-	-	+	-	nd	nd
L3-91	++	+++	++	-	-	+++	++	-	-	++	-	-	-	+	-	nd	nd

Table Interactions of ERR α selective peptides with other nuclear receptors

The interaction of the ERR α -selective peptides with other nuclear receptor (NRs) was tested in a mammalian-two-hybrid assay. The results of some representative peptides are shown. -: <10% of highest ERR/peptide activity, +: 10-25%, ++: 25-50%, +++: 50-75%, ++++: 75-100%, +++++: >100%, nd indicates the peptides were not tested against the receptor. ERR α -selective peptides with minimal interactions with other NRs are indicated by light or dark shading (light: 1-2 other NRs, dark: no other NR). Shown here are the interactions that occur between the peptides and receptors in the presence of the receptor-specific agonists, with the exception of ERR α , ERR β , ERR γ , ROR α , LRH1, and PPAR γ , for whom no ligand is required. HepG2 cells were transiently transfected with VP16-receptor expression vector in combination with peptide-Gal4DBD fusion construct, 5x-GAL4-luciferase reporter, and CMV- β -Gal control plasmid. Following transfection, cells were treated with vehicle or the following hormones: 100nM 17 β -estradiol for ER α and ER β , 100nM progesterone for PR-A, 100nM dexamethasone for GR, 1 μ M 5 α -dihydrotestosterone for AR, 100nM 9-*cis*-retinoic acid for RAR α and RXR α , 100nM triiodothyronine for TR β , 100nM 1,25-dihydroxyvitamin D3 for VDR, 10 mM 22R-hydroxycholesterol for LXR, and 50 mM chenodeoxycholic acid for FXR. After 24 h luciferase assays were performed, and each value was normalized to the β -galactosidase activity. Abbreviations: ERR: estrogen receptor-related receptor; ER: estrogen receptor; PR: progesterone receptor; GR: glucocorticoid receptor; AR: androgen receptor; RAR: retinoic acid receptor; ROR: RAR-related orphan receptor ligand binding domain; TR: thyroid receptor; VDR: vitamin D receptor; LXR: liver X receptor; FXR: farnesoid X receptor; LRH: liver receptor homolog 1; PPAR γ : peroxisome proliferator activated receptor gamma

Task 2 Development of ERR interacting peptides into high affinity peptide antagonists and assessment of the efficacy of ERR peptide antagonists in cell-based assays.

The ability of ERR selective peptides to inhibit ERR transcriptional activity was tested in transient transfection assays. The best peptides were capable of inhibiting the transcriptional activity of each of the ERRs by greater than 90% (Figure 1). The transcriptional activity of ER α was only minimally affected by addition of the peptides, supporting the data that the peptides are receptor selective. The peptides were able to inhibit ERR activity on multiple promoter/reporter constructs indicating that the effect of the peptides was not promoter dependent. To test the hypothesis that the peptides bind the coactivator binding pocket and thus prevent coactivator:receptor interactions, cofactor:peptide competition assays were conducted in which cells were transiently transfected with receptor, cofactor, peptide, and a reporter plasmid. These assays showed that the coactivators and peptides competed for binding to the receptor. However, differences between coactivator families were identified through these assays. Specifically, a single copy peptide could compete with members of the steroid receptor coactivator (SRC) family while it was necessary to express two copies of the peptides to inhibit coactivation by PGC-1 α (Figure 2). This data suggested that there were differences in receptor affinity between the coactivators. Indeed, using an *in vitro* phage ELISA assay we determined that ERR α binds to the PGC-1 α nuclear receptor interaction domain (NRID) with a dramatically higher affinity than it binds to the SRC2 NRID (Figure 3). Supporting this idea, we found that on some promoters, PGC-1 α was a better coactivator than SRC2 (data not shown). However, this effect was not present on all promoters suggesting that promoter specific sequences may determine cofactor recruitment, an finding that was previously reported for ER α (Hall 2002).

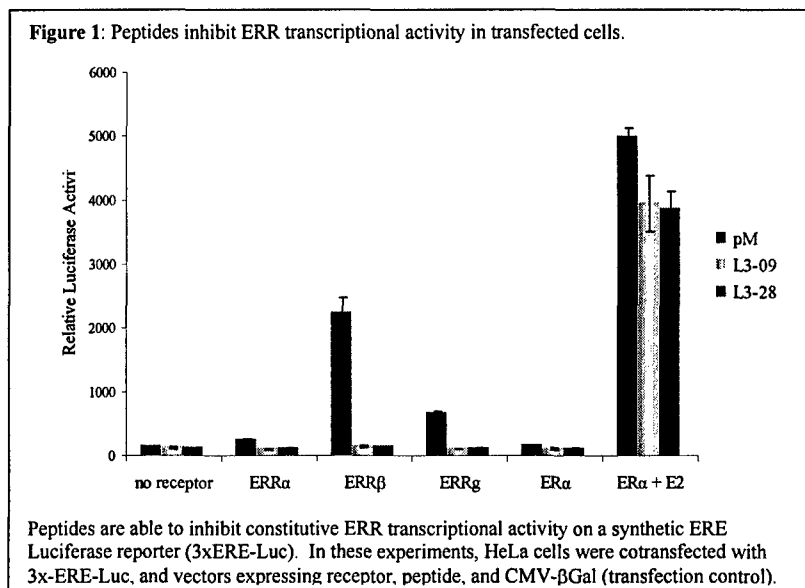


Figure 2: Coactivator enhanced transcription is blocked by the selective peptide antagonists

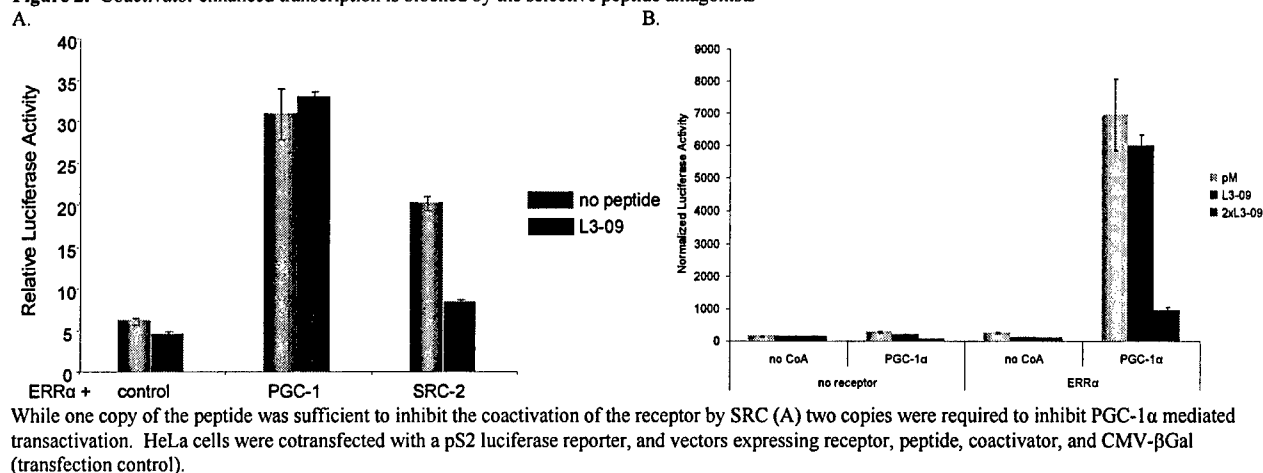
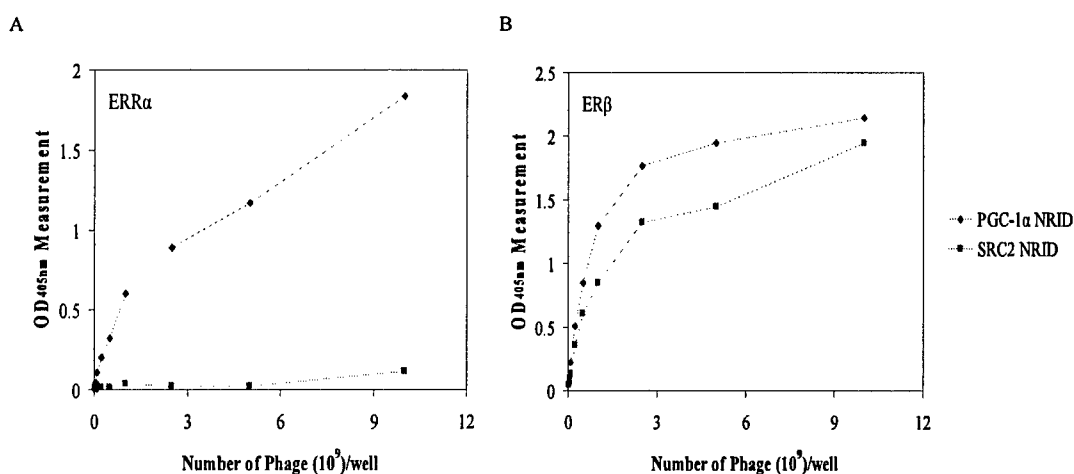


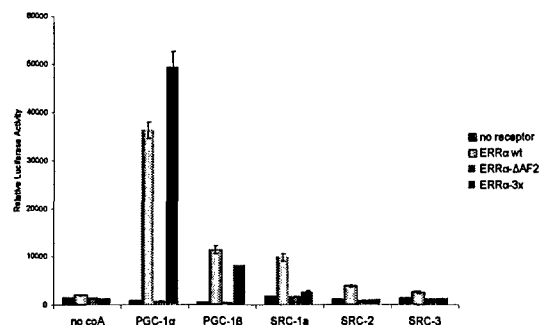
Figure 3: Assessment of coactivator binding affinity using T7 phage cDNA fragments



Phage ELISAs were used to measure the relative binding affinity of the PGC-1α NRID (nuclear receptor interaction domain) compared to the SRC2 NRID. Increasing concentrations of T7 phage expressing either the PGC-1α NRID or a SRC2 NRID cDNA fragment were incubated with either ERRα (A) or ERβ (B) to assess binding affinity of the fragment for the receptor. Wells were treated with neutravidin and biotinylated double-stranded DNA oligomers containing an ERE. Saturating concentrations of either purified ERRα (A) or ERβ (B) were incubated with the target DNA sequences. Indicated numbers of phage plaque forming units (pfu) were incubated in individual wells for 2 h. The interaction of the phage was quantitated using an anti-T7-HRP-conjugated antibody and the colorimetric HRP substrate, ABTS. The absorbance was measured at 405nm. The ERβ wells were treated with 1 μM 17β-estradiol during the protein-binding and phage-binding steps.

The differences in affinity between the cofactors for the receptor prompted us to identify the molecular determinants governing the interactions between ERRα and SRC2 versus PGC-1α. Our data show that the hydrophobic cleft is the primary site of interaction between ERRα and its known coactivators. We identified the C-terminal region containing an activation function

Figure 4: AF-2 point mutations selectively prevent SRC coactivation

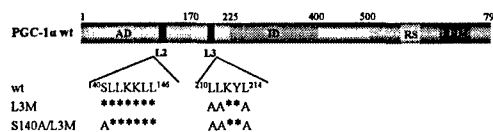


Mutations within the AF-2 domain that neutralize the "charge clamp" of helix 12 selectively interfere with SRC-mediated coactivation while leaving coactivation by PGC-1α and PGC-1β intact, suggesting that while the AF-2 domain is required for PGC-1 interaction, the positively charged pocket formed by helix 12 is not necessary. HeLa cells were cotransfected with a pS2 luciferase reporter, and vectors expressing receptor, peptide, coactivator, and CMV-βGal (transfection control).

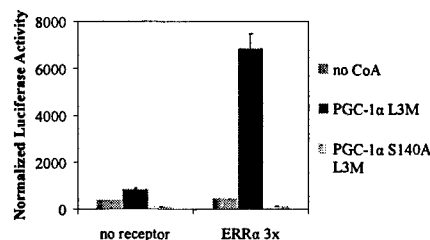
(AF2) domain of ERRα as an essential component of the binding surface for all currently known ERRα coactivators. Our data show that mutations of the hydrophobic cleft of ERRα differentially affect coactivator recruitment (Figure 4). The SRC:ERRα interaction is more sensitive to perturbations within the structure than the PGC-1α:ERRα interaction. PGC-1α does not require the charge clamp of the hydrophobic pocket to bind ERRα. This is similar to other studies that indicate that PGC-1α interactions with nuclear receptors are charge clamp independent (Tcherepanova 2000; Wu 2003; Wu 2002). The ability of PGC-1α to maintain interactions with ERRα in the absence of the charge clamp appears to be dependent on the presence of a serine upstream of the LxxLL motif (Figure 5).

Figure 5: Serine 140 of PGC-1α partially determines charge clamp dependency

A.



B.



A. Schematic representation of the mutations in PGC-1α to generate PGC-1α L3M and PGC-1α S140A L3M. * represents unchanged residues. AD: activation domain, ID: inhibitory domain, RS: arginine and serine rich region, RRM: RNA recognition motif. B. Transactivation assay testing the coactivation potential of PGC-1α L3M and PGC-1α S140A L3M on ERRα 3x. HeLa cells were cotransfected with a 3x-ERE-TATA luciferase reporter, and vectors expressing receptor, coactivator, and CMV-βGal (transfection control).

Through these studies we have developed a novel tool, peptide antagonists of an orphan nuclear receptor, that can be used to study ERR α biology. In addition, we have elucidated the molecular mechanisms of cofactor binding to this receptor.

Task 3 Evaluation of the biological consequences of blocking ERR-mediated transcription on ERR- and ER-regulated genes.

During this last year we focused on approaches to evaluate the biological consequences of blocking ERR-mediated transcription on ERR and ER-regulated genes. To this end we had proposed to identify novel target genes of ERR using microarray technology. In accordance with the outlined statement of work, we first developed an adenovirus designed to overexpress one of the peptide antagonists. While we were successful and the adenovirus-expressed peptide was able to interact with ERR in a mammalian-two-hybrid assay (data not shown), we were unable to use these peptides to determine the effect of inhibiting ERR α activity because the transcriptional activity of the endogenous receptor is difficult to measure, consistent with numerous reports [data not shown and (Huss 2002; Lu 2001; Schreiber 2003)]. Instead we designed an approach to activate ERRs using a coactivator that can selectively regulate ERR transcriptional activity.

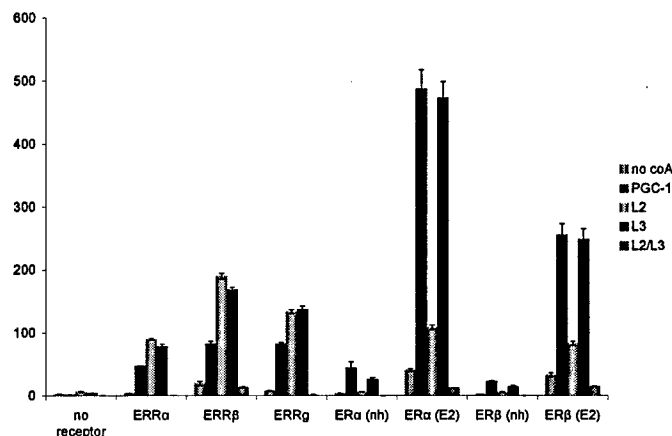
PGC-1 α is a tissue-specific inducible coactivator that has been shown to regulate the metabolic pathways involved in mitochondrial biogenesis, gluconeogenesis, and fatty acid oxidation (Lin 2003; Puigserver 1998; Wu 1999; Yoon 2001). In addition to its activity on a number of nuclear receptors, this coactivator binds to and strongly enhances the transcriptional activity of ERR α along with inducing increased expression of the receptor (Huss 2002; Schreiber 2003). Recent reports have suggested that ERR α mediates PGC-1 α -dependent stimulation of mitochondrial biogenesis and oxidative phosphorylation, as well as fatty acid oxidation (Huss 2004; Mootha 2004; Schreiber 2004). The studies above had indicated that ERR α has a high affinity for PGC-1 α and is strongly activated by this cofactor. Thus, we sought to use PGC-1 α as a "protein ligand" to activate ERR α and identify target genes. However, because PGC-1 α interacts with several NRs, we developed an approach to limit the specificity of the coactivator. In effect, we developed an ERR-selective coactivator that we could transduce into cells to activate the receptor and identify both immediate target genes and downstream biological processes.

Previous reports implicated two of PGC-1 α 's three LxxLL motifs as the critical points of interaction with ERR α (Huss 2002; Schreiber 2003). Consistent with these reports, mutation of both of the important LxxLLs is required to disrupt the ERR α coactivation potential of PGC-1 α (Figure 6). Mutation of each of the individual LxxLLs is not sufficient. This is in contrast to ER α which uses the L2 motif of PGC-1 α as its major site of interaction and mutation of this single motif disrupts the interaction with ER α .

We postulated that we may be able to limit the nuclear receptor interaction profile of PGC-1 α by replacing the LxxLL motifs within the nuclear receptor interaction domain with our ERR-selective peptides. Through a series of cloning steps, we created a PGC-1 α coactivator in which a 19 amino acid region surrounding each of the 2nd and 3rd LxxLLs were replaced with the ERR-selective peptides (19mer peptides). The N- and C-terminal sequences as well as the sequence separating the LxxLLs of PGC-1 α remain intact. The ERR-selective PGC-1 α retain

their coactivation potential and preferentially enhance the transcriptional activity of ERRs versus that of ER α or ER β (Figure 7 and data not shown). In transient transfection assays, the ERR-selective PGC-1 α is unable to coactivate other nuclear receptors, with few exceptions (i.e. HNF4 α). This data in conjunction with the PGC-1 α LxxLL mutation studies described above suggests that the LxxLL motifs and surrounding sequences are necessary and sufficient to target the coactivator to nuclear receptors. In effect, these sequences determine receptor preference.

Figure 6: Mutation of two LxxLL motifs in PGC-1 α required to disrupt coactivation

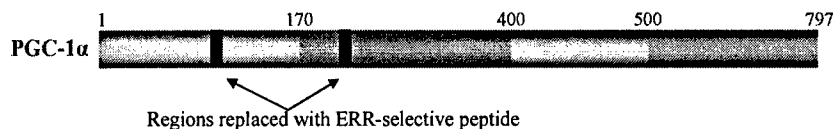


HeLa cells were cotransfected with a 3xERE-TATA-luciferase reporter, and vectors expressing receptor, peptide, coactivator, and CMV- β Gal (transfection control). L2: mutation of PGC-1 α L2 motif LxxLL to AxxAA, L3: mutation of PGC-1 α L3 motif LxxLL to AxxAA, L2L3: PGC-1 α in which the leucines of both L2 and L3 are mutated to alanines.

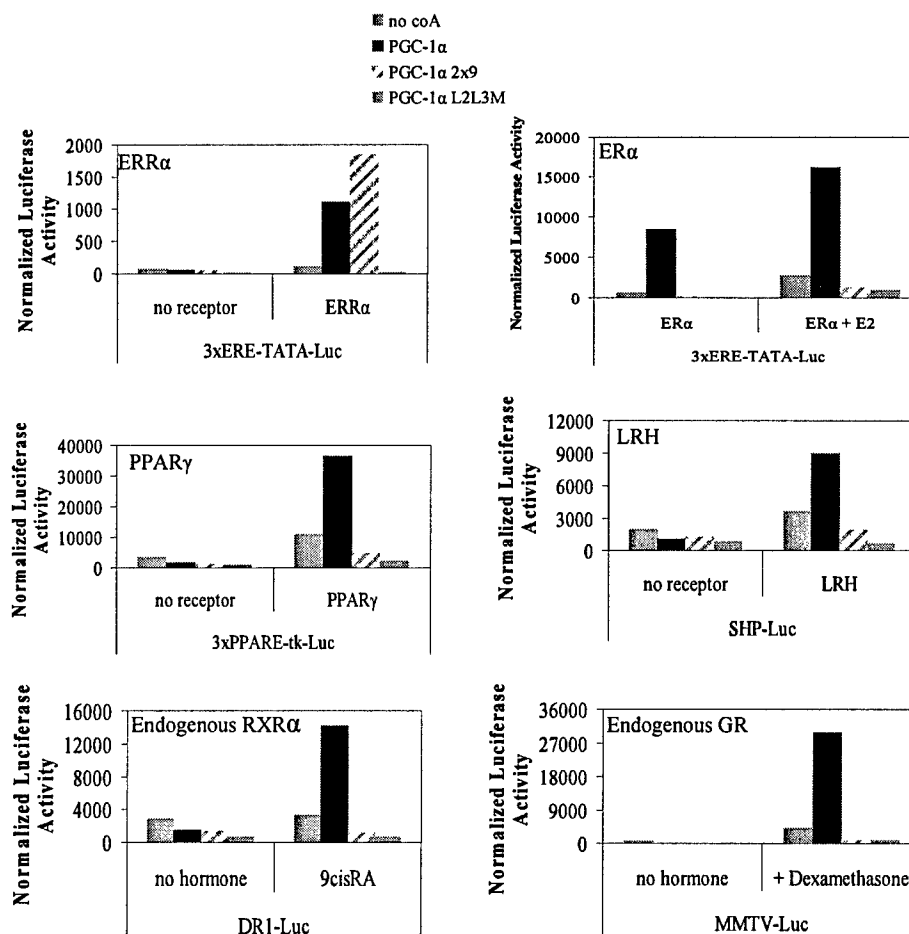
Recent studies suggest that the regulation of genes necessary for mitochondrial biogenesis, oxidative phosphorylation, and fatty acid oxidation are dependent on activation of ERR α by PGC-1 α (Huss 2004; Mootha 2004; Schreiber 2004). Therefore we tested whether our selective coactivator could be used to regulate these ERR α -dependent physiological processes. We first developed an adenovirus to deliver and overexpress the selective coactivator. The induction of ERR α target genes, including cytochrome c oxidase IV, ATP synthase β , isocitrate dehydrogenase 3A, and peroxisome proliferator activated receptor alpha, was determined using quantitative polymerase chain reaction assays (qPCR) (Figure 8). To test whether the induction of these genes by PGC-1 α was dependent on ERR α , we generated an adenovirus expressing an RNA interference sequence targeted to ERR α . With these approaches we have been able to demonstrate that genes regulating oxidative phosphorylation and fatty acid β -oxidation are indeed induced by PGC-1 α in an ERR α dependent manner in multiple cell lines. Furthermore, we have developed assays to measure mitochondrial number and oxygen consumption and have determined that in some cell lines PGC-1 α can induce these physiological processes by activating ERR α (Figure 9 and data not shown).

Figure 7: ERR-targeted PGC-1 α selectively activates ERR α

A

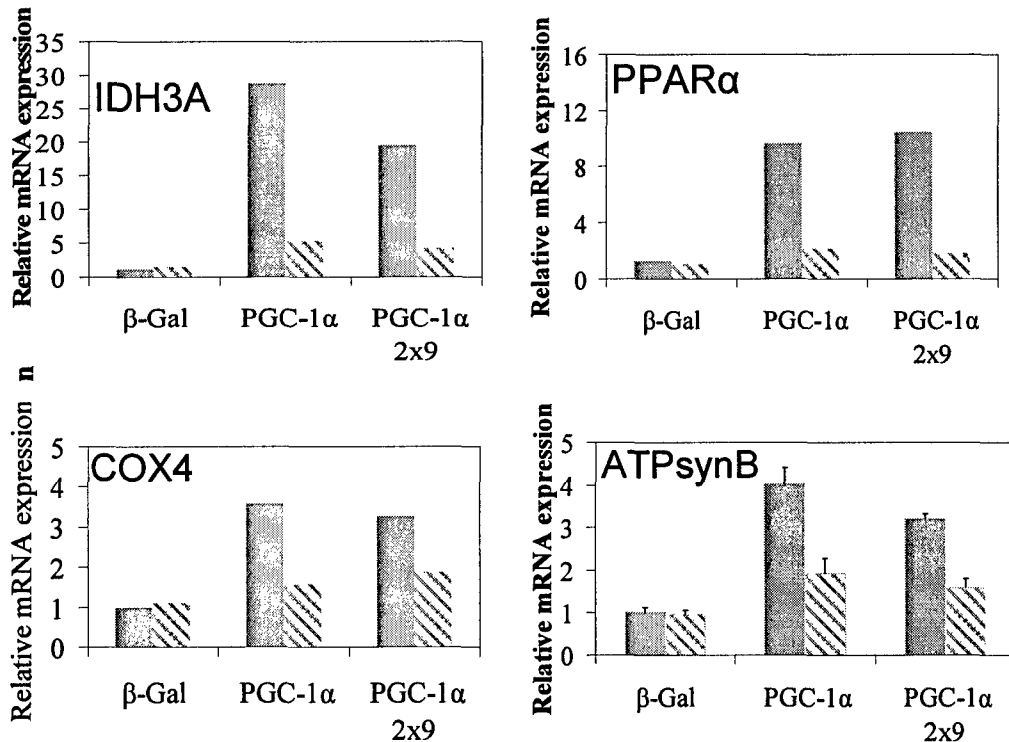


B



Transcriptional assays to determine whether the ERR-selective PGC-1 α can activate other nuclear receptors. HeLa cells were transfected with indicated reporter (Luc: luciferase), CMV- β -Gal (transfection control), vectors for the indicated receptor, and either wildtype PGC-1 α , an ERR-selective PGC-1 α (PGC-1 α 2x9), or a PGC-1 α LxxLL mutant that does not interact with nuclear receptors (PGC-1 α L2L3M). 100nM 17 β -estradiol (E2), 100nM 9cis retinoic acid (RA), and 100nM dexamethasone were used to activate ER α , RXR α and GR, respectively; no hormone corresponds to an equivalent volume of ethanol. Results are expressed as normalized luciferase activity (normalized with β -Gal for transfection efficiency) $\pm$ standard error of the mean (SEM) per triplicate sample of cells. These experiments were conducted with a dose response of PGC-1 α 2x9 expression plasmid and the results shown are with the highest concentration used.

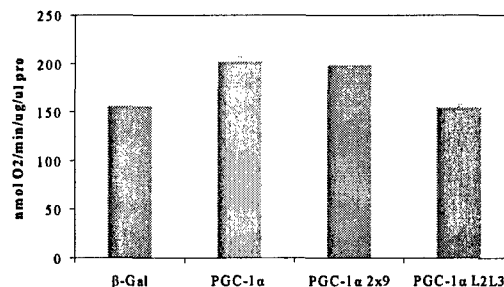
Figure 8: Activation of genes in an ERR α dependent manner by wildtype PGC-1 α and the ERR-selective PGC-1 α



Cells were infected for 48 h with adenoviruses expressing control (solid bars) or small interfering RNA targetted against ERR α (hashed bars) followed by a second infection with adenoviruses expressing β -Galactosidase (control), wildtype PGC-1 α , or ERR-selective PGC-1 α (PGC-1 α 2x9). RNA was isolated 48h after second infection. Levels of mRNA were measured by qPCR and normalized to 36B4 levels. The error bars represent the standard error of the mean (SEM) of three biological replicates. IDH3A: isocitrate dehydrogenase 3A, PPAR α : peroxisome proliferator activated receptor alpha; COX4: cytochrome C oxidase 4; ATPsynB: ATP synthase beta

Having validated this approach, we are now pursuing experiments to identify ERR α target genes in several cell models, particularly breast cancer cells. In preliminary studies we have identified many of the same genes regulating oxidative phosphorylation as targets of ERR α in MCF7 cells (data not shown). This finding may elucidate the mechanisms regulating the

Figure 9: Increased mitochondrial function induced by ERR-selective PGC-1 α



Measurement of oxygen consumption was measured using an oximeter 72 h after infection. HepG2 cells were infected with adenoviruses expressing β -Galactosidase (control), wildtype PGC-1 α , ERR-selective PGC-1 α (PGC-1 α 2x9), or a PGC-1 α unable to bind nuclear receptors (PGC-1 α L2L3). The measurements were performed in duplicate and normalized to the protein content of the cells.

altered metabolism that is characteristic of cancer cells. In addition, we have shown that the breast cancer marker pS2 (also known as trefoil factor 1 or TFF1), a known ERR α target gene, is induced in an ERR α -dependent manner (data not shown), consistent with previous reports that this gene is under the regulation of ERR α (Lu 2001). These studies suggest that this approach will be successful in identifying novel ERR α target genes and allow us to probe the biological role of this receptor in breast tumors.

In sum, we have developed two novel approaches to the study of orphan nuclear receptors: peptide antagonists and selective coactivators. These technologies will have broad applicability to the study of orphan NRs and will allow a specific determination of the biological consequences of any receptor:coactivator pair.

Key Research Accomplishments

- Purification of functional human ERR α protein expressed in a baculovirus system
- Identification of peptides that interact with ERRs through phage display screening of random peptide libraries
- In cell-based assays, identification of peptides which preferentially bind ERRs as compared to 12 other nuclear receptors
- Validation of the use of peptides as specific peptide antagonists (inhibition of ERR activity on the pS2 promoter)
- Identification of a differential interaction of coactivators with ERR α ; the requirement of the charge clamp for SRC coactivators to potentiate ERR activity; identified critical amino acids which determine the interaction
- Enhancement of inhibition by two copy peptides
- Development of systems for peptide antagonist expression: adenoviruses expressing peptides and a MCF7 cell line expressing the peptide under an inducible promoter.
- Development of ERR-selective PGC-1 α coactivator
- Validation of the use of the ERR-selective PGC-1 α coactivator in metabolic assays
- Development of ERR α siRNA expressing adenovirus

Reportable Outcomes

- Presentation of research at several conferences and seminars:
 - Medical Scientist Training Program Symposium *June 2003*
 - Endocrine Society Conference *July 2003*
 - Department of Pharmacology and Cancer Biology Annual Retreat *September 2003*
 - Biological Sciences Graduate Student Research Day *November 2003*
 - Keystone Symposium: Nuclear Receptors (Steroid Sisters) *March 2004*
 - Duke University Graduate Student Research Day *March 2004*
 - Sex and Gene Expression Conference *March 2004*
 - Medical Scientist Training Program Symposium *March 2004*
 - Cancer Biology Student Seminar Series *March 2004*
 - Department of Pharmacology and Cancer Biology Annual Retreat *October 2004*
 - Cancer Biology Student Seminar Series *January 2005*
 - Invited Speaker, Endocrine Society, Basic Science Student Award *June 2005*
 - Invited Speaker, Era Of Hope, Department Of Defense (DOD), Breast Cancer Research Program (BCRP) Meeting *June 2005*
- Awards received based on research:
 - Endocrine Society Travel Award *July 2003*
 - Best Poster Award, Biological Sciences Graduate Student Research Day *November 2003*
 - Florence P. Haseltine Award for the Outstanding Presentation by a New Investigator in Sex-Based Biology at the Fifth Annual Conference on Sex and Gene Expression Conference *March 2004*
 - AAAS/Science Program for Excellence in Science Award Recipient *March 2004*
 - Outstanding Poster Award, Biological Sciences Graduate Student Symposium *November 2004*

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- Endocrine Society Basic Science Student Award, Endocrine Society *June 2005*
 - Successfully defended Ph.D. dissertation in the Department of Pharmacology and Cancer Biology, Duke University *May 2005*
 - Reagents generated through this project:
 - Expression vectors for ERR specific peptide antagonists, including two-copy peptides
 - Adenovirus expressing peptide antagonist L3-09
 - MCF-7 cell line expressing peptide antagonist L3-09
 - ERR mutations: ERR α - Δ AF2, ERR α -3x, ERR α K244A, ERR α K244A/3x, ERR α M258R, ERR α M258R/3x
 - PGC-1 α mutations: PGC-1 α L2M, PGC-1 α L3M, PGC-1 α L2/L3M,
 - ERR selective PGC-1 α
 - ERR siRNA adenovirus

Training aspects of fellowship

This fellowship allowed the principal investigator to focus on research focused on understanding a nuclear receptor potentially involved in breast cancer development and progression. The fellowship funded the purchase of reagents necessary to conduct the experiments outlined in the report. Specific training included coursework at Duke University Medical Center along with attendance at Keystone and Endocrine Society conferences.

List of Personnel Receiving Pay from the Research Effort

Stéphanie Gaillard

Conclusions

Our project has been very successful in developing technologies for use in the dissection of the role of ERRs in regulating ER function in breast cancer cells. ERRs have traditionally been difficult to study because they are orphan nuclear receptors and there are no ligands which could be used to specifically modulate receptor activity. Through this project, we have developed two approaches to aid the study of orphan nuclear receptors. Peptide antagonists can be delivered to cells to inhibit the constitutive activity of receptors for which there are no ligands. In the second approach we can selectively activate individual nuclear receptors using a targeted coactivator, in effect acting as a "protein ligand". Thus, by both negatively and positively regulating receptor function, we can modulate the activity of receptors for which we previously had no tools. These approaches can easily be developed to target other otherwise intractable orphan nuclear receptors.

As a result of our current work, we have a better understanding of the binding of coactivators to $ERR\alpha$, the key regulatory mechanism determining the activity of the receptor. We have identified the critical regions of the receptor important for coactivator binding and defined differential binding requirements between coactivator families. We believe this distinction will be useful in the development of pharmaceutical agents to manipulate ERR activity by facilitating the recruitment of individual cofactors. These drugs may be beneficial treatments for diseases such as osteoporosis, diabetes, and breast cancer.

Another advancement through our work has been the ability to selectively activate target genes of individual receptors. With the selective coactivator, we have already identified several metabolic genes and processes as targets of $ERR\alpha$. In ongoing studies, we are continuing to elucidate these pathways, as well as connect novel targets to $ERR\alpha$. These targets may have implications for the study of the altered metabolism characteristic of cancer cells. Through future studies and animal models, we hope to illuminate the role of $ERR\alpha$ in tissue biology and advance our comprehension of breast cancer development and progression.

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