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CONTRACTING ORGANIZATION: Baylor College of Medicine
Houston, Texas 77030

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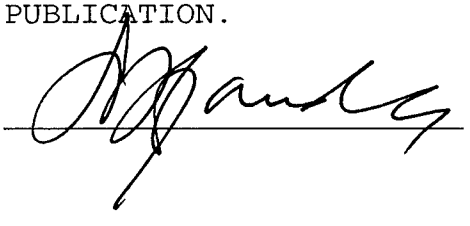

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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

The role of steroid receptor coactivator-1 (SRC-1) in breast cancer is primarily a function of (i) the expression patterns of nuclear receptors (NRs) and SRC family members in the tumor, and (ii) their inherent interaction affinities. An outstanding question is the extent to which SRC/NR pathways in breast cancer are uncoupled by administration of selective estrogen receptor modulator (SERM) regimens such as 4-hydroxytamoxifen. An appreciation of the role of SRC-1 and other SRCs in the selctive recruitment of NRs during the development of breast cancer is a prerequisite for the design of novel SRMs for treatment of breast cancer. We used BIAcore analysis to monitor interactions between SRCs and His-tagged NRs. Distinct affinities were noted for different NR-coactivator interactions, indicating an interaction preference spectrum for estrogen receptor-α (ERα) of SRC-3>SRC-1>SRC-2. The interaction of ERα with SRC family members was differentially influenced by different SERMs. In all cases, the interaction between ERα and SRCs was promoted by 17β-estradiol and inhibited by 4-hydroxytamoxifen, raloxifene and ICI 182, 780. The interaction kinetics of SRC family members with liganded NRs are consistent with a bipartite model involving a transitional intermediate. By providing accurate measurements of the modulation by SRMs of the interaction between NRs and SRC family members, we have created a model system within which rational design of SERMs for breast cancer treatment can be evaluated in a functional context.

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The Role of Steroid Receptor Coactivator-1 in Breast Cancer

DAMD17-98-1-8026

Annual Summary Report, 2000-2001

Table of Contents

Introduction

The nuclear receptor superfamily

Nuclear receptors (NRs) are ligand-inducible transcription factors which specifically regulate the expression of target genes involved in metabolism, development and reproduction. Their primary function is to mediate the transcriptional response in target cells to the sex steroids, adrenal steroids, vitamin D_3, thyroid and retinoid (9-*cis* and all-*trans*) hormones, in addition to a variety of other metabolic ligands (Tsai and O'Malley, 1994). The past five years have witnessed a growing appreciation of the complexity of NR-mediated signaling with the identification and characterization of a group of molecules, the coactivators, which mediate the functions of activated NRs at their cognate promoters (McKenna et al., 1999).

The SRC family

The cloning and characterization of steroid receptor coactivator-1 (SRC-1) by our laboratory was the first description of a common transcriptional mediator for NRs. Characterization of cDNAs encoding two more proteins with considerable sequence and functional similarity to SRC-1 established the existence of the SRC/p160 family, comprised of SRC-1/NCoA-1, TIF2/GRIP-1/SRC-2 and p/CIP/ACTR/RAC-3/AIB-1/TRAM-1/SRC-3. The SRC/p160 family is distinct from other coactivator classes, such as the p300/CBP cointegrators, TRAP/DRIP and others (McKenna et al., 1999).

NRs, SRCs and breast cancer

The intense scrutiny to which ERα, ERβ and, to a lesser extent, PR, have been subject in epithelial breast cancer research is evidence of the historical association between estrogens and the initiation and development of breast cancer. The fruits of this research have been realized in a 25% decline in breast cancer mortality rates in the UK and US over the last decade, a decrease attributed largely to the effects of ablative therapy using tamoxifen (Peto et al., 2000). Many breast tumors evade tamoxifen therapy however, and exhibit hormone-independent growth, a perennial problem in breast cancer treatment. To compound the problem, the little-understood intricate tissue-specific pharmacology associated with ER and PR ligands hampers their wider clinical application, a fact spelled out at the 2000 2nd Department of Defense Era of Hope Breast Cancer Research Program Meeting in Atlanta, Georgia.

To date, over fifty coactivators have been cloned, many with overlapping expression patterns in the mammary gland. Such rapid evolution of the coactivator field has turned the spotlight on a central question: since many tissues express an array of potential receptor-interacting partners, how do liganded NRs discriminate between SRC-1 and other SRC family members, and can we use affinity constants to construct models of coactivator recruitment in breast cancer in the presence of different selective receptor modulators, including selective estrogen receptor modulators (SERMs)? Having identified SRCs as important targets of liganded receptors in breast cancer cell lysates (see last report, 1999-2000) we wished to progress to quantifying the interactions of these factors with NRs in a model system. With this goal in mind, we developed an experimental approach designed to quantify the tripartite relationships between ligand, receptor and coactivator, with a view to clarifying the molecular role of SRC-1 and its related family members in breast cancer. We believe that this quantitative approach is essential to fully understanding the molecular role of SRC-1 in breast cancer, and its potential as a therapeutic target in the disease.

Body

Materials and Methods

Equipment and reagents

The BIAcore 2000 system, FLAG sensor chips, Tween-20, amine coupling and GST capture kits were obtained from BIAcore, Inc (Piscataway, NJ). 17-β-estradiol (E2) and 4-hydroxy-tamoxifen, progesterone and FLAG reagents were obtained from Sigma. Raloxifene was synthesized by Wyeth-Ayerst Medicinal Chemistry

and ICI-182,780 was provided by Zeneca Pharmaceuticals. The SRC-1 antibody has been described previously. PR antibody 1294 was a kind gift of Nancy Weigel and the SRC-3 antibody was provided by Jiemin Wong. ^{3}H-acetyl CoA was obtained from NEN Dupont.

Plasmids

Construction of the SRC-1, SRC-2 and SRC-3 Xenopus expression vectors was carried out using standard molecular biology techniques (data not shown).

Receptor purification

Sf9 cell pellets containing the full-length His-tagged human ERα and ERβ were a kind gift of Dean Edwards (University Health Science Center at Colorado, CO, USA). Whole cell extracts were prepared by dounce homogenization in a high salt buffer (0.4M KCl/10mM Tris-HCl/pH 8.0/0.2mM EDTA/1mM DTT/10% glycerol) containing a cocktail of protease inhibitors, in addition to 0.1mM PMSF. After an initial 15,000 rpm spin, clarified supernatants were incubated with an amount of Ni-nitrilotriacetate (NTA)-affinity resin (Qiagen, Chatsworth, CA) in the presence of 10mM imidazole. After elution of specifically bound protein with 100mM imidazole, protein concentration was determined by Bradford assay (Bio-Rad), and purity was estimated by Coomassie staining of aliquots separated by SDS/PAGE. Panvera Corp (Madison, WI), which was also an alternative source of ERα and ERβ.

Synthesis of full-length coactivators

N-terminal FLAG fusions of SRC-1, SRC-2 and SRC-3 were expressed in *Xenopus* oocytes by injection of *in vitro* synthesized mRNA encoding Flag-tagged SRCs into 1,000 stage VI *Xenopus* oocytes (5 ng of mRNA per oocyte). Oocytes were lysed as previously described [Liu, 1999 #10]. No further purification was necessary since FLAG-SRC fusions were purified by immobilization on a FLAG antibody-programmed BIAcore sensor.

BIAcore analysis

The buffer used in all experiments was 150mM NaCl/50mM Tris-HCl/pH 7.9/0.2mM EDTA/0.05% Tween-20/1mM DTT. FLAG protein immobilization on the BIAcore sensor chip surface was performed according to manufacturer's instructions. Anti-FLAG antibody was immobilized using an amine coupling kit. Crude *Xenopus* lysates containing FLAG-tagged coactivators were then immobilized. Unprogrammed oocyte lysate was used in each run to establish background binding to the FLAG antibody-coated sensor chip and generate a baseline for each individual assay. For NR binding, each cycle was performed with a constant buffer flow of 10μl/min at 25°C. Samples of receptor were injected across the surface using a sample loop. After the injection plug had passed the surface, complexes were washed with buffer for an additional 1000 sec. Following each injection, the surface was regenerated with one 10μl injection of 0.05% SDS. To remove immobilized FLAG coactivator, a 10μl injection of 10mM glycine pH 2.0 was used. Data were collected at 1Hz and analyzed using the BIAEvaluation program 3.1 (BIAcore, Inc.) on a PC. This program uses a global fitting analysis method for the determination of rate and affinity constants for each interaction. Refractive index differences for the ERs at different protein concentrations were adjusted using Sigma Plot 5.0 programs.

Results

Expression of SRC-1 and SRC family members in Xenopus oocytes

An area of significant difficulty encountered during the establishment of this project was the extreme sensitivity of the SRC family members to proteolytic degradation. I have alluded to this problem in previous reports in our attempts to examine the expression of truncated SRC variants in breast tumor samples. In this case, the objective was the over-expression of FLAG-tagged forms of SRC-1 and its family members in

Xenopus oocytes, with the purpose of quantifying their interaction with ER isoforms. FLAG-tagged SRC family members were generated in oocytes (Fig. 1A) and bound to a FLAG antibody-programmed sensor chip.

Selective recruitment by SRC-1 and other SRC family members of ERα

To examine quantitative aspects of the interactions between SRC family members and ERα in the context of full length molecules, we observed their association in real time using surface plasmon resonance analysis. His-tagged ERα was injected across the surface of a FLAG-SRC programmed chip and binding events were monitored by surface plasmon resonance. Fig. 1B shows overlaid time course sensograms for SRC-ERα at ERα concentrations ranging from 13nM to 0.4μM. A saturable interaction was detected for all three SRC family members, indicating the specific interaction of ERα with each of the three coactivators. Fig.1C shows, for each of the three coactivators, the amount of ERα bound at the end of each injection plotted as a function of log [ERα]. In the case of SRC1 and SRC2, there is a significant increase in the amount of bound ERα as a function of [ERα]. In the case of SRC3, the differences in the amount of ERα bound at different ERα concentrations are significantly smaller than for SRC-1 and SRC-2. This result indicates that the SRC3/ERα system is close to saturation at the lowest ERα concentration, suggesting that the affinity of the ERα-SRC3 interaction is higher than for ERα-SRC1 and/or ERα-SRC2.

Our data were analyzed using the BIAevaluation software to generate values for association constants for the interaction of 17β-estradiol-bound ERα with each of the SRC-1 family members (Fig. 1D).

Recruitment by SRC family members of ERβ

To evaluate the interactions between SRC family members and ERβ, we repeated the surface plasmon resonance analysis described above. His-tagged ERβ was injected across the surface of a FLAG-SRC programmed chip and binding events were monitored by surface plasmon resonance. Fig. 2 shows overlaid time course sensograms for SRC-ERβ at ERβ concentrations ranging from 13nM to 0.4μM. A saturable interaction was detected for the SRC-2 and SRC-3 family members, indicating the specific interaction of ERβ with each of the three coactivators. The failure of SRC-1 to attain saturation may be attributable either to a lower affinity of ERβ for SRC-1. Work is currently underway to more fully evaluate the kinetics of the interaction between ERβ and SRC family members in the presence of the breast cancer therapeutic drug tamoxifen.

Interaction of SRC-1 with ERα is influenced by SERM breast cancer therapeutic ligands

We next used the BIAcore apparatus to analyze the effect of different SERMs on the interaction between ERα and SRC family members. Fig. 3 shows overlaid sensograms of the interaction between chip-bound SRC-1 and ERα bound to its agonist 17-β estradiol, two SERMs used in the treatment of breast cancer, 4-OH-tamoxifen and raloxifene, the pure antiestrogen ICI 182,780, or no ligand. Binding of agonist resulted in significantly increased formation of the ERα-SRC-1 complex, to the extent that $k_{on}1$ was enhanced typically up to 3-fold over unliganded ERα. Binding of 4HT, raloxifene and ICI 182, 780 resulted in a 3-fold reduction in ERα affinity for SRC-1 relative to unliganded ERα. Essentially similar effects were observed for the interaction of ERα with SRC-2 and SRC-3.

The SRC-1-ERα interaction fits a two-step interaction model

We next used the BIAevaluation software to test several possible interaction models for the ERα-SRC-1 interaction. The results obtained do not fit adequately in to a simple one-step interaction model ($A+B \rightarrow AB$). Rather, the kinetics of the interaction are consistent with a two-step model (Fig. 4), described by $A+B \leftrightarrow A:B \rightarrow A-B$. At each receptor concentration in Fig.4, two lines are present, one experimental and one theoretical, corresponding to the progress of a reaction following ideal bimolecular reaction kinetics. The close correlation between each pair of lines indicates a good fit with a two-step reaction model. Specifically, rapid initial ERα binding ($k_{off}1$ =) appears to result in the formation of an unstable transitional intermediate which then

assumes a more stable conformation at a significantly slower rate ($k_{off}2$ =). Similar kinetics, consistent with a two-step reaction model, were observed for SRC-3 and ERα (Fig. 5).

Deviation from the Statement of Work & Difficulties Encountered

Technical difficulties beyond my control were responsible for abandonment of the attempt to create stable MCF-7 breast cancer cell lines overexpressing SRC-1 FL and SRC-1 DN forms. I was unable to create stable lines for either of these forms, presumably since cell lines are not viable expressing these forms due to their apparent toxicity in large amounts. For the same reason, attempts to generate transgenic breast cancer mice expressing the regulator and SRC-1-FL or SRC-1-DN forms ran into difficulty. Work is continuing to identify the identity of members of the SRC-1 complex in breast cancer cells.

In the pursuit of my original goal, the role of SRC-1 in breast cancer, I have developed a keen interest in the role of NR ligands in influencing the discriminatory relationships between SRC coactivators and NRs, and the extent to which these molecular interactions influence, and can be manipulated in the treatment of, breast cancer.

Key Accomplishments

- *Xenopus* expression vectors were constructed for production of full length SRC coactivators in Xenopus oocytes.
- Full length ERα and ERβ proteins were purified from harvested baculovirus-infected Sf9 cells
- BIAcore analysis was carried out to quantify the interactions between ERα, ERβ and SRC-1, in addition to other SRC family members.
- The SERM dependency of the interactions between ERα and SRC family members was established.
- The interaction between ERα and SRC-1 was investigated and shown to correlate with two step reaction kinetics.
- A commentary article highlighting research presented at the 2nd Era of Hope Meeting of the Department of Defense Breast Cancer Program was published in *Nature Medicine*.

Reportable outcomes

Manuscripts/papers

- **McKenna, N.J.** and O'Malley B.W. (2001) Redefining coactivators: a complex task. (accepted by *Science*, subject to revision).

  McKenna, N.J. and O'Malley, B.W. Nuclear receptors, coregulators and SERMs: making sense of the patchwork quilt. *Annals of the New York Academy of Sciences. In Press.*

- **McKenna, N.J.**, Liu, Z., Cheskis, B. Lyttle, C.R. and O'Malley, B.W. (2000) Association constants determine hierarchical interactions between nuclear receptors and steroid receptor coactivator (SRC) family members *in vitro*. (In preparation).

- **McKenna, N.J.** and O'Malley B.W. (2000) An issue of tissues: divining the split personalities of selective estrogen receptor modulators. *Nature Medicine*. **6,** 960-962

McKenna, N.J. and O'Malley, B.W. (2000) From ligand to response: generating diversity in nuclear receptor coregulator function. *J. Steroid Biochem. Mol. Biol.* **74,** 351-356.

Funding applied for based on work supported by this award

NIH P01: In the summer of 2000 I, along with several faculty members in the Department of Molecular and Cellular Biology, assembled and submitted a 5-year Program Project Award application entitled "Genetic and Metabolic Fingerprints of Coactivators". The award was predicated upon a hypothesis that coactivators play a central role in regulating developmental and metabolic responses to steroid hormones and other signaling pathways in both normal and disease states, including breast and prostate cancer. The application scored in the 3.5 percentile and was funded in its entirety. Project 1 will extend the initial results summarized in this annual report to build up an extended database of the differential affinities between NRs and SRCs in normal breast tissue and in breast cancer.

Conclusions

In order to understand the molecular role of SRC-1 in breast cancer, it is essential to measure not only the expression levels of SRC molecules, but also the inherent affinities which govern NR-coactivator interactions. Current models of the role of SRCs and other coactivators in breast cancer have suggested that their overexpression might be a stimulus for unregulated cellular proliferation in the mammary gland, but no studies exist which have established the inherent affinities between native SRC family members and receptors, and the extent of discrimination, if any, in these interactions.

The premise behind the current studies on the role of SRC-1 and related family members in breast cancer was that they would benefit from an approach in which the disparate observations on NR-coactivator interactions were drawn together in the context of a quantitative, standardized assay. In addition, the value of such an assay in establishing the fundamental effects of novel candidate SERMs for the treatment of breast cancer on ER/SRC interactions can be readily appreciated.

My initial observations have provided evidence that a well-defined hierarchy governs interactions between ERs and SRC family members, and give every reason to predict that such discriminatory associations will be a general rule for other receptors. Firstly, the fact that saturable binding events were observed for all ER-coactivator pairs examined reiterates the specific nature of these interactions. It should be noted that the experimental results obtained represent those obtained after many rounds of receptor purification, coactivator preparation and assay optimization. Any further testing of the validity of my observations will require the construction of a rigorously controlled *in vivo* ligand response model, in which the role of SRCs and NRs in the transcriptional response in breast cancer cells can be evaluated.

A significant limitation of our assay is its inability to recapitulate the tissue-specificity known to influence the transcriptional response to SERMs in the mammary gland and other tissues. For example, while the effects of the SERMs tamoxifen and raloxifene on ER activity are known to vary significantly between the mammary gland, uterus and bone, they are uniform antagonists in this assay. Refinement of the assay to account for such intricate pharmacologies will require a more lucid understanding of the factors which influence variations in ligand interpretation by the ER during the development of breast cancer. Several groups are pursuing the hypothesis that spatiotemporal fluctuations in coactivator/corepressor equilibrium might contribute to these effects, and it is hoped that our assay might incorporate similar variables in future studies.

I anticipate that we will be in a position to extend this work to purified SRC complexes in order to determine the affinity of their interaction with NRs. In addition, similar analyses on the interaction of NRs with the breast cancer-associated coactivator SRA (see 1999-2000 annual report) will be carried out.

References

1. Tsai MJ, O'Malley BW 1994 Molecular mechanisms of action of steroid/thyroid receptor superfamily members. *Annu Rev Biochem* 63:451-486
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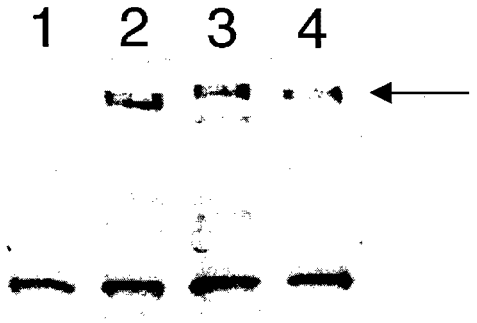
3. Peto, R., Boreham, J., Clarke, M., Davies, C. & Beral, V. UK and USA breast cancer deaths down 25% in year 2000 at ages 20-69 years. *Lancet* **355**, 1822 (2000).

APPENDICES

APPENDIX 1

Figure 1A



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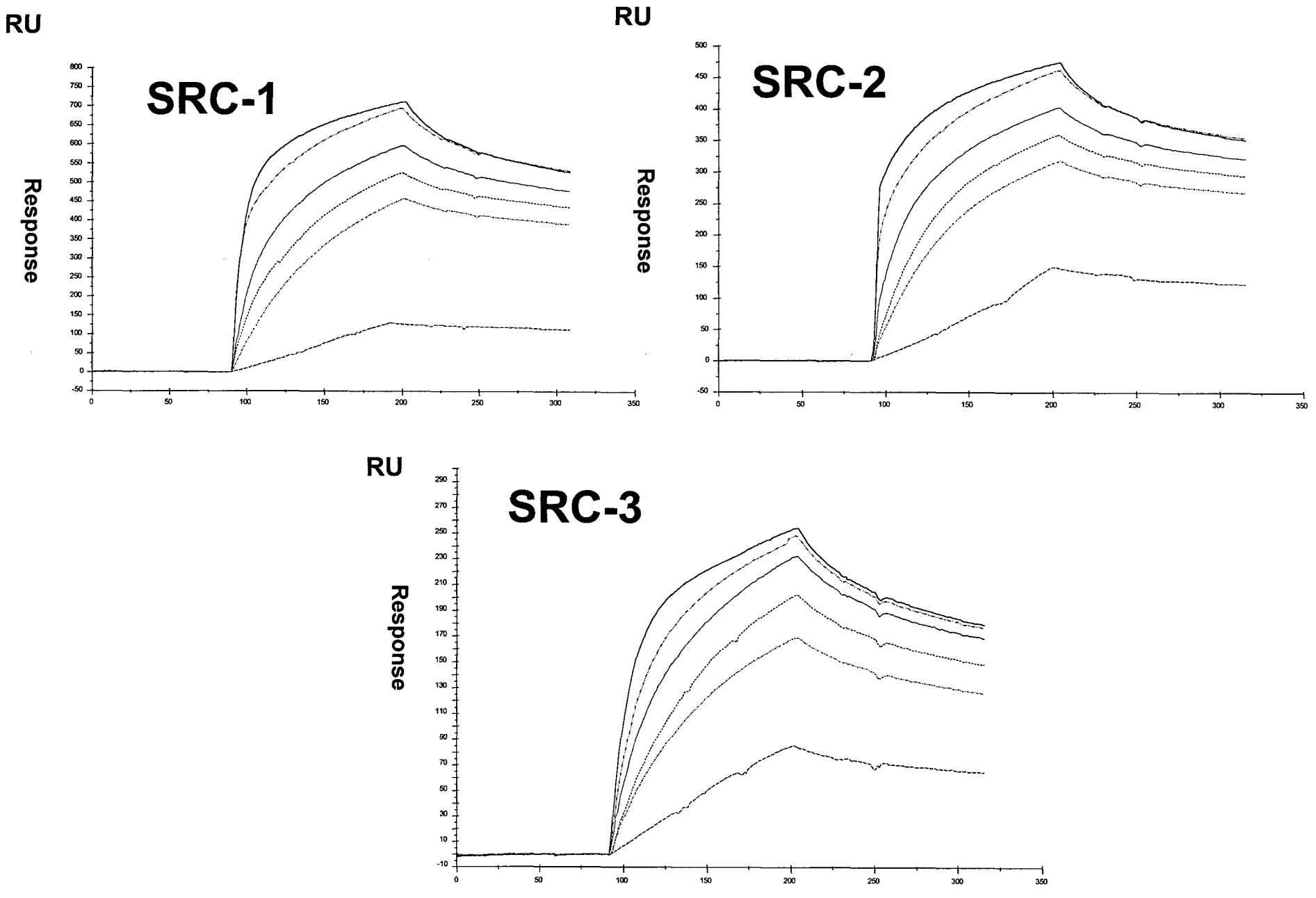

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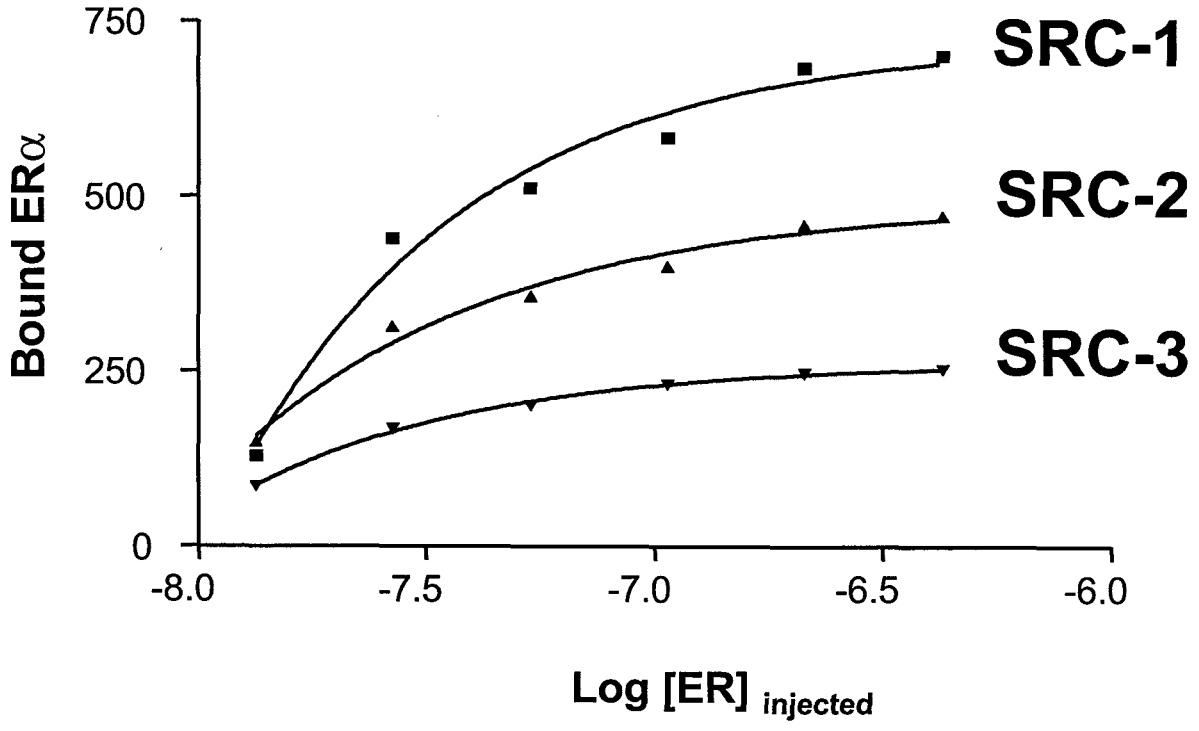
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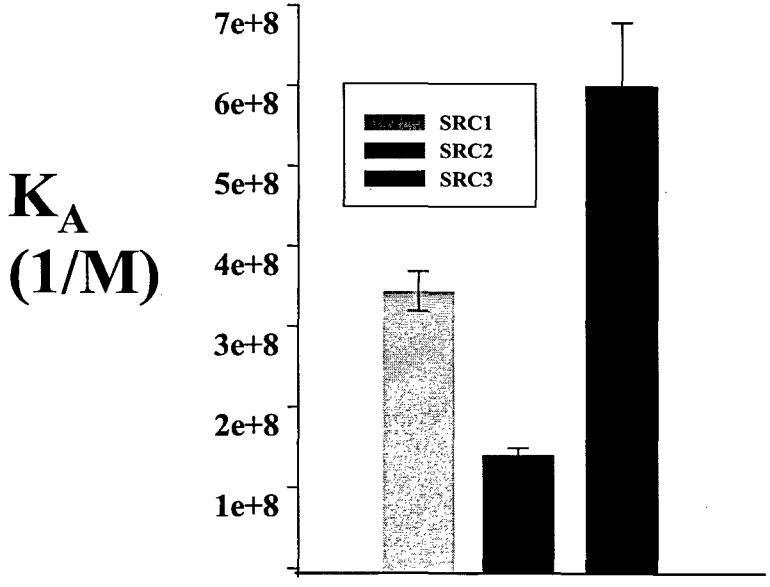
Figure 1D



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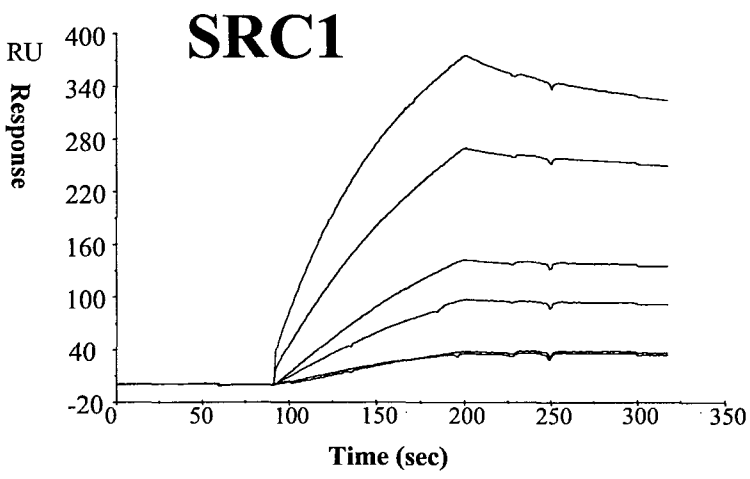
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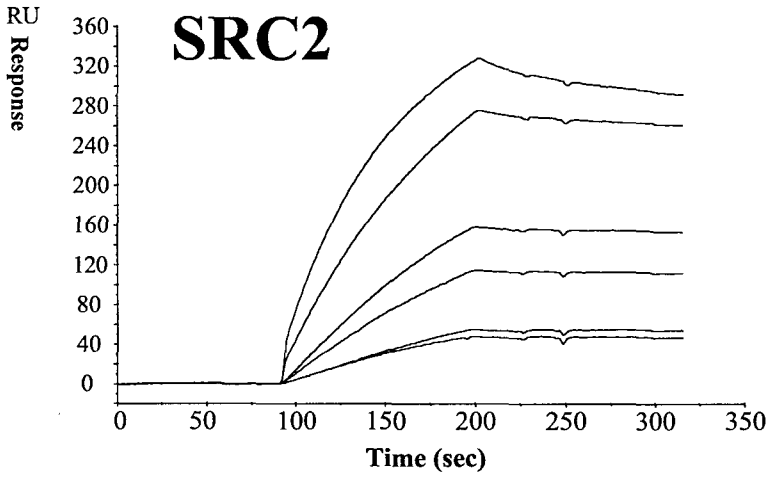
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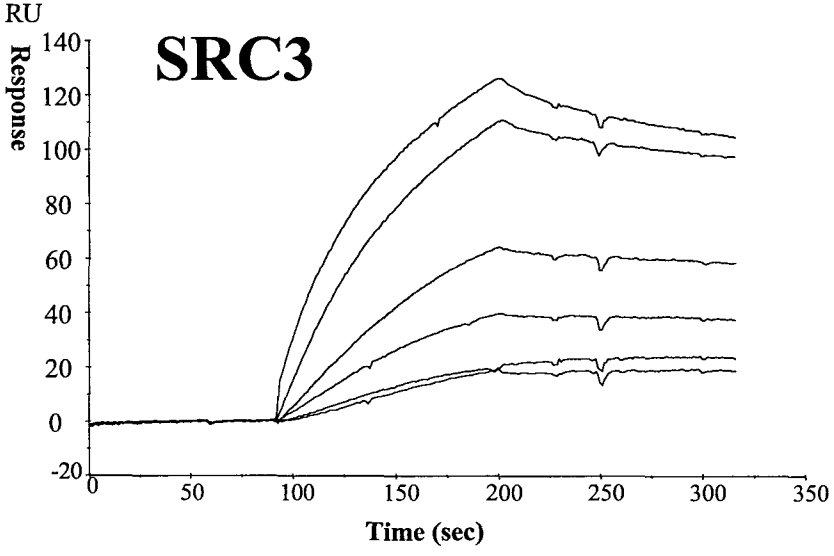
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Figure 3

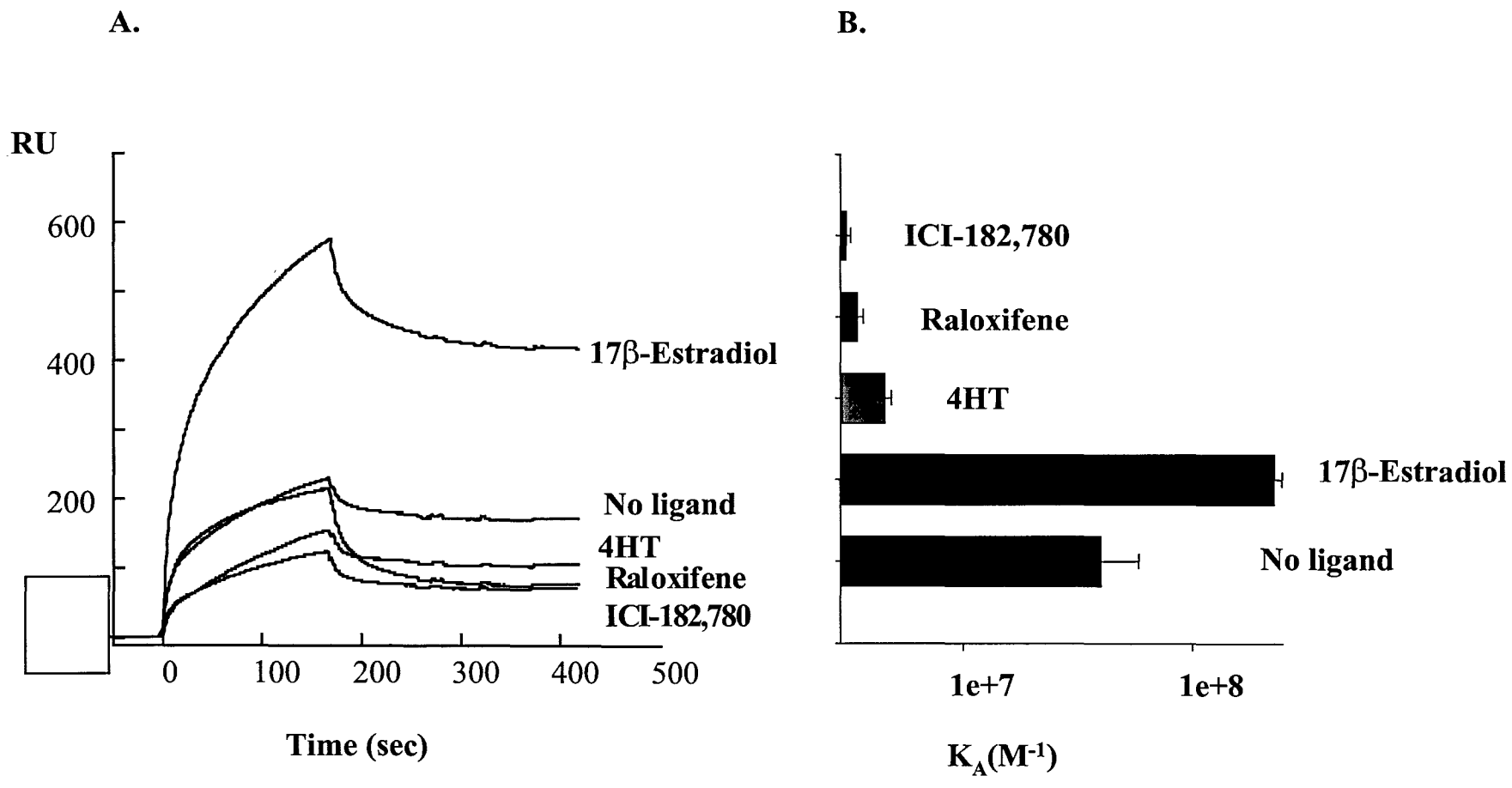
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Figure 4

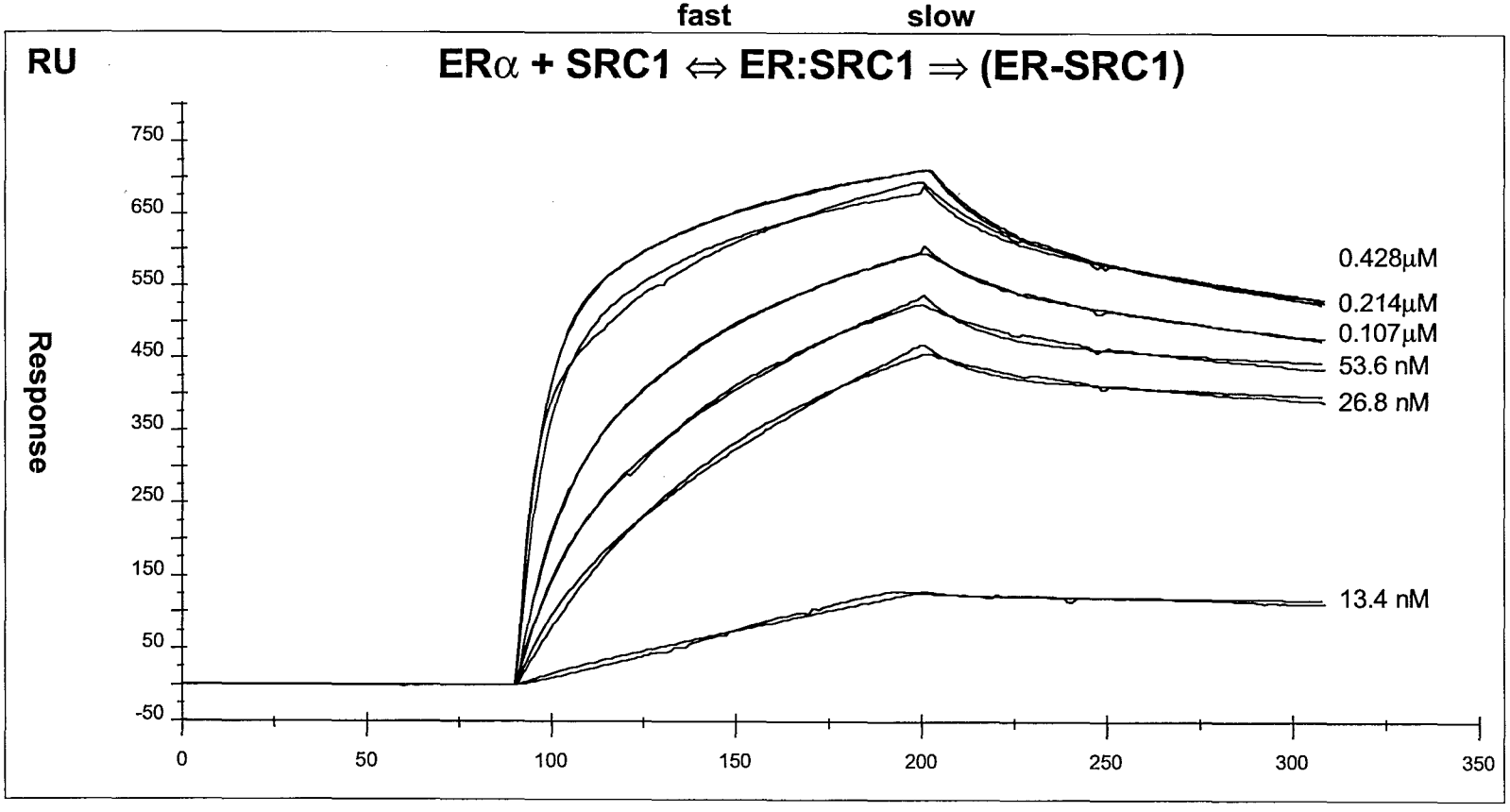

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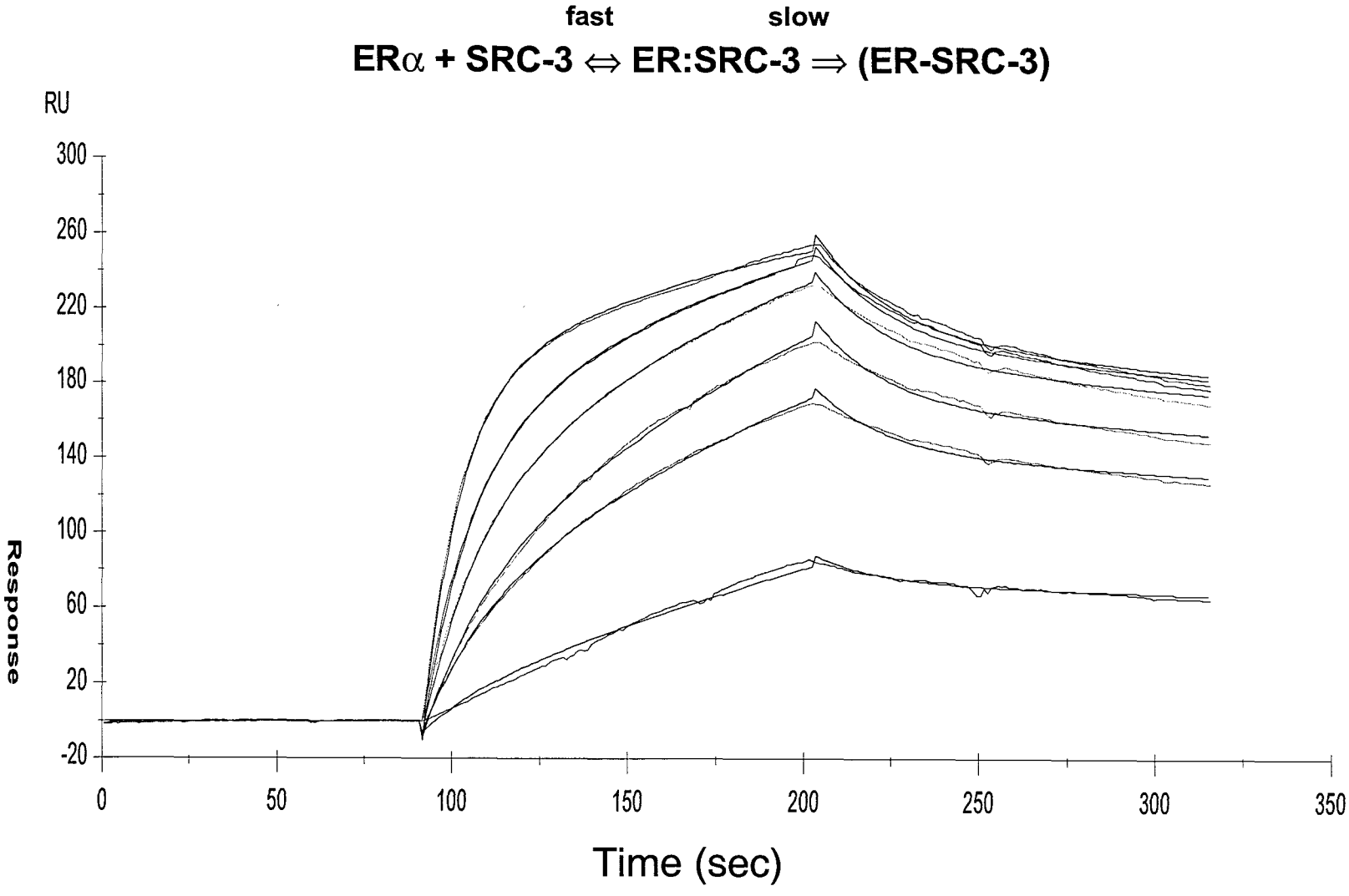
Figure 5


fast
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ERα + SRC-3 ⇔ ER:SRC-3 ⇒ (ER-SRC-3)
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Time (sec)

Figure Legends

Fig. 1. Selective interaction of SRC family members with ERα. (A) FLAG antibody immunoblot analysis of Xenopus-expressed SRC family members showing unprogrammed lysate (lane 1), SRC-1 (lane 2), SRC-2 (lane 3) and SRC-3 (lane 4). The lower band represents a cross-reacting species. (B) Titration of SRC family members with ERα. FLAG chip-immobilized SRCs were incubated with a range of concentrations of ERα. ERα concentrations were (from bottom): 13.4 nM; 26.8nM; 53.6nM; 0.107μM; 0.214μM; and 0.4μM. Prior to injection, ERα was incubated with 1μM (final concentration) 17β-estradiol at room temperature. (C) Amount of ERα bound to SRC-1, SRC-2 and SRC-3 at the end of each injection as a function of log[ER] injected. (D) Apparent affinity rate constants for the interactions between ERα and each of the SRC family members. Constants were calculated using by global fitting analysis using the BIAevaluation software (BIAcore).

Fig. 2. Selective interaction of SRC family members with ERβ. Titration of SRC family members with ERβ. FLAG chip-immobilized SRCs were incubated with a range of concentrations of ERβ. ERβ concentrations were (from bottom): 13.4 nM; 26.8nM; 53.6nM; 0.107μM; 0.214μM; and 0.4μM. Prior to injection, ERβ was incubated with 1μM (final concentration) 17β-estradiol at room temperature.

Fig. 3. Comparison of the effects of SERMs on ERα binding to SRC family members. Overlaid sensograms of 30μl injections of unliganded ERα and ERα liganded with 17β-estradiol, 4HT, raloxifene and ICI-182,780. Prior to injection, ERα was incubated with the indicated ligands at 1μM.

Fig. 4. The ERα - SRC-1 interaction fits a two-step reaction model. Interactions of immobilized SRC-1 with different concentrations are as described in Fig. 1. Two lines are shown for each receptor concentration, one experimental and the other theoretical, corresponding to the progress of an ideal bimolecular interaction.

Fig. 5. The ERα - SRC-3 interaction fits a two-step reaction model. See Fig. 4.

The discovery of coregulators and other recent advances in our understanding of the molecular biology of nuclear receptor action have generated expectations that these exciting basic advances will be translated into new diagnostic and therapeutic approaches for endocrine diseases such as breast cancer.

An issue of tissues: divining the split personalities of selective estrogen receptor modulators

NEIL J. MCKENNA & BERT W. O'MALLEY

Tamoxifen is the most popular prototype for a new arsenal of drugs, termed selective estrogen receptor (ER) modulators (SERMs), which elicit a complex array of tissue-specific effects. The ER belongs to the nuclear receptor (NR) superfamily—the largest group of metazoan transcription factors—whose members mediate the developmental, metabolic and physiological effects of steroid, thyroid, retinoid and vitamin D_3 hormones. The intense scrutiny to which ERα—and more recently, ERβ—have been subject in breast cancer research attests to the historical association between estrogens and development of the disease. The fruits of this research have been realized in a 25% decline in breast cancer mortality rates in the UK and US over the last decade[1], a decrease attributed largely to the effects of ablative therapy using tamoxifen. Despite this success, a large number of breast tumors evade tamoxifen therapy and proceed to hormone-independent growth, a perennial problem in breast cancer treatment. Furthermore, as was discussed at great length in the recent 2nd Department of Defense Era of Hope Breast Cancer Research Program Meeting[2], the elaborate pharmacology of the SERMs presents obstacles to their broader clinical applications. By evaluating selected current models of ER pharmacology in breast cancer etiology, we will seek in this commentary to sketch the clinical implications of recent developments in NR signaling.

Estrogens bring their potent mitogenic stimuli to bear in the G_1 phase of the cell cycle. Recent evidence indicates that key factors in two pathways, namely c-Myc and cyclin D1, are central to these effects. A multistep pathway, involving phosphorylation of pRB by cyclin-dependent kinases and release of E2F transcription factors required for DNA synthesis, culminates in progression from the G_1 to the S phase of the cell cycle. ER earmarks a primary breast tumor for an initially positive prognosis and the probability of a good response to hormone ablation therapy. Indeed, a striking pathogenetic symmetry exists between the role of the ERs in breast cancer and that of another NR superfamily member, the androgen receptor, in prostate cancer. Of the ER-positive breast tumors selected for tamoxifen treatment, roughly 70% will respond initially. As treatment progresses however, a considerable proportion of tumors will acquire hormone resistance and fail to respond to tamoxifen. To compound matters, tamoxifen is a two-edged sword. Although it opposes estrogen activity in the breast, it is an estrogen mimetic in the uterus, an important consideration in its prophylactic use against breast cancer. Conversely, raloxifene, an FDA-approved second-generation SERM, although estrogenic in its reduction of the severity of postmenopausal osteoporosis, is, broadly speaking, antiestrogenic in the breast and uterus.

Three central issues are raised by these clinical observations. First, what mechanisms in the breast tumor uncouple cell cycle progression from estrogen regulation? Second, how can the opposing effects of SERMs in different tissues in the body be reconciled, and how can these characteristics be manipulated to improve prospects for their future therapeutic applications? Third, can we accurately predict the tissue-specific effects of new NR ligands? Although full answers to these questions are beyond the scope of this commentary, we will provide clues that are now emerging from a rapidly developing chapter in NR action: co-regulators. Their characterization is fleshing out the linear depiction of NR function, which existed until the middle of the last decade, and is establishing a model in which the perplexing pharmacology of SERMs might finally be resolved.

The tripartite structure of NRs is defined by a central se-

APPENDIX 2

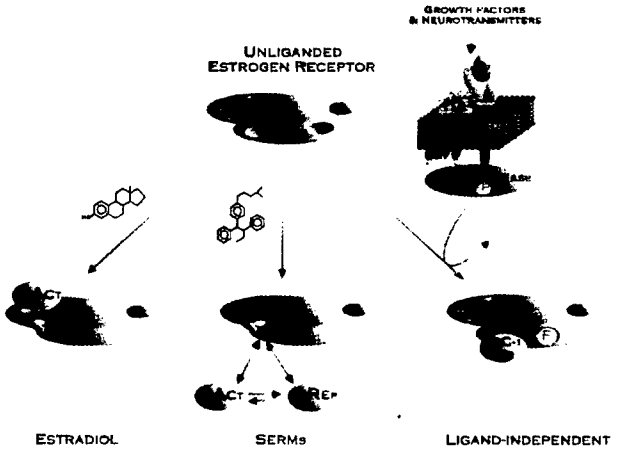

Fig. 1 Nuclear receptor co-regulators modulate the transcriptional potential of estrogen receptor activated by estradiol, SERMs or growth factors. SERM-specific variation in the topography of AF-2, particularly in the case of helix 12, seems to be an important influence on the selectivity of ER-co-regulator interactions. A variety of intermediate conformations of helix 12 elicited by binding of different SERMs is likely to contribute to the spectrum of tissue-specific effects of these molecules. In addition, tissue-specific differences in steady-state co-activator/co-repressor levels may also be key factors in SERM pharmacology. Ligand-independent activation of ER by growth factor pathways is potentially mediated in part by co-regulator interactions with AF-1.

quence-specific DNA binding domain, an amino terminal constitutive activation function (AF-1) and a distinct carboxy terminal, ligand-dependent activation function, AF-2. NR co-regulators are defined as cellular factors recruited by the AF-1 and AF-2 domains of NRs for efficient transcriptional control of promoters regulated by their cognate DNA response elements[3]. Intensively studied co-activators include members of the SRC-1 family (SRC-1/NCoA-1; GRIP-1/TIF2/SRC-2; and p/CIP/ACTR/AIB-1/RAC-3/SRC-3), PBP/TRAP220 and CREB-binding protein (CBP), whereas in the case of co-repressors, the functions of N-CoR and SMRT have been well characterized. Generally speaking, co-activators mediate the functions of activated receptors, whereas co-repressors mediate the repressive effects of inactive receptors. Receptor-recruited co-regulator complexes are thought to subject chromatin, receptor—and each other—to a sequence of post-translational modifications that furnish the target promoter for the expression or silencing of the gene, depending upon the activation state of the receptor.

The hormone-dependent recruitment of co-activators by NRs has been attributed principally to the apposition of their signature amphipathic helices, or NR boxes, with a ligand-induced hydrophobic surface in AF-2 of NRs. At the recent Era of Hope Meeting[2], Geoffrey Greene of the University of Chicago presented intricate crystallographic snapshots of ER bound to various SERMs. Tamoxifen and raloxifene seem to oppose the action of estrogen by sequestering a critical AF-2 helix, helix 12 (blue cylinder on AF-2 in Fig. 1), in a conformation sterically inhospitable to co-activator binding. Recent evidence suggests however that silencing of the tamoxifen-bound receptor may be enhanced by binding of co-repressors[3]. It seems that binding of tamoxifen to ER does not seal its inactivity; rather, an ER-tamoxifen complex, although kinetically challenged, is still primed to recruit co-activators, conceivably through AF-2 surfaces other than helix 12. In other words, the activity of ER bound to a specific SERM may be sensitive ultimately to the competing pool of co-activators and co-repressors in that tissue, itself a function of the tissue expression fingerprint of co-regulators and their intrinsic affinity for that SERM-bound receptor. In this model, it is implied that SERM-specific manipulation of ER AF-2 topography, particularly in the case of helix 12, is an important determinant of co-regulator selectivity.

Bearing in mind the checkered clinical performance of SERMs, it is appropriate to consider the role of the co-repressor:co-activator ratio in a breast tumor in influencing both its initial response to tamoxifen, and its progression to hormone resistance. Specifically, can selective pressure bring about changes in steady-state amounts of co-activators and co-repressors as tumors progress from hormone-dependency to hormone-independency? Can the 'anti-estrogen' tamoxifen be interpreted as an estrogen in the breast? Recent data suggest that alterations in co-activator:co-repressor equilibrium might underlie such a phenomenon. N-CoR levels are significantly decreased in invasive ductal carcinomas compared with intraductal carcinomas[4], and SMRT levels are down regulated tenfold in hormone-resistant MCF-7 cells transplanted in nude mice[5]. In addition, decreased levels of N-CoR have been correlated with tamoxifen resistance in a mouse breast cancer model[6]. Of further interest in this regard is the role of REA, an ER-selective co-repressor that suppresses SRC-1-mediated co-activation of ER by direct competition, and potentiates the inhibitory effect of anti-estrogens[7]. Incorporating these effects into the above model, it is reasonable to speculate that spatio-temporal fluctuations in expression of REA, or a similar co-regulator, might underlie differential responses to tamoxifen in breast tumors.

Are co-activators likewise implicated by association in the role of NRs in breast cancer? Initial insight into this question has arisen from targeted deletion of the SRC-1 gene in our laboratory. The SRC-1 null phenotype is characterized by substantially compromised hormone-dependent proliferation of mammary ductal branches[8]. Commensurate with this observation, data implicating co-activator over-expression in breast cancer pathogenesis, whether as a cause or effect, have been steadily accumulating. Elevated levels of TIF2 and CBP have been observed in intraductal carcinomas when compared with normal mammary tissue[4], and amplification and over-expression of the PBP/TRAP220 gene have been observed in breast tumors[9]. Perhaps most critically, the SRC-1 family member AIB-1/SRC-3 is over-expressed in 60% of primary breast tumors, and amplification of the gene seems to be a consistent feature of up to 10% of breast tumors[10,11].

Although we have until now focused upon the AF-2-specific functions of co-regulators, an alternative model of hormone-independent tumor progression invests ER AF-1 with increased functional significance. A central issue is whether NR-regulated pathways can mediate ligand-independent cell-cycle progression in breast tumors. Historical evidence for the role of such a pathway has documented the activation of several steroid receptors, including ERα and ERβ, by non-steroidal factors including mitogens, growth factors and PKA activators. The confluence of several protein phosphorylation cascades at the ER, including those of the Ras-MAP kinase and EGF pathway, has suggested a potential role of ER in mediating their cellular effects, at least in part. Once again, co-activators provide clues

as to a possible mechanism. Although tamoxifen effectively uncouples SRC-1 potentiation of the ER AF-2 function, it has no such effect on AF-1 activity. Furthermore, targeted phosphorylation of ER by Ras, EGF and IGF-1 enhances the interaction of ERβ with SRC-1 (ref. 12). In a separate study, MAP kinase-induced activation of the ER AF-1 was concomitant with recruitment of the AF-1 co-activator p68 (ref. 13).

How does this relate to the AF-2-compromised receptor bound to anti-hormone? As proof of principle, the AF-1 co-activator SRA potentiates progesterone receptor bound to the antiprogestin RU486 (ref. 14), pointing to a possible role for SRA in mediating the functions of other antagonist-bound steroid receptors. In summary, growth factor-mediated phosphorylation of AF-1 might supplant AF-2 as the primary route of ER recruitment of co-activators during activation of ER target genes in tamoxifen-resistant cells. Implicit in our perspective is the potential for NR co-activator over-expression to enhance flux through ligand-dependent and -independent ER signaling pathways to confer a selective advantage in tumor progression.

What lies ahead? A primary objective should be the rigorous evaluation of the potential of tumor levels of co-regulators as prognostic indices[10,15], particularly in determining response to SERMs. A wealth of evidence argues for a role in breast cancer pathogenesis of a host of ER variants[16], whose relationship with co-regulators, and ability to mediate tissue-specific SERM action, are issues as yet unresolved. Given the data emerging on aberrant expression levels of co-activators and co-repressors in breast tumors[11,17], it will be instructive to test the effect of co-activator gene-specific anti-sense probes in established *in vitro* models of breast cancer, particularly in models of hormone resistance. Progressing to *in vivo* models of breast cancer, crossing of null mutants of SRC-1 family members and of other co-activators into well-characterized breast cancer models will yield interesting information. In addition, the construction of a comprehensive microarray database of tissue expression fingerprints of co-regulators will be of great benefit in predicting tissue-specific responses to individual ligands and to selective NR modulators. Such information would find application not only in the classic hormone-dependent cancers of the breast, ovary and prostate, but also in disorders of carbohydrate and lipid metabolism, key players in which cytokines and JAK/STATs—are known to interface with NR signaling pathways.

In clinical terms, NR co-regulators are a largely unknown quantity. Unlike the NRs themselves, whose discovery was rooted in decades of painstaking endocrinology, little historical physiology or pathology preceded the somewhat dizzying pace of the cloning and characterization of NR co-regulators. Epidemiologist Malcolm Pike of USC (Los Angeles) anticipated at the Era of Hope Meeting[2] that an important goal of clinical endocrinology will be the careful evaluation of the biology of SERMs and other selective NR modulators, and we anticipate the importance of NR co-regulators in achieving that goal. As the joining of the mechanistic dots in this chapter of the NR saga progresses, we await deeper exploration of its clinical and biological ramifications, and can only hope that more questions are answered than are raised.

Acknowledgments

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From ligand to response: generating diversity in nuclear receptor coregulator function

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Abstract

Nuclear receptor ligands regulate diverse developmental and physiological processes by activating intracellular members of the nuclear receptor superfamily. Activated nuclear receptors mediate the expression of distinct gene networks in vivo by an as yet unspecified mechanism. Central to the process is the recruitment by these receptors of coactivators, a functionally diversified set of factors shown to be required for efficient transcriptional regulation by activated receptors. This article will highlight recent advances in selected mechanistic aspects of receptor function, as well as discussing the potential of coactivators to act as mediators of the intricate pharmacology of nuclear receptor ligands. © 2000 Published by Elsevier Science Ltd.

Keywords: Coregulators; Coactivators; Gene expression; Steroid receptors; Nuclear receptors; Steroid hormones; Ligands; Acetylation

1. Introduction

Steroid and thyroid/retinoid hormones are important signaling molecules in metazoans whose biological effects are manifest in processes as diverse as organogenesis during development, to governing cyclicity in reproductive tissues. Their myriad physiological functions occur as a result of specific interactions with target tissue intracellular receptors, which collectively constitute the nuclear receptor superfamily [1]. These receptors bind ligand in high affinity interactions, which generally speaking, are concomitant with their apposition to enhancer elements in the proximity of promoters of their target genes. Central to the efficient orchestration of events which lead to transcriptional activation at these promoters is the recruitment by receptors of coregulators–coactivators or corepressors — defined as factors, which interact with and affect transactivation by, nuclear receptors [2].

Recent research efforts have been directed towards establishing the basis of the discriminatory effects of nuclear receptor ligands, and have been aimed in particular at determining the potential of coactivators to mediate their distinct biological effects. This commentary will consider selected themes which enhance our current understanding of the pivotal role of coactivators in nuclear receptor function. It will be divided into two parts: the first will discuss current understanding of selected molecular events surrounding the interaction of liganded receptor with multiple coactivator complexes at hormone regulated promoters. The second will seek to place these events in a wider biological context by discussing the role of coregulators in mediating DNA element- and ligand specific functions of nuclear receptors. For a detailed discussion of the characterization of individual coactivators, the reader is referred to selected reviews [2,3].

2. Multiple coactivators and the dynamic promoter

To date, nearly thirty different coactivators or coactivator complexes have been implicated in transcriptional activation by nuclear receptors. The physiological relevance of what are predominantly in vitro observations can ultimately be answered only in knockout models of individual coactivators, such as that of SRC-1 [4]. While the abundance of coactivators is reflected in part by tissue-specific expression fingerprints for different coactivators, the coexpression of multiple coactivators

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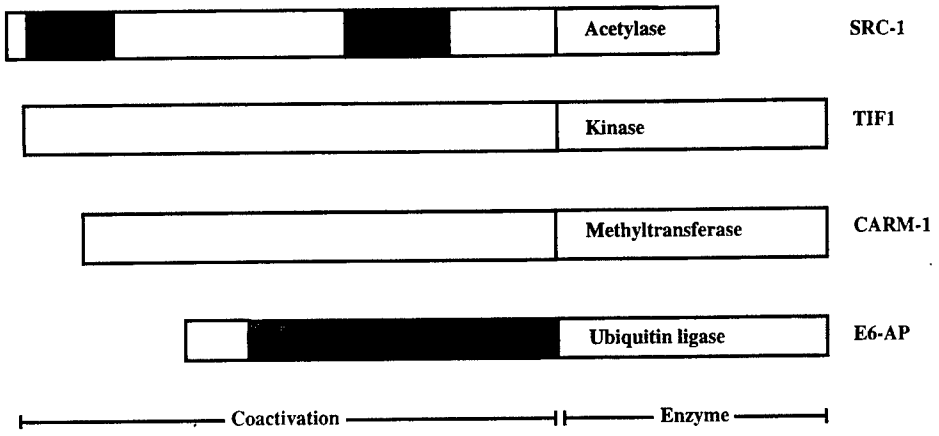

Fig. 1. Structural analogies among coactivators. An emerging theme in several coactivators is the juxtaposition of distinct coactivation functions and enzymatic activities. Some activation functions are better defined than others and are indicated by shading.

in a single tissue appears to be a general rule. Given the thermodynamic constraints upon the simultaneous interaction of these factors with a liganded receptor dimer, it can be presumed that, after binding of a specific ligand to a specific receptor, an ordered series of sequential receptor-coactivator interactions culminates in transcriptional initiation. Two important goals are: (i) to establish an order for such discrete interactions, which can account for the recruitment by nuclear receptors of functional domains as diverse as ATPases [5], acetyltransferases [6,7], methyltransferases [8], ubiquitin ligases [9], and an RNA coactivator [10]; and (ii) to discern the molecular signals which orchestrate this sequence of events.

A simplified sequence of events at hormone regulated promoters envisages initial targeting of chromatin modifying complexes such as SWI/SNF and the PCAF, p300 and SRC family[1] histone acetyltransferase (HAT) activities, resulting in nucleosome disruption. Subsequent recruitment of complexes thought to forge a direct link with general transcription complexes, such as DRIP/ARC and Mediator, results in transcriptional activation. While the exact mechanism behind this sequence of events is currently unclear, recent evidence has hinted that sequentiality and dynamism at the promoter may be driven at least in part by defined post-translational modifications of a variety of substrates, mediated by specific coactivator domains. These domains are enzymatic in nature and appear to be functionally autonomous with regard to their associated activation domains. In particular, HAT activity, initially identified as primarily directed towards histones, has more recently been proposed as a context and substrate-dependent mechanism for promoter dynamism.

Although p300 was initially characterized as a HAT [11], evidence for a role of p300 acetylase activity at a step subsequent to nucleosome disruption [12] suggests its function in this regard should be viewed in a broader context. It was shown that the acetyltransferase activity of p300 was required not for initial chromatin disruption but rather was required for transcriptional activation from a disrupted chromatin template. The study provided evidence that p300 targets the general transcription factors TFIIF/RAP74 and TFIIEβ for acetylation, casting acetylase activity in a more general role at an activated promoter. Indeed, p300-catalyzed acetylation has been shown to target lysine residues in the vicinity of the central LXXLL motifs of ACTR/hSRC-3, a modification which has been suggested to uncouple the intera ction between ACTR/hSRC-3 and estrogen receptor (ER) [13]. This event was placed in the context of sequential interactions between receptor and distinct nuclear receptor-recruited assemblies such as SRC-1 complexes, CBP complexes and other mediator-like complexes such as DRIP/ARC. Such results raise the possibility that targeted acetylation of the basal transcription machinery and other factors by p300 [12] may have a role in topological alterations at the promoter, but this is yet to be established.

Dissection of the functional domains of the coactivators characterized to date suggests that many coactivators are at least bifunctional, containing enzymatic activities in addition to coactivation domains, as classically defined in reporter gene assays (Fig. 1). The results alluded to above point to a possible rationale for the coupling of distinct activation and enzymatic domains in coactivators. Coactivation domains may mediate as yet undefined links between receptor and

[1] In the unifying SRC family nomenclature [2,29], the prefix 'h' is used for all human clones and the prefix 'm' identifies mouse clones, such that 'hSRC-1' represents SRC-1 and 'mSRC-1' corresponds to NCoA-1 [30]. The clone GRIP1 [31] is synonymous with mSRC-2 and hSRC-2 identifies TIF2 [32]. hSRC-3 is an umbrella term for the clones ACTR [7], RAC3 [33], AIB1 [34], TRAM-1[35] and SRC-3 [36]; p/CIP [37] is referred to as mSRC-3. Throughout this commentary, discussions of individual clones refer to original clone name name under proposed nomenclature, e.g. GRIP1/mSRC-2.

downstream targets while their associated enzymatic activities serve as engines for promoter refurnishing. Domains harboring acetyltransferase, methyltransferase, kinase and ubiquitin ligase activities target histones, receptor, cofactor complexes and basal transcription factors, facilitating the entry of subsequent complexes and priming the promoter for initiation of transcription.

Recent studies have emphasized the fact that coactivators exist primarily in large molecular weight complexes, an important fact when considering the ability of coactivators to negotiate the local enhancer/promoter architecture. Such complexes may serve a structural role, to maneuver a single active subunit into the precise three-dimensional geometry of a specific promoter — much as a crane overcomes thermodynamic and physical constraints to maneuver a girder into its correct position in a building superstructure. To illustrate this idea, only half of the eight common subunits of human SWI/SNF are required to catalyze the bulk of its known enzymatic reactions [14]. An equally plausible scenario is that ancillary subunits may serve in a 'co-coregulatory' capacity to modulate the activity of the functional core of the complex.

These observations can be assembled into a general sequential model of receptor activation (Fig. 2). The model poses important questions concerning the order of recruitment of chromatin remodeling complexes, which are thought to create a transcriptionally permissive environment at hormone-regulated promoter. Recent data on the *S. cerevisiae HO* promoter suggest that binding of the SWI/SNF ATPase complex is required to observe increased acetylation of the promoter, which is concomitant with binding of SAGA [15], the yeast homolog of the PCAF complex [16]. Binding of both these complexes is required for transcriptional activation at the *HO* promoter. Nucleosome acetylation is increasingly being cast in the role of augmenting ATPase-dependent chromatin remodeling, helping to 'fix' the chromatin in an active state [17]. In Fig. 2, a liganded receptor initially recruits a member(s) of the SRC family and attendant protein/RNA coactivators, followed by targeting of a chromatin remodeling complex to the promoter. This in turn is required for binding of histone-targeting acetylase complexes, primarily the PCAF complex. Specific acetylation events directed towards non-histone targets then result in dissociation of SRC-1 family members and facilitate recruitment of complexes contacting the basal transcription machinery, such as Mediator and DRIP/ARC, which may also maintain contact with chromatin-remodeling activities to maintain the 'open' conformation of the promoter [18]. Components of the ubiquitination pathway would engage dissociating complexes for reprocessing by protein degradation pathways (Fig. 2). Within this model, cell context, enhancer and promoter-specific effects will influence both the composition of the recruited complexes and the sequence of events, which precede transcriptional activation (see below).

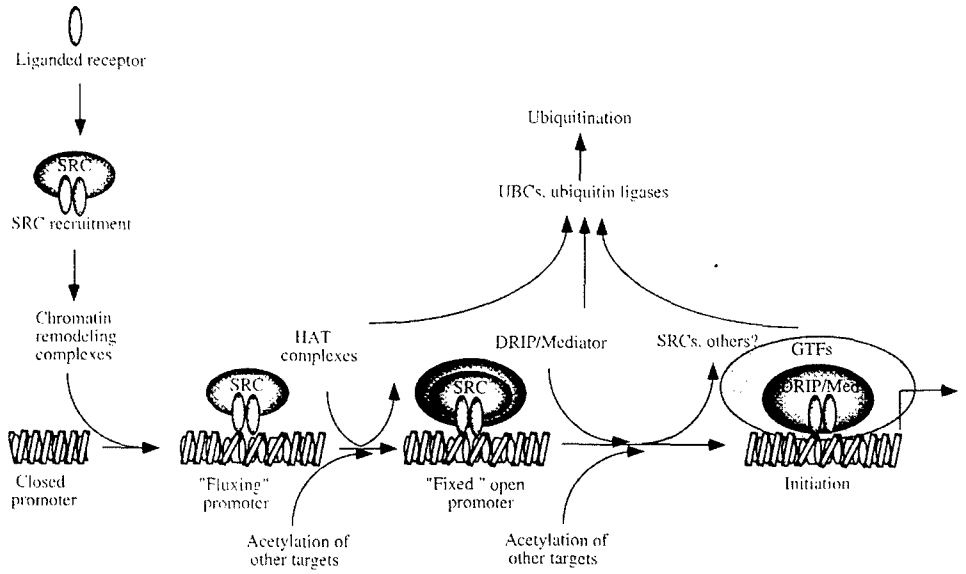

Fig. 2. General dynamic model of assembly and disassembly of distinct coactivator complexes at a hormone-regulated promoter. Acetylase activity and other targeted postranslational modifications may be act as catalysts for promoter fluidity and sequential recruitment of functional domains as required. Ubiquitination appears dominant in cessation of function of the transcriptional complex.

3. Coregulators as determinants of ligand specificity

3.1. Enhancer/promoter context

Promoter and enhancer context have emerged as important determinants of the nature of the nuclear receptor complexes which may be efficiently recruited by diverse transcription factors [19]. Such selectivity is starkly illustrated by transcriptional regulation of positive and negative thyroid response elements by thyroid hormone receptor (TR) and its coregulators [20]. While isolated DR4 (positive) elements permit binding of TR and CBP in the presence of ligand, negative TREs selectively recruit TR-HDAC2 (histone deacetylase) complexes in a ligand-dependent manner. Their results illustrate not only the ability of the promoter to discriminate between, and select for, the identity of coregulator complexes bound to ligand-bound TR, but suggest also that events other than binding of ligand by LBD are necessary to furnish the C-terminal activation function (AF-2) for interaction with coactivator complexes.

3.2. Ligand

Of particular interest currently is the possibility that multiple ligands for nuclear receptors may influence the biological activity of the receptor by influencing selective recruitment of coregulator complexes. It is becoming apparent that the type of agonist bound to a specific receptor is an important determinant of its affinity for a particular subset of coactivator complexes, thereby ultimately influencing the biological response to the ligand. The vitamin D_3 receptor (VDR) has been the focus of particular attention in this regard. Takeyama et al. [21] showed that the ability of vitamin D derivatives to elicit distinct biological responses might be a function of differential affinity of ligand-bound VDR for coactivator complexes in vivo. For example, whereas 1α,25-dihydroxyvitamin D_3 promoted interaction between VDR and all three SRC family members, 22-*oxa*-1α,25-dihydroxyvitamin D_3 (OCA) efficiently induced interaction of VDR only with TIF2/hSRC-2, and only TIF2/hSRC-2 was capable of potentiating VDR transactivation in the presence of OCA.

This concept has been extended to DRIP, a complex originally isolated using the VDR ligand-binding domain bound to 1,25 dihydroxyvitamin D_3 (D_3)[22]. While GRIP-1/mSRC-2 and DRIP-205 were recruited with comparable affinity by D_3-bound VDR, C-20 stereoisomers of D_3 (20-epi analogues) induced recruitment of DRIP205 by VDR at concentrations 100 fold lower than those at which D_3 or other derivatives, such as OCA, induced the VDR/DRIP 205 interaction. Interestingly, GRIP-1/mSRC-2 was not stably recruited by 20 epi-bound VDR until ligand concentrations were 100-fold in excess of those at which DRIPs were efficiently bound. Twenty epi-analogs of D_3 have for some time been known to modify proteolytic cleavage patterns of ligand-bound VDR, presumably by inducing specific conformational changes in the LBD. It was suggested that such specific alterations might result in structures which preferentially accommodate DRIP over SRC-1 or SRC-2-containing complexes [23]. Interestingly, DRIP205, a component of the DRIP complex, has been shown interact with glucocorticoid receptor (GR), suggesting that DRIP may have a more general role for both type I and type II receptors than was previously considered [24]. It remains to be established whether different GR agonists have a comparable effect upon recruitment of coactivator complexes by GR.

To place these observations in a physiological and clinical context, one can cite the biological activity of a burgeoning group of designer ER ligands, the selective ER modulators (SERMs). Through high affinity interactions with the ER, SERMs run the gamut of tissue-selective responses such that in certain tissues their effects are 'estrogenic' (estrogen-like) while in others they oppose the classic estrogen response-an 'antiestrogenic' effect. For example, while both raloxifene and tamoxifen oppose the action of estrogen in breast cells, only tamoxifen induces an estrogenic effect in the uterus, whereas raloxifene elicits an estrogenic response in bone which is more potent than that of tamoxifen [25]. Current models for SERM action postulate that tissue-specific coregulator fingerprints influence the net transcriptional flux in response to the ligand as a result of their selective interaction with different ER-SERM complexes. For example, L7/SPA enhances ER transactivation in response to certain partial ER agonists but does not influence transactivation by pure agonist-bound ER [26]. Furthermore, REA, (repressor of estrogen activity), is recruited by ER in an estrogen and antiestrogen-dependent manner to down-regulate the agonist effect of the respective ligand [27]. While the ability of L7-SPA and REA-like factors to mediate selective SERM effects in vivo remains to be established — for example, by a tissue specific expression profile-their potential to do so can be readily appreciated. Inherent in both these models is the notion that the changes in AF-2 similar to, but distinct from, those elicited by agonist binding can be effected by different SERMs [28]. Given its malleable structure, AF-2 might serve as a highly discriminate docking site for additional corepressors or coactivators, depending upon the cellular context.

A diagram summarizing the contribution of coactivators to the diversity of the biological response to ligand is shown in Fig. 3. In this scenario, binding of a specific ligand to a specific receptor in a given tissue commits the receptor to interaction with a particular subset of coactivators which activate a specific subset of genes in

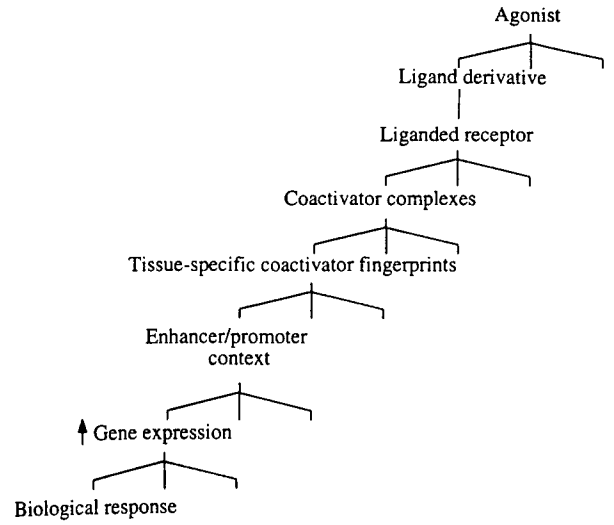

Fig. 3. Activation of specific gene networks in vivo by nuclear receptors resulting from multiple potential responses to ligand.

an enhancer and promoter-specific context. The potential for selective responses at each step ensures a flexible and adaptable response to physiological stimuli. Not included for the sake of clarity are the numerous post-translational modifications, some possibly instigated by parallel signal transduction pathways, which may further serve to modulate the amplitude and scope of the biological response to ligand.

4. Concluding remarks

Nuclear receptor ligands are exquisite pharmacological agents, eliciting a selective physiological response at a specific time, in a confined area for a specified period. This commentary has sought to highlight those lines of research which address the biological basis for their highly discriminate effects, focussing in particular on the profusion of coactivators known to be required by activated receptors for efficient transcriptional activation. To be sure, there is strength in numbers, and partial functional redundancy is known to exist between different coactivators, the phenotype of the SRC-1 mouse being the most obvious testament in this regard. The striking functional diversity of the coactivators characterized to date however argues for alternative explanations for their abundance. The discriminate in vivo effects of synthetic ligands has provided evidence at least for the existence of similar molecules in vivo, if not yet proving their physiological relevance. By influencing tissue-specific interpretation of ligand, diverse coregulator expression profiles can augment the pharmacological repertoire of individual ligands through successive developmental stages or during cyclic alterations in the composition of a specific organ, for example during pregnancy. Furthermore, in vitro evidence has established enhancer and promoter context as a significant factor in determining the nature and composition of receptor-coactivator complexes, which may be assembled at a given gene. Future work will place the relative contribution of these and other factors in a physiological context to more accurately assess their importance in the biological response to ligand.

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