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The estrogen receptor (ER) is a critical diagnostic marker and therapeutic target for a large number of breast cancers. The elucidation of the mechanisms by which ER controls the growth of certain breast cancers may provide new targets of intervention that would disrupt estrogen signaling in hormone-dependent breast cancers. SHP and Dax-1 are members of the nuclear receptor superfamily of ligand activated transcription factors that have been shown to interact with other nuclear receptors in a hormone dependent manner. In support of grant number DAMD17-99-1-9163 the findings presented here describe interactions between ER and DAX-1 and also ER and SHP. These interactions have been determined to be both direct and hormone-dependent. Functional studies of this interaction indicate that both DAX-1 and SHP act as negative regulators of ER signaling. Mutational analysis has identified regions in the amino-terminus of SHP and DAX-1 that are required for the interaction with ER. These analyses have demonstrated that subtle variations of a leucine-rich motif LXXLL, found within SHP and DAX-1 mediate the interaction. These studies suggest that SHP and DAX may act as negative regulators of ER signaling that may compete with the p160 coactivators and help to recruit co-repressors.

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Introduction

The estrogen receptor (ER) is a critical diagnostic marker and target for chemo-therapy and chemo-prevention of a large number of breast cancers. The elucidation of the mechanisms by which ER controls the growth of certain breast cancers may provide new targets of intervention that would disrupt estrogen signaling in hormone-dependent breast cancers. SHP and Dax-1 are members of the nuclear receptor superfamily of ligand activated transcription factors that have been shown to interact with other nuclear receptors in a hormone dependent manner. In support of grant number DAMD17-99-1-9163 the findings presented here describe interactions between ER and DAX-1 and also ER and SHP. These interactions have been determined to be both direct and hormone-dependent. Functional studies of this interaction indicate that both DAX-1 and SHP act as negative regulators of ER signaling. Mutational analysis has identified regions in the amino-terminus of SHP and DAX-1 that are required for the interaction with ER. These analyses have demonstrated that subtle variations of a leucine-rich motif LXXLL, found within SHP and DAX-1 mediate the interaction. These studies suggest that SHP and DAX may act as negative regulators of ER signaling that may compete with the p160 coactivators and help to recruit co-repressors.

Body

SHP and DAX-1 interact with the Estrogen Receptor (ER) in a hormone-dependent manner.

By two separate experimental approaches we have observed an physical interaction between ER and either SHP or DAX-1. First, in a yeast-based two hybrid assay in which SHP was tethered to DNA by the LexA DNA binding domain, ER was shown to interact with SHP in the presence of estrogen. This interaction was measurably stronger in the presence of estrogen. In addition several deletion mutations were introduced in the SHP portion of the LexA-SHP fusion and used to determine the region of SHP that mediates the interaction bewteen SHP and ER. These studies indicated that a region of SHP from amino acid 72 to 148 was required for the hormone-dependent interaction with ER. These studies indicate that there is an in vivo association bewteen ER and SHP. In order to independently confirm these findings we measured the ability of ER to interact with ^{35}S-SHP in vitro. These experiments were carried out by generating a glutathione-s-transferase to ER fusion protein and immobilizing it on glutathione-linked sepharose beads. This complex was the incubated with ^{35}S-SHP in the presence or absence of estrogen. A similar GST-fusion protein that carried the nuclear receptor, RXR was used as a positive control since it had arlready been demonstrated that RXR interacts with SHP in a ligand-dependent manner. Following the incubation, the retained fraction was washed and resolved on a 12% SDS PAGE. These studies indicated that like RXR, ER was capable of interacting with SHP in a hormone-dependentent manner. Taken together, these studies support the conclusion that ER interacts with SHP in a hormone-dependent manner and that this interaction is mediated by a region of the SHP portein that ranges from amino acid 72 to amino acid 148.
The significant homology between SHP and DAX-1 suggested that DAX-1 may also be able to interact with ER in a hormone-dependent manner. To test this idea, ^{35}S-DAX-1 was produced by in vitro translation and incubated, in the presence and absence of estrogen, with the GST-ER fusion protein bound to glutathione-linked-agarose. In these studies, but the GST-ERα and GST-ERβ were tested for their ability to interact with DAX-1. In these studies estrogen was able to stimulate an interaction between DAX-1

and ERα however the interaction between DAX-1 and ERβ appeared to be independent of exogenous hormone. This in vitro result may reflect a fundamental difference in the biological activities of ERα and ERβ. Current efforts are underway to test this result using an in vivo system such as the yeast or mammalian two-hybrid system.

SHP and DAX inhibit the transcriptional response of ER to estrogen.

The observation of physical interactions observed between ER and either SHP or DAX-1 suggested that SHP and DAX may be able to modulate the transcriptional activity of ER. To test this idea transient transfection assays were carried out in the ER-neagitve cell line U2OS. An estrogen responsive reporter construct consisting of two copies of the estrogen response element form the rat vitellogenin gene fused to a minimal promoter and to the cDNA encoding luciferase (ERE_2-tk-luc) was transfected in the presence or absence of and ER expression plasmid and an expression plasmid encoding either SHP or DAX-1. In these studies, it was observed that in the absence of ER there was no measurable transcriptional response to estrogen. Against this low background, over expression of either ERα or ERβ was sufficient to potentiate a transcriptional response that was measured to be 200-fold and 100-fold respectively. Under these conditions it was observed that overexpression of DAX-1 had little effect on the basal activity of the reporter, but

significantly inhibited the ligand-dependent transcriptional activity of both ER isoforms. In similar experiments this inhibition was shown to be dependent on the amount of DAX-1 transfected into the system. These studies suggest that DAX_1 is a hormone-dependent inhibitor of ER-mediated transcriptional activation and that the inhibition by DAX-1 of ER signaling is dose-dependent. Similar transfection experiments in which DAX-1 was replaced by SHP-1 indicated that SHP-1 is behaving in a manner that is very similar to DAX-1. SHP was capable of inhibiting estrogen-dependent transcriptional activation by either ERα or ERβ. Additionally this effect was dosage-dependent in that increasing amounts of the SHP expression plasmid could be correlated with increased inhibition of estrogen signaling. Take together these transfections establish that there is a functional consequence to the physical interaction described above, and suggest that both SHP and

DAX are capable of blunting the transcriptional response that is mediated by either ERα or ERβ.

The interaction domains of SHP and DAX-1 may compete with those of the p160 family of nuclear receptor coactivators.

The p160 family of nuclear receptor coactivators consists of three members that were identified based upon their ability to interact with a variety of nuclear receptors. Each of these family members has several copies of the amino acid sequence LXXLL, which were shown to physically interact with the ligand binding domain of a nuclear receptor in a hormone-dependent manner. The amino terminal half of both SHP and DAX-1 are composed of three extended repeats of approximately 65 amino acids each. It was interesting to note that in each of these repeats an amino acid sequence similar to the LXXLL was identified in both SHP and DAX. These observations lead to the hypothesis that the interaction between either SHP or DAX-1 with ER might be mediated by the same protein/protein interaction interface as that which mediates the interaction between ER and the p160 family of coactivators. To test this idea we measured the ability of ER to interact with DAX-1 in the presence and absence of a peptide which represents one of the nuclear receptor interaction domains NRID of the p160 family member, GRIP1. This sequence (KHKILHRLLQDSS) had previously been shown to be sufficient for the interaction between GRIP1 and horomone bound nuclear receptors. Additionally this peptide had been shown to compete with full-length GRIP1 for binding to a nuclear receptor. This peptide and a corresponding double-alanine mutant (KHKILHR**AA**QDSS) were then used as competitors in the binding of DAX-1 to the hormone-bound GST-ER. In these experiments it was observed that the ER/DAX interaction was interrupted by the wild-type NRID and not by the mutant. This experiment suggests that DAX-1 bind to the same region of the hormone bound ER as do the p160 family of receptors and it raises the intriguing possibility that ligand-dependent corepressors by directly compete with ligand-dependent coactivators. These experiments are being repeated and further evaluation will be needed tin order to draw this conclusion.

Additional Initiatives

There is abundant evidence that a woman's risk of breast cancer is in direct correlation with the levels and duration of estrogen exposure. Since our data suggest that

the role of SHP and DAX-1 may be to inhibit estrogen signaling, we sought to investigate if the actions of DAX-1 might be implicated in the chemo-preventative setting. In order to test this idea, a mammary epithelial cell line was developed that is immortalized but not transformed. This line was derived by overexpressing the catalytic subunit of human telomerase in primary human mammary epithelia. Our characterization of these cells has indicated that they resemble many of the pre-cancerous mammary lesions that fall into the broad categorization of "atypical hyperplasia." Currently we are testing the hypothesis that these cells are in fact pre-cancerous. This is being done by introducing known oncogenes and into the cells and evaluating them for their ability to grow in soft agar and also in nude mice. The development of tumors in this setting will validate this system for the modeling of pre-cancerous lesions. It will then be of great interest to study the effect of estrogen signaling in these cells and the ability of SHP and/or DAX-1 to modulate these effects. We anticipate being able to carry out these studies in the first half of 2001.

Key Research Accomplishments

1. We have demonstrated by several different experimental strategies that SHP and DAX-1 are both capable of interacting with the estrogen receptor in the presence of estrogen. These demonstrations have been made both in vitro and in vivo.

2. We have demonstrated that both SHP and DAX-1 functionally interact with the estrogen receptor. These studies have indicated that SHP and DAX-1 function as hormone-dependent transcriptional co-repressors.

3. We have crudely identified the domains of SHP and DAX-1 that mediate the interaction with the hormone-bound estrogen receptor.

4. We have demonstrated that the same interaction domain used by the p160 family of transcriptional coactivators is capable of competing with SHP and DAX-1 for binding to the estrogen receptor.

Reportable Outcomes

1. We have demonstrated by several different experimental strategies that SHP and DAX-1 are both capable of interacting with the estrogen receptor in the presence of estrogen. These demonstrations have been made both in vitro and in vivo.

2. We have demonstrated that both SHP and DAX-1 functionally interact with the estrogen receptor. These studies have indicated that SHP and DAX-1 function as hormone-dependent transcriptional co-repressors.

3. We have crudely identified the domains of SHP and DAX-1 that mediate the interaction with the hormone-bound estrogen receptor.

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Modified Statement of Work

This document is being included to reflect the fact that the proposed work in this grant remains the same and was unaffected by the Principal Investigator's move to Dartmouth Medical School.

Inhibition of Estrogen Receptor Action by the Orphan Receptors, SHP and DAX-1

The original Statement of Work attached to this grant consisted of four tasks. These are outlined below.

Task 1. To analyze the interaction patterns of SHP and DAX-1 with ERα and ERβ.

1. Analysis of interaction patterns both in vivo and in vitro.
2. Mapping the interaction domains of ER and DAX-1
3. Co-immunoprecipitation from whole cell extracts to demonstrate an in vivo association extracts.

Task 2. Investigate the expression patterns of both SHP and DAX at both the mRNA and protein levels in conjunction with ER expression.

RNA and Protein analysis:

1. Isolation of RNA from primary mammary epithelia and breast cancer cell lines.
2. Analysis of SHP, DAX-1 and ER expression by northern blot and RT-PCR.
3. Development of SHP monoclonal antibody
4. Analysis of protein levels in primary mammary epithelia and breast cancer cell lines by western blotting.

Task 3. To study the effects of SHP and DAX on estrogen signaling

1. Analyze the effects of SHP and Dax-1 on the transcriptional activity of ERα and ERβ by transient co-transfection.
2. Analyze the effects of SHP and Dax-1 on the ability of estrogen to promote cell cycle progression.

Task 4. Screen for SHP homologues expressed in mammary gland.

1. Informatic based screen of published genomic data for SHP and DAX-1 homologues
2. Degenerate PCR approach to identifying novel family members

Portion of work to be completed:

Task 1. Section 3: Progress has been made at demonstrating the interaction of SHP and ER in whole cell extracts using a commercially available antibody directed against DAX-1 and ER. Efforts to identify antibodies directed against SHP-1 are ongoing.

Task 2 Section 3: Two previous efforts to develop monoclonal antibodies against SHP have been unsuccessful. Our hypothesis is that the small size of SHP results in decreased immungenicity in the mouse. Current efforts will be to conjugate SHP to other proteins resulting in fusion-proteins that are larger and more immunogenic.

Task 3 Section 2: Using the MCF-7 model for hormone-dependent cell cycle progression we are currently establishing the experimental parameters for over expression of wild-type and mutant SHP and DAX-1 and their effects on G1/S progression in response to estrogen.

Task 4: To date no significant homologues of SHP-1 and DAX-1 have been identified using a degenerate PRC strategy to amplify such products from the cDNA of several breast cancer cell lines. Our ongoing effort is aimed at optimizing this RT-PCR strategy and also at scanning the public genetic databases for homologues.

It is the opinion of the PI that the recent move to Dartmouth will have minimal effects on the progress of this project. All of the necessary lab space, equipment and reagents will be available at the time of the transfer from Dana Farber to Dartmouth. In addition to the physical space and equipment, the faculty of the Department of Pharmacology and Toxicology at Dartmouth Medical School has a wealth of knowledge on topics relating to the regulation of gene expression by the nuclear receptor superfamily.

Signature of Principle Investigator

Oct 5 2001
Date

Conclusions

In support of Grant # DAMD17-99-1-9163 we are now able to draw the following conclusions which are reiterated int he **Reportable Outcomes** section of this report. First, there is a highly specific physical interaction between the estrogen receptor and both SHP and Dax. This interaction is strictly dependent upon the presence of estrogen. These observations have been confirmed both in vitro and in vivo. Second, we conclude that both SHP and Dax are capable of repressing ER-mediated transcriptional activation which suggests that they act as hormone dependent transcriptional co-repressors. Third, we conclude that SHP and Dax utilize a similar protein-protein interaction domain as that of the p160 family of nuclear receptor coactivators and that these factors compete with SHP and Dax for binding to the hormone-bound ER. Taken together, we believe that these studies suggest that the expression patterns of SHP and Dax may contribute to the cell and tissue specific responses to estrogen and also to the selective estrogen receptor modulators (SERMs).