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13. Abstract (Maximum 200 Words) (abstract should contain no proprietary or confidential information) The estrogen receptor (ER) is involved in breast cancer development and progression. We have isolated a novel 97 kDa DEAD box RNA helicase (DP97) that interacts with the estrogen receptor alpha (ERa) in the presence of either estradiol or trans-hydroxytamoxifen. DP97 represses the transcriptional activity of the estradiol-occupied estrogen receptor and this repression can be relieved with Trichostatin A(TSA), a selective histone deacetylase inhibitor. DP97 represses the activity of a constitutive SV40 promoter when it is recruited as a Gal-4 DNA binding domain fusion protein. DP97 also interacts with, and represses the activity of, other nuclear receptors such as the progesterone receptor b, glucocorticoid receptor, and retinoic acid receptor a. However, DP97 does not repress the activity of either p53 or VP16. We show that the N-terminal helicase region of DP97 is dispensable for its activity as a transcriptional repressor. Furthermore, a BLAST homology search of the remaining sequence of p97 reveals that this protein has a small region of homology with the SMRTe(NCoR2) corepressor protein. This small region is also able to repress a constitutively active SV40 promoter when it is recruited to it as a Gal-4 DNA binding domain fusion protein. Therefore, DP97 functions as a RNA helicase, a nuclear receptor interacting protein, and as a transcriptional coregulator.				
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INTRODUCTION

Nuclear receptors act as a conduit for cognate hormonal ligands that activate or repress gene transcription in a manner that is dependent on the nature of the coregulator proteins (coactivators or corepressors) that are recruited to the ligand-receptor complex. While many coactivator proteins have been identified, there appear, by contrast, to be relatively few corepressor proteins (1). Coactivators include the SRC/p160 family of proteins, which function as coactivators by recruiting CREB binding protein (CBP) and/or p300 complexes that up-regulate nuclear receptor activity through their histone acetyltransferase activity. ATP-dependent chromatin remodeling complexes, such as BRG1/hBrm, and the TRAP-DRIP-ARC complex, which act sequentially or combinatorially, also enhance gene transcription by facilitating RNA polymerase II recruitment to promoters (3-5).

The corepressors NCoR (nuclear receptor corepressor) and SMRT (silencing mediator of retinoic and thyroid receptor) function as major negative regulators of several members of the nuclear receptor family, including thyroid and retinoic acid receptors, and they appear to exert their repressive activities through recruitment of histone deacetylases to promote a repressive chromatin state. In the case of the estrogen receptor (ER), two additional negative coregulator proteins have been identified; these are the repressor of estrogen receptor activity (REA) (6,7) as well as a repressor of tamoxifen transcriptional activity denoted RTA (8). To search for other factors that are involved in

mediating the activity of estrogen agonist and antagonist ligands, we used 2-hybrid interaction screening with antiestrogen liganded-ER as bait. Through this screen, we have isolated a novel DEAD box RNA helicase that acts as a corepressor of the liganded ER, and of other nuclear hormone receptor superfamily members.

Two other DEAD box RNA helicase proteins have been shown to be specifically involved in modulating nuclear receptor transcriptional activity. p68 RNA helicase interacts with the N-terminal A/B region of ER α and selectively potentiates the activity of ER α ; this coactivator does not interact with or serve as a coactivator of either ER β or other nuclear receptors (9, 10). Another DEAD box RNA helicase, DP103, has been shown to interact with a specific repression domain in the orphan nuclear receptor, steroidogenic factor-1 (SF-1) and potentiate the transcriptional repression activity of SF-1 (11). In contrast, DP97 interacts with the C-terminal hormone binding region of ER α , ER β and other nuclear receptors and functions more broadly as a corepressor of these receptors. For all three proteins, the DEAD box motifs are not required for their role as nuclear receptor coregulators.

The recruitment of DP97 to ligand-occupied nuclear receptors results in suppression of their transcriptional activity. DP97 also has intrinsic repressive activity of a constitutively active promoter when it is recruited as a GAL4 DNA binding domain fusion protein. In fact, DP97 has a small region that shows significant homology to a repression domain in the known corepressor NCoR2

(SMRTe.) This repression domain is necessary and sufficient for the transcriptional repression activity of DP97.

In this study, we have characterized the role of a novel DEAD box RNA helicase protein, DP97, in the transcriptional activity of nuclear receptors. The DEAD box motifs are dispensable for the repression activity of DP97. Also, DP97 has intrinsic transcriptional repression activity and a transcriptional repression domain that shows homology to NCoR2. A selective histone deacetylase protein, Trichostatin A, is able to relieve the repression caused by DP97. Finally, knocking down endogenous DP97 protein levels in cells makes them respond more strongly to estrogens.

BODY

In the third year of this work, we describe the identification of a novel DEAD box RNA helicase, DP97, that interacts in a hormone-dependent manner with the estrogen receptor and other nuclear receptors and has several interesting biological properties. DP97 is an ATP-dependent RNA helicase; it interacts with and represses the activity of nuclear receptors; and it has a small region of sequence homology with NCoR2 (SMRTe) that is responsible for its intrinsic transcriptional repression activity. Analysis of the repression activity of truncated forms of the DP97 protein show that the DEAD box motifs are dispensable for DP97 repression activity, indicating that its RNA helicase and corepressor activities represent distinct and separable functions.

Nuclear receptor corepressors, such as NCoR1 or NCoR2, typically have separable functional domains for nuclear receptor interaction and for transcriptional repression (12, 13). Using GST pull-down analysis, we have shown that the C-terminal part of DP97(663-865) is the region that interacts with the C-terminal portion of nuclear receptors encompassing the ligand binding/AF-2 regions. Although DP97 contains 3 NR boxes (LXXLL motifs) through which many coregulators interact with nuclear receptors, these NR boxes are in the N-terminal portion of DP97, not in the C-terminal region that interacts with nuclear receptors. This indicates that other sequences in DP97 are responsible for its receptor interaction. This is consistent with findings from peptide phage display and studies with other coregulators (6-8) that have shown that peptide

sequences in addition to LXXLL motifs (and CoRNR boxes) can interact with nuclear receptors with high affinity (14, 15).

We have shown that DP97 has an intrinsic active transcriptional repression region. Like DP97, several other transcriptional repressor proteins have small transferable regions with active transcriptional repression activity. These include Nab-1, a direct acting corepressor of the NGFI-A family of zinc finger transcription factors (16), Sim-2, a protein involved in midline development in mice (17), and AREB6, a zinc-finger homeodomain transcription factor (18).

It is of note that the repression region of DP97 shows considerable homology with a repression domain in the well known corepressor, NCoR2 (SMRTe(13,19,20,21)). As shown in Figure 8B, DP97(589-631) has homology to two regions in NCoR2, amino acids 498-534 and amino acids 813-839. NCoR2(813-839), is within the second repression domain (RD2) of NCoR2 (19). NCoR2(498-534) encompasses a polyglutamine and acidic-basic region (20) that is between SANT(SWI3, ADA2, NCoR, TFIIIB: <http://www.uib.no/aasland/SANT.html>) domains A and B. Since this region has only been examined as a Gal4 fusion along with other repressive regions of NCoR2 such as the SANT domains and the region of high similarity between NCoR and NCoR2 (33), it is not known whether NCoR2(498-534) has intrinsic repression activity on its own. Our observations with DP97 expand the utilization of related motifs for nuclear receptor regulation beyond SMRT/NCoR to a new corepressor protein, DP97.

With the exception of the small repression region (amino acid 589-631) of DP97, DP97 shows no structural resemblance to NCoR or SMRT or to two other proteins known to be negative coregulators for nuclear receptors (repressor of estrogen receptor activity (REA) and repressor of tamoxifen transcriptional activity (RTA)). Like DP97, REA preferentially interacts with antiestrogen-liganded ER but both also interact with estrogen-occupied ER. However, in sharp contrast to DP97, REA is an ER-selective coregulator (6,7,22). RTA binds to ER as well as other nuclear hormone receptors, as does DP97, but RTA interacts through the N-terminal region of the receptors in a ligand-independent manner, whereas DP97 interaction with receptor is via the hormone binding/activation function-2 domain and is ligand regulated. Of interest, RTA contains RNA recognition motifs that are required for its repressor function, indicating a role for RNA binding in regulation of nuclear receptor activity (8). For DP97, its repressor activity is physically and functionally separable from its RNA helicase activity.

In this work, we identify a novel RNA helicase from a human breast cancer cDNA library, that interacts in a hormone-dependent manner with nuclear hormone receptors and represses their transcriptional activity. Since RNA helicases are known to be involved in many aspects of RNA processing, including RNA transcription, processing and transport and ribosome biogenesis, it is tempting to speculate that there is perhaps a linkage between RNA processing and transcriptional activity of the nuclear hormone receptors. DP97

may be the beginning of some regulatory feedback mechanism that could have positive components (p68) and negative components (DP97), as well as receptor specific and receptor nonspecific factors. Further understanding to the role of RNA binding proteins and helicases will better allow for the role of these proteins in nuclear receptor transcriptional activity.

Key Research Accomplishments for 9/1999 to 9/2002

- Obtained full-length clone of a novel protein originally isolated from a yeast-two hybrid screen
- Performed ATP hydrolysis assays of p97 protein to characterize its biochemical ATP dependent RNA helicase activity
- Upon further characterization, the 97 kDa novel protein was found to act as a repressor of the estrogen receptor in many different estrogen-responsive promoter gene constructs such as pS2, thymidine kinase, and complement C3.
- Determined that the C-terminal region of p97 interacts with the DEF region (and not the ABC region) of the estrogen receptor in a ligand dependent manner
- Determined that DP97 is able to work as an intrinsic repressor on constitutively active promoters
- Linked the ability of DP97 to repress transcriptional activity of the estrogen receptor to histone deacetylase activity
- Determined that the N-terminal DEAD box motifs containing region of p97 is not required for the repression of the estrogen receptor
- Determined that p97 is able to work as an intrinsic repressor on constitutively active promoters and that p97(413-663) is able to maintain this intrinsic repression activity

- Found a region of DP97 with a high homology to SMRTe that is necessary and sufficient for the intrinsic repression of DP97
- Linked the ability of p97 to repress transcriptional activity of the estrogen receptor to histone deacetylase activity

Reportable Outcomes for 9/1999 to 9/2002

TRAVEL GRANTS

Endocrine Society Travel Grant Award Recipient, 2001

Keystone Symposia-Nuclear Receptor Superfamily Travel Grant Award Recipient, 2002

PUBLICATIONS

- Rajendran, R.R.**, Nye, A.C., Frasor, J., Balsara, R.D., Martini, P.G.V., and Katzenellenbogen, B.S. (2002) Regulation of Nuclear Receptor Transcriptional Activity by a Novel DEAD box RNA Helicase (DP97). in preparation.
- Nye, A.C., **Rajendran, R.R.**, Stenoien, D.L., Mancini, M.A., Katzenellenbogen, B.S., and Belmont, A.S. (2002) The estrogen receptor alters large scale chromatin structure. *In press*, Molecular and Cellular Biology
- Nye, A.C., **Rajendran, R.R.**, Belmont, A.S. (2002) The cell. Encyclopedia of the Human Genome; Nature Publishing Company in press.
- Nye, A.C., **Rajendran, R.R.**, Katzenellenbogen, B.S., Belmont, A.S. (2001) Movie: The estrogen receptor alters large-scale chromatin structure. Trends in Cell Biology GFP in Motion CD-ROM, volume 2, ed. B Ludin and A Matus.
- Sun, J., Meyers, M.J., Fink, B. E., **Rajendran, R.R.**, Katzenellenbogen, J.A., Katzenellenbogen, B.S. (1999) Novel ligands that function as selective estrogens or antiestrogens for estrogen receptor- α or estrogen receptor- β . Endocrinology, 140:800-804.

PRESENTATIONS

- Rajendran, R.R.**, Nye, A.C., Balsara, R.D., and Katzenellenbogen, B.S. (2002) Regulation of Nuclear Receptor Transcriptional Activity by the DEAD Box RNA Helicase DP97, Era of Hope meeting for the Department of Defense (DOD) Breast Cancer Research Program (BCRP), Orlando, Florida.
- Rajendran, R.R.**, Nye, A.C., Balsara, R.D., Katzenellenbogen, B.S. (2002) Regulation of Nuclear Receptor Transcriptional Activity by the DEAD Box RNA Helicase p97, Keystone Symposia-Nuclear Receptor Superfamily. Snowbird, Utah
- Rajendran, R.R.**, Nye, A.C., Martini, P.G., Katzenellenbogen, B.S. (2001) p97: A novel DEAD box RNA helicase that acts as a nuclear receptor coregulator. 83rd Annual Meeting of the Endocrine Society. Denver, Colorado

- Katzenellenbogen, B.S., Martini, P.G.V., **Rajendran, R.R.**, Delage-Morrour, R., Sun, J., Choi, I., Kraichley, D.M., Katzenellenbogen, J.A. (2000) Estrogen Receptor Alpha and Beta: Selective Ligands and Coregulator Modulation of Receptor Activity. Keystone Symposia-Nuclear Receptors 2000. Steamboat Springs, Colorado
- Nye, A.C., Stenoien, D.L., **Rajendran, R.R.**, Katzenellenbogen, B.S., Mancini, M.A., Belmont, A.S. (2000) The estrogen receptor alters large scale chromatin structure. Dynamic Organization of Nuclear Function. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York
- Nye, A.C., **Rajendran, R.R.**, Katzenellenbogen, B.S., Belmont, A.S. (1999) The estrogen receptor induces decondensation of large scale chromatin structure. Chromatin Structure and DNA function: Twenty-Five Years of the Nucleosome, College Station, Pennsylvania

CONCLUSIONS

We have identified a novel DEAD box RNA helicase (97 kDa, DP97) from a breast cancer cDNA library that interacts in a hormone-dependent manner with nuclear receptors and represses their transcriptional activity. DP97 has RNA-dependent ATPase activity, and mapping studies have localized the interacting regions to be between the hormone binding/activation function-2 region of estrogen receptors (ER) and several other nuclear receptors to the C-terminal region of DP97. The region of DP97 that is responsible for repression maps to a small central region (amino acids 589-631) that has homology to a repression domain in the corepressor protein NCoR2/SMRTe. This region of DP97 is necessary and sufficient for intrinsic repression activity, repressing the activity of a constitutive SV40 promoter when it is recruited as a Gal4 DNA binding domain fusion protein. The N-terminal helicase region of DP97 is dispensable for its transcriptional repressor activity. Neutralization of endogenous cellular DP97 by expression of antisense DP97 results in significant enhancement of estradiol-ER stimulated transcription, implying that endogenous DP97 normally suppresses transcriptional activity of the receptor. Thus, DP97, a novel DEAD box RNA helicase, functions as a hormone-dependent nuclear receptor interacting protein and transcriptional coregulator.

REFERENCES

1. Rosenfeld, M. G., and Glass, C. K. (2001) *J. Biol. Chem.* 276, 36865-36868
2. Hermanson, O., Glass, C. K., and Rosenfeld, M. G. (2002) *Trends Endocrinol. Metab.* 13, 55-60
3. Alland, L., Muhle, R., Hou, H., Jr., Potes, J., Chin, L., Schreiber-Agus, N., and DePinho, R. A. (1997) *Nature* 387, 49-55
4. Heinzl, T., Lavinsky, R. M., Mullen, T. M., Soderstrom, M., Laherty, C. D., Torchia, J., Yang, W. M., Brard, G., Ngo, S. D., Davie, J. R., Seto, E., Eisenman, R. N., Rose, D. W., Glass, C. K., and Rosenfeld, M. G. (1997) *Nature* 387, 43-48
5. Nagy, L., Kao, H. Y., Love, J. D., Li, C., Banayo, E., Gooch, J. T., Krishna, V., Chatterjee, K., Evans, R. M., and Schwabe, J. W. (1999) *Genes Dev.* 13, 3209-3216
6. Montano, M. M., Ekena, K., Delage-Mourroux, R., Chang, W., Martini, P., and Katzenellenbogen, B. S. (1999) *Proc. Natl. Acad. Sci. U.S.A.* 96, 6947-6952
7. Delage-Mourroux, R., Martini, P. G., Choi, I., Kraichely, D. M., Hoeksema, J., and Katzenellenbogen, B. S. (2000) *J. Biol. Chem.* 275, 35848-35856
8. Norris, J. D., Fan, D., Sherk, A., and McDonnell, D. P. (2002) *Mol. Endocrinol.* 16, 459-468
9. Endoh, H., Maruyama, K., Masuhiro, Y., Kobayashi, Y., Goto, M., Tai, H., Yanagisawa, J., Metzger, D., Hashimoto, S., and Kato, S. (1999) *Mol. Cell. Biol.* 19, 5363-5372
10. Watanabe, M., Yanagisawa, J., Kitagawa, H., Takeyama, K., Ogawa, S., Arai, Y., Suzawa, M., Kobayashi, Y., Yano, T., Yoshikawa, H., Masuhiro, Y., and Kato, S. (2001) *EMBO J.* 20, 1341-1352
11. Kraus, W. L., Montano, M. M., and Katzenellenbogen, B. S. (1993) *Mol. Endocrinol.* 7, 1603-1616
12. Horlein, A. J., Naar, A. M., Heinzl, T., Torchia, J., Gloss, B., Kurokawa, R., Ryan, A., Kamei, Y., Soderstrom, M., Glass, C. K., and et al. (1995) *Nature* 377, 397-404
13. Ordentlich, P., Downes, M., and Evans, R. M. (2001) *Curr. Top. Microbiol. Immunol.* 254, 101-116
14. Norris, J. D., Paige, L. A., Christensen, D. J., Chang, C. Y., Huacani, M. R., Fan, D., Hamilton, P. T., Fowlkes, D. M., and McDonnell, D. P. (1999) *Science* 285, 744-746
15. Paige, L. A., Christensen, D. J., Gron, H., Norris, J. D., Gottlin, E. B., Padilla, K. M., Chang, C., Ballas, L. M., Hamilton, P. T., McDonnell, D. P., and Fowlkes, D. M. (1999) *Proc. Natl. Acad. Sci. U.S.A.* 96, 3999-4004
16. Swirnoff, A. H., Apel, E. D., Svaren, J., Sevetson, B. R., Zimonjic, D. B., Popescu, N. C., and Milbrandt, J. (1998) *Mol. Cell. Biol.* 18, 512-524
17. Moffett, P., Reece, M., and Pelletier, J. (1997) *Mol. Cell. Biol.* 17, 4933-4947

18. Ikeda, K., Halle, J. P., Stelzer, G., Meisterernst, M., and Kawakami, K. (1998) *Mol. Cell. Biol.* 18, 10-18
19. Ordentlich, P., Downes, M., Xie, W., Genin, A., Spinner, N. B., and Evans, R. M. (1999) *Proc. Natl. Acad. Sci. U.S.A.* 96, 2639-2644
20. Park, E. J., Schroen, D. J., Yang, M., Li, H., Li, L., and Chen, J. D. (1999) *Proc. Natl. Acad. Sci. U.S.A.* 96, 3519-3524
21. Wu, X., Li, H., Park, E. J., and Chen, J. D. (2001) *J. Biol. Chem.* 276, 24177-24185
22. Martini, P. G., Delage-Mourroux, R., Kraichely, D. M., and Katzenellenbogen, B. S. (2000) *Mol. Cell. Biol.* 20, 6224-6232