Forkhead Homologue in Rhabdomyosarcoma Functions as a Bifunctional Nuclear Receptor-interacting Protein with Both Coactivator and Corepressor Functions*

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In a search for novel transcriptional intermediary factors for the estrogen receptor (ER), we used the ligand-binding domain and hinge region of ER as bait in a yeast two-hybrid screen of a cDNA library derived from tamoxifen-resistant MCF-7 human breast tumors from an in vivo athymic nude mouse model. Here we report the isolation and characterization of the forkhead homologue in rhabdomyosarcoma (FKHR), a recently described member of the hepatocyte nuclear factor 3/forkhead homeotic gene family, as a nuclear hormone receptor (NR) intermediary protein. FKHR interacts with both steroid and nonsteroid NRs, although the effect of ligand on this interaction varies by receptor type. The interaction of FKHR with ER is enhanced by estrogen, whereas its interaction with thyroid hormone receptor and retinoic acid receptor is ligand-independent. In addition, FKHR differentially regulates the transactivation mediated by different NRs. Transient transfection of FKHR into mammalian cells dramatically represses transcription mediated by the ER, glucocorticoid receptor, and progesterone receptor. In contrast, FKHR stimulates rather than represses retinoic acid receptor- and thyroid hormone receptor-mediated transactivation. Most intriguingly, overexpression of FKHR dramatically inhibits the proliferation of ER-dependent MCF-7 breast cancer cells. Therefore, FKHR represents a bifunctional NR intermediary protein that can act as either a coactivator or corepressor, depending on the receptor type.

The nuclear hormone receptors (NRs) play an important role in a variety of physiological functions such as cell growth, development, differentiation, and homeostasis (1, 2). The NR superfamily is often divided into steroid and nonsteroid receptor subfamilies, which show different features in DNA binding and dimerization and a different effect on the basal transcriptional activity of the target (2, 3). The estrogen receptor (ER), a member of the steroid receptor family, is critical for the development and progression of breast cancer, and it is a useful diagnostic and therapeutic target (4–8). Like other NRs, ER contains two distinct transactivation function domains (AFs): the ligand-independent (AF-1) and ligand-dependent (AF-2) activation domains (4, 8). A large number of ER-interacting proteins have been identified that modify ER activity. Several coactivators have been characterized recently including SRC-1, GIP1/TIF2, RIP140, Tripl, CBP/P300, SPA/L7, and AIB1/ACTR/RAC3/p/CIP (9–12). In addition, several corepressors have also been identified including N-CoR and SMRT (13). The relative expression and/or activity of coactivators and corepressors in a particular environment may modulate the agonistic/antagonistic activities of the partial ER antagonist, tamoxifen (Tam) (14–17). Most recently, two bifunctional NR intermediary proteins, TIF1 and NSD1, have been described that can regulate transcription either positively or negatively, depending on both the promoter context and the cell type (18, 19).

To identify novel transcriptional intermediary factors for ER that might contribute to estrogen-dependent cell proliferation, we used the AF-2 and hinge region of ER as bait in a yeast two-hybrid screen of a cDNA library derived from Tam-resistant breast tumor tissues from an MCF-7 athymic nude mouse model (20, 21). Here we report the isolation and characterization of FKHR (forkhead homologue in rhabdomyosarcoma), a previously described member of the hepatocyte nuclear factor 3/forkhead homeotic gene family (HNF3/FKH), as a novel bifunctional NR-interacting protein that displays corepressor activity on steroid receptors and coactivator activity on nonsteroid receptors (22, 23). Consistent with these observations, overexpression of FKHR in MCF-7 cells, an estrogen-dependent human breast cancer cell line, dramatically inhibits their proliferation. FKHR has been shown recently to be an important player in several signal transduction pathways regulated by the AKT protein kinase (24–32).

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§ The abbreviations used are: NR, nuclear hormone receptor; ER, estrogen receptor; AF, transactivation function domain; Tam, tamoxifen; FKR, forkhead homologue; FKH, FKH in rhabdomyosarcoma; HNF, hepatocyte nuclear factor; E2, estrogen; DBD, DNA-binding domain; β-gal, β-galactosidase; TR, thyroid hormone receptor; RAR, retinoic acid receptor; GST, glutathione S-transferase; ERE, estrogen response element; AD, transactivation domain; PR, progesterone receptor; GR, glucocorticoid receptor; h, human.
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EXPERIMENTAL PROCEDURES

Yeast Two-Hybrid Screen—The plasmid pAS2–1-ER (DEF), coding for a fusion protein containing the GAL4 DBD and the AF-2 and hinge regions of hERα, was constructed from the vector pAS2–1 (CLONTECH) and used as bait. The cDNA library was prepared using Tam-resistant breast tumor tissues from an MCF-7 athymic nude mouse model constructed in pGAD10 by CLONTECH (20, 21). The screen was performed in the presence of estrogen (E2). The positive clones were identified by inserting the hinge and AF-2 regions of hERα (amino acids 402–629) in the absence and presence of ligand.

GST Pull-down Assay—The constructs GST-TR and GST-RAR were provided kindly by Michael G. Rosenfeld (33). GST-ER (DEF) was generated by inserting the hinge and AF-2 regions of hERα into the BamHI/EcoRI sites of the pGEX-2kt vector (Amersham Pharmacia Biotech). The plasmid pcDNA3.1/AIB1 was provided kindly by Paul S. Melzer (9), and pcDNA3/FKHR was constructed by subcloning the full-length FKHR into pcDNA3 at the Klenow-filled XhoI sites. The GST pull-down assay was performed as described previously (34).

Transfections, Luciferase, and Growth Inhibition Assays—The reporter genes 2×ERE-Tk-Luc (35), PRE/GRE-TATA-Luc (36), pC3-Tk-Luc (37), TRE-Tk-Luc, and RARE-Tk-Luc (38) were described previously. Monkey kidney-derived COS-1 (ATCC) and human hepatocyte carcinoma HepG2 cells (ATCC) were used as test cells. For transfections, the DNA was cotransfected with a plasmid containing the luciferase gene. The total amount of DNA was kept constant in all transfections by the addition of empty vector DNA as carrier. Twelve hours later, the cells were treated with different ligands for 24 h and harvested for β-galactosidase assay (34).

RESULTS

Using ER as bait, the yeast two-hybrid screen of the cDNA library derived from Tam-resistant breast tumors was utilized to identify novel ER-interacting proteins. A 0.8-kilobase open reading frame DNA fragment obtained from this screen showed 100% identity with the C-terminal one-third of FKHR (amino acids 402–629), a member of the HNF3/FKH transcription factor family (22, 23). The sequence of FKHR protein exhibits certain interesting features (22). As shown in Fig. 1A, the protein contains at its N terminus a forkhead domain that is highly homologous among the HNF3/FKH family and is necessary for DNA binding, and at its C terminus a proline-rich and acidic serine/threonine-rich transactivation domain (AD). Other motifs in FKHR include an SH3 binding site and an alanine-rich region that has been associated with transcriptional repression in other proteins. FKHR and two other closely related members, FKHR1 and AF (41, 42), are relatively divergent from other HNF3/FKH family members both within and outside the forkhead region. As shown in Fig. 1B, their forkhead domain lacks the N-terminal KPPY motif common to most HNF3/FKH family genes and contains a novel 5-amino acid insert (DKGDS) instead. Interestingly, at their C termini an NR-interacting domain or LXXLL motif is highly conserved among the HNF3/FKH family and is necessary for DNA binding and, at its C terminus a proline-rich and acidic serine/threonine-rich transactivation domain (AD).

The interaction between FKHR and ER was defined further using the yeast two-hybrid and GST pull-down assays. In the yeast two-hybrid assay (Fig. 2A), in the presence of E2, co-expression of two chimeric proteins (GAL4 AD-FKHR (amino acids 402–629) and GAL4 DBD-hERα) resulted in good cell growth on selection medium, and the colonies turned blue within 1 h in the β-gal filter lift assay (Fig. 2A, lane 1), whereas a minimal growth was seen either in the absence of ligand (Fig. 2A, lane 2), or the presence of anti-estrogen (Fig. 2A, lane 3). Surviving colonies in the absence of ligand or with Tam did not turn blue within 16 h in a β-gal filter lift assay. In the GST pull-down assay, FKHR only weakly interacted with ER both in the absence of hormone (Fig. 2B, lane 2) and the presence of
FKHR interacts with ER in an E2-dependent fashion and represses ER-mediated transactivation. A, the yeast two-hybrid assay. The yeast strain Y190 was cotransfected with two chimeric promoters containing GAL4 DBD-ER (DEF) and GAL4 AD-FKHR (amino acids 402–629), plated on the selection medium containing either no hormone (No ligand), E2 (10⁻⁷ M), or Tam (10⁻⁸ M) and followed by a β-gal assay. B, GST pull-down assay. Aliquots (10 μl) of in vitro translated 35S-labeled FKHR or AIB1 were incubated with glutathione-Sepharose beads loaded with bacterially expressed GST alone or GST-hERα (DEF) in the presence of vehicle alone (−H) or vehicle plus E2 (10⁻⁷ M) or Tam (10⁻⁸ M). The retained radiolabeled FKHR or AIB1 was eluted from the beads and resolved by SDS-polyacrylamide gel electrophoresis followed by autoradiography. The input lanes show the amounts of FKHR and AIB1, respectively, used for each reaction. The large number of bands in the AIB1 in vitro translated sample is caused by the large numbers of open reading frames in the AIB1 cDNA sequence. C and D, FKHR represses ER-mediated transactivation on both artificial ERE promoter (C) and natural ERE promoter (D). HepG2 cells were cotransfected with 1 μg of either 2xERE-Tk-Luc (C) or p3C3-Tk-Luc (D), along with 100 ng of pCMX-βGal and 25 ng of hERα, otherwise as indicated, and increasing amounts of FKHR as indicated. Transfected cells were then treated with vehicle alone (no ligand) or vehicle plus E2 (10⁻⁷ M) or Tam (10⁻⁸ M) 12 h after transfection. The cells were harvested 24 h later for β-gal and luciferase activities as described under “Experimental Procedures.”

FKHR antagonizes the coactivator activity of AIB1. HepG₂ cells were cotransfected with the 2xERE-Tk-Luc reporter and hERα as described in the Fig. 2C legend along with either a constant amount of AIB1 (250 ng) plus increasing amounts of FKHR as indicated (B) or a constant amount of FKHR (250 ng) plus increasing amounts of AIB1 as indicated (A). Transfected cells were treated with different ligands and harvested for β-gal and luciferase activities.
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**Fig. 4. Effect of FKHR on other NR-mediated transactivation.** A and B, FKHR represses PR- and GR-mediated transactivation. COS-1 cells were cotransfected with PRE/GRE-TATA-Luc reporter (1 μg), either PR (A) or GR (B) (25 ng), and increasing amounts of FKHR as indicated. Transfected cells were then treated with vehicle alone (no ligand) or vehicle plus progesterone (10−7 M) or cortisol (10−7 M) for PR and GR, respectively, and harvested for β-gal and luciferase activities. C, FKHR interacts with RAR and TR in vitro in a ligand-independent manner. Aliquots (10 μl) of in vitro translated 35S-labeled FKHR were incubated with glutathione-Sepharose beads loaded with bacterially expressed GST alone, GST-hRARα, GST-hTRβ, or GST-hERα (DEF) in the presence of vehicle alone (−H) or vehicle plus all-trans-retinoic acid (−RA) (10−6 μM), thyroid hormone (T3) (10−6 M), or E2 (10−7 M) for RAR, TR, and ER, respectively. The retained radiolabeled FKHR was eluted from the beads and analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography. The input lane shows the amount of FKHR used for each reaction. D and E, FKHR enhances TR- and RAR-mediated transactivation. COS-1 cells were cotransfected with either the RARE-Tk-Luc (D) or TRE-Tk-Luc (E) reporter (1 μg) along with either hRAR (D) or hTR (E) (25 ng) and increasing amounts of FKHR as indicated. Transfected cells were then treated with either vehicle alone (no ligand) or vehicle plus at-RA (10−7 M) or thyroid hormone (10−7 M) for RAR and TR, respectively, and harvested for β-gal and luciferase activities.

and RAR both in the absence and presence of their cognate ligands, indicating the ligand independence of the interaction. In contrast to steroid receptors, cotransfection of FKHR resulted in 2–3-fold stimulation, rather than repression, of RAR- and TR-mediated transactivation both in the absence and presence of their cognate ligands (Fig. 4, D and E).

To determine the physiologic relevance of the interaction of FKHR with ER, we tested the effect of FKHR on the growth of an estrogen-dependent human breast cancer cell line, MCF-7. Using a single colony reduction assay (Fig. 5A), we observed that empty vector-transfected cells had a significantly different distribution in the cell number per colony or cluster than FKHR-transfected cells (p < 0.0001). Clusters containing single cells or doublets were much more common in cells transfected with FKHR, whereas clusters containing more than 10 cells were common in control dishes (Fig. 5A). This difference in colony size is more apparent when comparing the median cell number per colony for pcDNA-transfected cells (seven cells/colony) with the FKHR-transfected cells (two cells/colony). In the colony reduction assay (Fig. 5B), in comparison with vector alone, overexpression of FKHR greatly reduced colony formation (50% reduction). Similarly, overexpression of p21, a known negative regulator for cell growth, resulted in a 70% reduction of colony formation (40).

Finally, Western blot analysis revealed the differential expression pattern of FKHR in different human tissues (Fig. 6). FKHR is expressed in most of the tissues tested with higher levels in ovaries and testes and intermediate levels in brain, heart, kidneys, liver, and skeletal muscle. There is very low FKHR expression in lungs and no detectable levels in placenta and spleen. Interestingly, there is a doublet band in muscle (heart and skeletal muscle) but not in other tissues tested, although we do not know currently the nature of this doublet. The tissue differences in FKHR protein levels may play a role in tissue specificity of NR-mediated responses to various hormones or antihormones.

**DISCUSSION**

We have shown that FKHR interacts with several members of the NR superfamily including ER, RAR, and TR, although the effect of ligand on this interaction varies by receptor type. Its interaction with ER is enhanced by estrogen, whereas its interaction with TR and RAR is ligand-independent. The characteristic features of the interaction of FKHR with ER compared with TR and RAR are similar to those described for the previously described NR intermediary protein NSD1 (19). The two distinct NR-interacting domains identified in NSD1 could be also present in FKHR. Alternatively, the different binding features of FKHR could also reside within the structure of the NRs themselves. As mentioned, steroid and nonsteroid receptors show distinct features in their DNA binding and dimerization and in their effects on the basal transcriptional activity of target genes. First, steroid receptors form homodimers in their active state, whereas nonsteroid receptors heterodimerize with RXR upon the addition of ligand. Conceivably, FKHR could interact differently with homodimer versus heterodimer partners. Second, unliganded steroid receptors are complexed with chaperone proteins and remain in an inactive state, whereas unliganded nonsteroid receptors are bound to DNA and are complexed with corepressors, resulting in the repression of basal transcription of target genes. FKHR could bind with different affinities and/or mechanisms to DNA-bound versus free NRs. Because there is no DNA or promoter present in our in vitro assay, the interaction seen in vitro may not necessarily reflect the in vivo situation.

In addition to its different binding properties with steroid and nonsteroid NRs, FKHR differentially regulates the transactivation mediated by different NRs. Co-expression of FKHR
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**FIG. 5.** FKHR inhibits the growth of MCF-7 breast cancer cells. A, single cell proliferation assay. MCF-7 cells maintained in improved Eagle’s medium plus 10% fetal bovine serum were cotransfected with 5 μg of pcDNA3 (n = 100) or pcDNA3/FKHR (n = 102) plus 0.5 μg of pcMV-β-gal. After 3–4 doublings, the cells were fixed and stained for β-gal in situ. Colonies containing blue cells were scored for the number of blue cells per colony and subjected to biostatistical analysis. B, colony formation assay. MCF-7 cells maintained in improved Eagle’s medium plus 10% fetal bovine serum were transfected with 1 μg of pcDNA3, pcDNA3/FKHR, pcDNA/p21, or mock-transfected and subjected to G418 selection 48 h after transfection. The surviving colonies were stained and counted after 14 days of selection. The number of colonies on each plate was counted and graphed as averages ± S.E. from triplicates.

**FIG. 6.** The expression pattern of FKHR in different human tissues. Aliquots (50 μg) of total cellular extract from different human tissues (CLONTECH) as indicated were analyzed on SDS-polyacrylamide gel electrophoresis and detected by Western blot using an anti-FKHR antibody. The extreme left lane (IVT) is in vitro translated FKHR as a positive control.

in mammalian cells (HepG2 and COS-1) dramatically represses transactivation mediated by ER, PR, and GR. In contrast, FKHR stimulates rather than represses transactivation mediated by RAR and TR. The differential effects of FKHR on the transactivation of different NRs might be explained by the presence of both coactivation and corepression domains in the FKHR molecule, as described for NSD1 (19). Sequence analysis of the FKHR gene has shown a transactivation domain at its C terminus as well as a repression region at its N terminus (22). Binding to different receptor types could result in conformational changes, exposing either the transactivation or repression domains of FKHR. In addition, homodimers and heterodimers of NRs might recruit different sets of transcriptional components that might then be differentially regulated by FKHR. Finally, because its regulatory function depends on the nature of the receptor, it is possible that FKHR activates transcription functions of TR and RAR by either sequestering TR- and RAR-specific corepressors or blocking the histone deacetylase activity associated with these corepressors. Further experiments are required to address these possibilities.

The HNF3/FKHR transcription factor family has been implicated in diverse biological functions varying from embryonic development to adult tissue-specific gene expression (46–48). In addition, variants of several genes of this family (especially the FKHR subfamily) have shown oncogenic potential (46, 49, 50). FKHR was originally cloned from a rhabdomyosarcoma because of its aberrant fusion with another transcription factor, PAX3, resulting from a unique chromosomal translocation t(2;13) (22, 23). The resulting fusion protein PAX3-FKHR is a hallmark of these tumors (51–54) and is thought to play a crucial role in muscle cell transformation and evolution to rhabdomyosarcoma. Little is known about the underlying mechanism of this transformation process (55–57). Characterization of FKHR as an NR transcriptional intermediary protein should provide clues about the biological function of FKHR and possibly the oncogenic mechanism of PAX3-FKHR. It is possible that the chromosomal translocation in rhabdomyosarcoma results in not only the activation of PAX3 but also disruption of functional FKHR, which may be essential for RAR-dependent muscle cell differentiation. This loss of a differentiation function of FKHR, rather than a gain of function by the PAX3-FKHR fusion, could conceivably contribute to the development of rhabdomyosarcoma. In addition, FKHR has been shown recently to play a role in several signal transduction pathways (24–32). Further studies on the mechanism of the regulatory function of FKHR and its biological relevance, especially its effect on hormone-dependent cell proliferation and differentiation, may help reveal the role of FKHR in the development and progression of cancers such as breast cancer, leukemia, and rhabdomyosarcoma.

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REFERENCES

1. Beato, M., Herrlich, P., and Schutz, G. (1995) Cell 83, 851–857
2. Mangelsdorf, D. J., Thummel, C., Beato, M., Herrlich, P., Schutz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P., and Evans, R. M. (1995) Cell 83, 835–839
3. Tsai, M., and O’Malley, B. W. (1994) Annu. Rev. Biochem. 63, 451–486
4. Hansen, R. K., and Fuqua, S. A. W. (1998) in Breast Cancer: Molecular Genetics, Pathogenesis, and Therapeutics (Bowcock, A. M., ed) pp. 1–30, Humana Press, Inc., Totowa, NJ
5. Macgregor, J. I., and Jordan, V. C. (1998) Pharmacol. Rev. 50, 151–196
6. Osborne, C. K., Ellerd, R. M., and Fuqua, S. A. W. (1996) Sci. Am. Sci. Med. 3, (suppl.) 31–41
7. Osborne, C. K. (1995) N. Engl. J. Med. 339, 1609–1618
8. Tonetti, D. A., and Jordan, V. C. (1995) Anticancer Drugs 6, 498–507
9. Anzick, S. L., Kononen, J., Walker, R. L., Azorsa, D. O., Taneer, M. M., Guan, X., Sauter, G., Kallioniemi, O., Trent, J. M., and Meltzer, P. S. (1997) Science 277, 965–968
10. Chen, H., Lin, R. J., Schitz, R. L., Chakravarti, D., Nash, A., Nagy, L., Privalsky, M. L., Nakatani, Y., and Evans, R. M. (1997) Cell 90, 569–580
11. Li, H., Gomes, P. J., and Chen, J. D. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 8479–8484
12. Torchio, J., Rose, D. W., Inostroza, J. Y., Kamel, S. W., Glass, C. K., and...
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