Retinoblastoma-binding protein 2 (Rbp2) was originally identified as a retinoblastoma protein (RB) pocket domain-binding protein. Although Rbp2 has been shown to interact with RB, p107, TATA-binding protein, and T-cell oncogene rhombotin-2, the physiological function of Rbp2 remains unclear. Here we demonstrate that Rbp2 not only binds to nuclear receptors (NRs) but also enhances the transcription mediated by them. Rbp2 interacts with the DNA-binding domains of NRs and potentiates NR-mediated transcription in an AF-2-dependent manner. Both the N-terminal and C-terminal domains of Rbp2 are critical for the transactivation activity of Rbp2 on NRs. The C terminus is the NR-interacting region. In addition, RB functions in maximizing the effect of Rbp2 on the transcription by NRs. These results suggest that Rbp2 is a coregulator of NRs and define a potential role for Rbp2 in NR-mediated transcription.

The retinoblastoma protein (RB) plays a pivotal role in the control of cell proliferation, development, and differentiation (1). The loss of RB function leads to a variety of cancers and defects in the development of certain cell types. RB has been shown to have the ability to block cell cycle progression from G1- to S-phase (2, 3). This inhibition of cell proliferation is to some extent through the binding of RB to the E2F family of transcription factors, which regulate numerous genes involved in S-phase entry, such as N-myc (4–6). Phosphorylation of RB by cyclin and cyclin-dependent kinases leads to dissociation of RB from E2F, allowing progression into the S-phase (7).

The region of RB required for growth suppression has been studied extensively. The region consists of a domain called the pocket domain, a domain targeted by viral transforming proteins such as adenovirus E1A protein, simian virus 40 (SV40) T antigen, and human papillomavirus E7 protein (reviewed in Ref. 8). Mutations in this region are often tumorigenic (9). A number of cellular proteins were identified based on their abilities to associate with the RB pocket domain. These RB-binding proteins, like viral E1A, T antigen, and E7, share a stretch of amino acid sequence LXXCXE within their RB-binding domains. Some of the RB-binding proteins have been shown to cooperate with RB in inducing cell cycle arrest (10), usually through the inactivation of E2F-responsive transcription (11). Retinoblastoma-binding protein 1 and 2 (Rbp1 and Rbp2) were originally isolated based on their ability to associate with the RB pocket domain (12–16).

Nuclear receptors (NRs) represent a superfamily of structurally and functionally related ligand-inducible transcription factors that are involved in diverse biological events including development, differentiation, and homeostasis (reviewed in Refs. 17–20). Members of the family include the receptors for thyroid hormones, steroids, retinoids, and vitamin D that activate transcription in response to their ligands by binding to cognate elements in the promoters of their target genes. All NRs have a modular structure with regions termed A–F. The N-terminal A/B regions contain the activity activation function 1 (AF1) that activates transcription constitutively. The central C region is the DNA-binding domain (DBD) that associates with specific DNA elements in target genes. Region D is a variable hinge region. The C-terminal region E contains a ligand-binding domain (LBD), a dimerization surface, and the ligand-dependent transcriptional activation function 2 (AF2). Region F is a variable region at the extreme end of the C terminus (reviewed in Refs. 17–20).

Ligand-dependent gene expression mediated by NRs involves the displacement of corepressors and subsequent recruitment of transcriptional coactivators to the LBD (21–24). These coactivators modify local chromatin structure by acetylating histones, and they directly assemble and/or stabilize the transcription preinitiation complex. A few families of coactivators have been identified through their abilities to bind directly to the LBD and enhance AF2 function. These are the p160 coactivators, which include SRC1/NcoR1 (25), TIF2/GRIP1/NcoR2 (26–28), pCIP/RAC3/ACTR/AIB1 (29–33), CREB-binding protein (CBP), p300 (34), and p/CAF (35). They acetylate histones and cause changes in nucleosome structure that are associated with gene activation (31, 36–37). In addition, NRs and CBP/p300 have been shown to interact directly or indirectly with TFIIB, TATA-binding protein (TBP), and components of the basal transcriptional machinery (26, 38). A short sequence motif LXXLL (where L is leucine and X is any amino acid), present in some coactivators like p140, SRC1, and CBP/p300, is necessary and sufficient for mediating the interaction of the coactivators with NRs (39).

Although Rbp2 was originally isolated as an RB pocket domain-binding protein and has been shown to interact also with TBP, p107, and T-cell oncogene rhombotin-2 (15–16), its role in transcription remains obscure. In this study, we show that...
Rbp2 not only interacts with NRs but also enhances their transcription activity. The presence of Rbp2 is required for the transcription mediated by NRs and RB is important for maximal activation. Both the C terminus and N terminus of Rbp2 play an important role in its activity.

**EXPERIMENTAL PROCEDURES**

**Materials**—pGem-4Z-Rbp2 was a gift from Deborah Defeo-Jones. Estrogen receptor (ER), retinoic acid receptor (RAR), rexinoid X receptor, and vitamin D receptor (VDR) were from Shen Cai Lin, and VDRE-LUC was from Victor Yu of the Institute of Molecular and Cell Biology, Singapore. GST-YKT6 was a gift from the Laboratory of Cell Biology, Roche Molecular and Cell Biology. RSV-CBP plasmid was a gift from Richard Goodman. Constructs of Rb and E7 were from Paramjeet Singh. Mouse ER, RAR, RXR, and MAPK6 were from Promega. Mouse anti-HA and anti-VSVG were purchased from Roche Molecular Biochemicals.

**Cell Culture, Transfection, and Luciferase Assay**—HeLa cells and C3A cells were grown in Eagle's minimal essential medium supplemented with 10% fetal bovine serum. COS-7 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. LipofectAMINE (Life Technologies, Inc.) was used for transfections. Transfections were done in 10-cm plates with 3 × 10⁶ cells using 10% charcoal-stripped serum, and the cells were induced with ligands according to the manufacturer's instructions. After transfection, the cells were transfected by using LipofectAMINE (Life Technologies, Inc.) in COS-7 cells in 10-cm plates. Cells were induced with ligands for 30 min at 4 °C, and the supernatant was mixed with 0.5 ml of glutathione-Sepharose 4B (Amersham Pharmacia Biotech) for 2 h at 4 °C. The resin was subsequently washed four times with 1 ml of phosphate-buffered saline. Bound proteins were either stored at 4 °C or eluted with 5 volumes of 10 mM reduced glutathione in 50 mM Tris (pH 8). The pure GST fusion proteins were stored at 4 °C or −20 °C.

**Protein-Protein Interaction in Vitro**—Protein-protein affinity chromatography with purified GST-RAR (amino acids 251–595), GST-RAR (amino acids 465–792), GST-RB (amino acids 373–792), and GST-YKT6 or GST alone bound to glutathione-Sepharose (5 μg/25 μl of resin), and 5 μl of [35S]methionine labeled in vitro translated Rbp2 protein was done in the presence or absence of appropriate ligands in binding buffer containing 50 mM Tris-Cl (pH 7.6), 150 mM NaCl, 0.1% Nonidet P-40, 0.5 mM EDTA, 1 mM diethylthiourea, and protease inhibitors mixture (Roche Molecular Biochemicals) in a total volume of 500 μl at 4 °C for 2 h. The resin was subsequently washed four times with 1 ml of phosphate-buffered saline. Bound proteins were released in SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer and then analyzed by electrophoresis under denaturing conditions and autoradiography.

**Immunoprecipitation**—An equal amount of each cDNA encoding HA-ER and VSVG-Rbp2 was introduced with LipofectAMINE (Life Technologies, Inc.) in COS-7 cells in 10-cm plates. Cells were induced with 17β-estradiol for 18 h before harvesting at 48 h posttransfection and lysed with 1 ml of lysis buffer containing 150 mM NaCl, 50 mM Tris-Cl (pH 7.6), 0.2 mM EDTA, 0.1% Nonidet P-40, 1 mM diethylthiourea, and protease inhibitors mixture (Roche Molecular Biochemicals). Cell extracts were centrifuged at 14,000 for 30 min at 4 °C, and the supernatant was collected and preincubated with protein G-Sepharose (Amersham Pharmacia Biotech) for 4 h. Precleared extracts were incubated with 30 μg of HA monoclonal antibody and 60 μl of a 50% slurry of protein G-Sepharose overnight. Immunoprecipitated HA-tagged protein complexes were washed five times with lysis buffer and eluted by boiling in 2× sample buffer. Eluted proteins were subjected to SDS-PAGE with a 7.5% polyacrylamide gel, and proteins were then transferred to nitrocellulose membrane (Amersham Pharmacia Biotech) and were probed with either anti-VSV or anti-ER (Santa Cruz Biotechnology) or withhorse-radish peroxidase conjugated goat anti-mouse or anti-rabbit secondary antibody (Pierce). Binding was detected by superpossignal chemiluminescent substrate (Pierce).

**Binding of Rbp2 to the ER-DNA Complex**—The binding assay was performed as described (35). Briefly, 30 pmol of 45-base pair double-stranded DNA fragment containing two copies of ERE labeled with biotin at the 5′ end of one strand was conjugated to streptavidin beads.
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RESULTS

Rbp2 Interacts with NR—Visual examination of Rbp2 amino acid sequence led to the identification of two LXXLL motifs (residues 725–729 and 945–949). To examine the potential association of Rbp2 with NRs, ER (amino acids 251–595) was fused to GST to generate GST-ER and incubated with 35S-labeled Rbp2 in the presence of absence of 17β-estradiol. 35S-Labeled Rbp2 was retained on GST-ER beads in a ligand-independent manner (Fig. 1A, lanes 4 and 5) but not on GST or GST-YKT6 beads (40), a protein involved in vesicle transport (Fig. 1A, lanes 3 and 12). 35S-Labeled Rbp2 was retained by the GST-RB pocket (amino acids 373–792) (Fig. 1A, lane 13), serving as a positive control. GST fusion proteins containing RAR (amino acids 143–462), GR (amino acids 465–795), and VDR (amino acids 66–427) were also produced and tested for interaction with 35S-labeled Rbp2. All these have the ability to retain Rbp2 in the presence and absence of ligands, although with different efficiencies (Fig. 1A, lanes 6–11). These results suggest that Rbp2 may directly interact with NRs in vitro in a ligand-independent manner.

To determine whether Rbp2 and NRs can physically interact in mammalian cells, COS-7 cells were transfected with HA-tagged ER and VSVG-tagged Rbp2 expression vectors. Cell lysates were first immunoprecipitated with anti-HA antibody, and the bound proteins were subsequently analyzed by immunoblotting with anti-VSVG antibody to detect Rbp2 that was coimmunoprecipitated. Rbp2 protein was clearly detected in the immunoprecipitates pulled down by anti-HA antibody, which recognized HA-tagged ER, from cells transfected with both ER and Rbp2, but not in the nontransfected cells (Fig. 1B, upper panel). The interaction between Rbp2 and ER is again ligand-independent. The membrane blot was stripped and immunoblotted again with anti-ER antibody to show the presence of ER protein in the anti-HA immunoprecipitates pulled down by anti-HA antibody (Fig. 1B, bottom panel). These results indicate that Rbp2 indeed interacts with ER in the cells.

The LXXLL Motifs of Rbp2 Are Not Required for Its Interaction with NRs—Rbp2 contains two LXXLL motifs in the central region (residues 725–729 as NR box 1 and residues 945–949 as NR box 2). LXXLL motifs are found in a large variety of LBD/NR box 2. Since the LXXLL motifs were tested. The LXXLL motifs were altered to LXXAA, a change influencing the binding of coactivators to receptors. Three LXXAA mutants of Rbp2 were constructed as shown in Fig. 2A. Rbp2M1 has an LXXAA mutation in the first NR box (amino acids 725–729). Rbp2M2 contains a LXXAA mutation in the second NR box (amino acids 945–949), whereas Rbp2M12 has both the NR boxes mutated. These mutants were compared with wild type Rbp2 for interaction with ER in vitro GST pull-down experiments. GST-ER interacts with Rbp2 and each of the Rbp2 mutants with comparable efficiencies (Fig. 2B). These results indicate that the LXXLL motifs of Rbp2 are not required for its interaction with NRs.

Rbp2 Interacts with the DBDs of NRs—Since the LXXLL motifs of Rbp2 are not required for binding to NRs (Fig. 2B), it is possible that Rbp2 binds to regions of NR other than LBD. To delineate the Rbp2-binding domain of ER, different regions of ER were expressed as fusion proteins with GST to produce GST-ER(A/B), GST-ER(DBD), and GST-ER(LBD), as shown in Fig. 3A. These various fusion proteins were used to examine their ability to retain Rbp2 from HeLa cell lysates. Proteins retained by these beads were analyzed by immunoblotting. As shown, Rbp2 was bound by GST-ER(DBD) and GST-ER (positive control) affinity beads (Fig. 3B, lane 4 and 7) but not by GST, GST-ER(A/B), or GST-ER(LBD) (Fig. 3B, lanes 2, 3, 5, and 6). Similarly, the DNA-binding domain of RAR binds Rbp2 (data not shown). These results indicate that Rbp2 interacts with the DBDs of NRs.

Since Rbp2 interacts with the DBDs of NRs, it is important to examine whether Rbp2 could form a ternary complex with NR bound to DNA. To test this possibility, a binding assay using ER that had been bound to the estrogen-responsive element (ERE) conjugated to agarose beads was performed. Fig. 3C shows that in vitro translated 35S-labeled ER bound to ERE beads in a dose-dependent manner (lanes 5–10). Similar to the reported ligand-independent interaction of RAR and retinoid X
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**FIG. 2.** The LXXL motifs of Rbp2 are not required for interaction with NRs. A, a schematic diagram showing the NR boxes of Rbp2 and their various LXXLL→LXXAA mutants. The two LXXLL motifs of Rbp2 are boxed, and the various LXXLL→LXXAA mutants are denoted. B, interaction of Rbp2 NR box mutants with ER in vitro. GST pull-down experiments were carried out as above by incubating 35S-labeled, in vitro translated Rbp2 or the mutants with immobilized GST or GST-ER (amino acids 251–595) either in the presence (+) of absence (−) of 17β-estradiol. The bound fractions were analyzed as above. 5% of the in vitro translated products used in binding is loaded in lane 1.

To investigate the requirement of Rbp2 for NR-mediated transcription, we used an antisense approach to determine whether depletion of Rbp2 would prevent NRs from activating NR-dependent transcription. The antisense construct of Rbp2(Rbp2AS) was generated and shown to reduce effectively the amount of Rbp2 protein in cells by up to 90% (Fig. 5C, upper panel, lane 3). The antisense construct of Rbp2 significantly inhibited the transcriptional functions of ER and RAR, either in the presence or absence of ligands (Fig. 5, A and B). The inhibition of transcription by Rbp2AS on NRs does not seem to be due to a general effect on transcription because transcriptions mediated by SV40 early promoter was not significantly inhibited by Rbp2AS. SV40-LUC activity are also increased by Rbp2 at different degrees. These results indicate that Rbp2 is an NR coactivator.

**A**

**B**

**C**

**D**

**E**

**F**

**G**

**H**

**I**

**J**

**K**

**L**

**M**

**N**

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**II**

**JJ**

**KK**

**LL**

**MM**

**NN**

**OO**

**PP**

**QQ**

**RR**

**SS**

**TT**

**UU**

**VV**

**WW**

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results demonstrate that Rbp2 is required for NR activation.

RB Is Necessary for Maximal Potentiation of ER-mediated Transcription by Rbp2—Since Rbp2 was originally identified as an RB pocket-binding protein, it is possible that the function of Rbp2 may depend on the presence of RB. To determine whether RB plays a role in Rbp2-enhanced NR transactivation, an RB-negative cell line, C33A, was chosen to test the requirement of RB on Rbp2 function. Fig. 6 showed that without the presence of RB, Rbp2 slightly potentiated the transcription activity of ER. Similarly, RB alone also potentiated ER-mediated transcription slightly. With the presence of RB and Rbp2 together, the effect on ER-mediated transcription was dra-

Fig. 3. Rbp2 interacts with DBDs of NRs. A, schematic diagram of full-length ER and various regions of ER expressed as GST fusion proteins. B, Rbp2 specifically interacts with the DBD of ER. HeLa cell extracts were incubated with immobilized GST (lane 2), GST-ER(A/B) (lane 3), GST-DBD (lane 4), or GST-LBD (lane 5 and 6) either in the presence (+) or absence (−) of 17β-estradiol. After washing, the bound proteins were analyzed by SDS-PAGE and immunoblotting with rabbit anti-Rbp2 antibody. GST-RB (amino acids 373–792) was used as positive control (lane 7). 10% of the HeLa cell extracts used in the pull-down experiments is loaded in lane 1. C, Rbp2 interacts with the ER-ERE complex. The indicated amount of in vitro translated, 35S-labeled ER or RAR were bound to ERE-conjugated agarose beads in the presence (+) or absence (−) of ligands 17β-estradiol or all-trans-retinoid acid (ATRA). The beads were washed and subsequently incubated with the indicated amount of in vitro translated, 35S-labeled Rbp2 in the presence and absence of ligands. The bound proteins were detected by autoradiography. The in vitro translated, 35S-labeled products of RAR, Rbp2, and ER used for binding were loaded in lanes 1–3. Extracts added to the ERE-conjugated beads without receptors are shown in lane 4.
matically enhanced. E7 protein, a viral protein shown to interact with RB and inhibit its function, was used to demonstrate further the requirement of RB in Rbp2 function. The synergistic effects of RB and Rbp2 on ER function, as well as the ones by RB or Rbp2 alone, were significantly reduced in the presence of E7 protein (Fig. 6). These data indicate that RB is required for the maximal potentiation of NR-mediated transcription by Rbp2.

N-terminal and C-terminal Domains of Rbp2 Are Critical for Potentiating NR-mediated Transcription—There is an ARID and three PHD motifs present in Rbp2, and these two types of motifs have been functionally defined to be involved in protein-protein interactions (41, 42). To characterize the importance of these motifs in the NR-mediated transcription by Rbp2, different deletion constructs of Rbp2, as shown in Fig. 7A, were generated to test for their abilities to activate ER-mediated transcription. All these constructs expressed the respective mutants efficiently in transfected cells (Fig. 7B). HeLa cells were transfected with ER, ERE-LUC, and the indicated deletion constructs individually in the presence or absence of ligand, and luciferase activities were measured. Fig. 7C showed that full-length Rbp2 induced ER-mediated transcription as shown previously (Fig. 5A). Surprisingly, once the N-terminal domain (amino acids 1–324) was deleted, the transactivation activity of Rbp2 on ER-mediated transcription was reduced to half that of full-length Rbp2 (Fig. 7C, compare lanes 3–6 with lane 2). Similarly, once the C-terminal part of Rbp2 was deleted (amino acids 1320–1722), the transactivation activity of Rbp2 was lost (Fig. 7C, compare lanes 7–10 with lane 2). This is consistent with the above data that the C-terminal domain of Rbp2 is the NR interaction domain. The requirement of the N-terminal domain of Rbp2 suggests that interaction with NR alone is not enough for the observed effect of Rbp2 on NR-mediated transcription. When Rbp2 was expressed with an increasing amount of either Rbp2(dN4) or Rbp2(dC4), the transactivation activities of Rbp2 on ER were reduced (Fig. 7C, lanes 12 and 13), indicating that these deletion mutants can inhibit normal activity of full-length Rbp2 in a dominant-negative fashion. These results suggest that the N-terminal and C-terminal regions of Rbp2 are both important for the activity of Rbp2 on NRs.

The Potentiation of NR-mediated Transcription by Rbp2 Is Dependent on the AF-2 Function of NR—Rbp2 interacts with DBDs of NRs in a ligand-independent manner, and the trans-
activation of Rbp2 on NR-mediated transcription is dependent on ligands. To confirm further that Rbp2 exerts its effect on the ligand-dependent NRs, the transactivation activity of Rbp2 on different deletion mutants of ER was performed. The different forms of ER were generated as shown in Fig. 8A. As expected, the full-length ER induced an ER-responsive reporter in a ligand-dependent manner (Fig. 8B, lane 1). The transcriptional activity of ER was enhanced further by coexpression of Rbp2 (Fig. 8B, lane 2). In the absence of ligand, Rbp2 has no significant effect on ER-mediated transcription. Interestingly, Rbp2 has a stronger transactivation activity on ER(CDEF), which is devoid of the N-terminal region A/B (Fig. 8B, lanes 3 and 4). In

![image]
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Fig. 6. RB is necessary for maximal potentiation of NR-mediated transcription by Rbp2. RB-negative C33A cells were transfected with ERE-LUC reporter plasmid and ER, along with the indicated expression constructs or vector in the presence and absence of 17β-estradiol. Luciferase activities were measured as described in Fig. 5.

In contrast, neither the AF1 activity encompassing the ER regions A and B (ER(ABC)), and the DBD (ER(C)) nor the AF-2 mutant (ER(CDF**)) of ER were stimulated by coexpression of Rbp2 (Fig. 8B, lanes 5–10). These results suggest that Rbp2 exerts its effect on the AF-2 function of NR, and this explains why the NR-mediated transcription by Rbp2 is ligand-dependent.

Since the N-terminal and C-terminal domains of Rbp2 behaved in a dominant-negative fashion on the transcription mediated by NRs (Fig. 7C) and Rbp2 potentiation on NR transactivation is AF-2-dependent, it is possible that these two domains of Rbp2 could exert a dominant-negative effect on other NR coactivators. To examine this possibility, we transfected HeLa cells with ER and ERE-LUC along with either Rbp2, CBP, Rbp2(dC4), Rbp2(dN4), or Rbp2 and CBP, or CBP with increasing amount of either Rbp2(dN4) or Rbp2(dC4), and luciferase activities were measured (Fig. 8). Rbp2 (lane 2) and CBP (lane 3) induced ER-mediated transcription. Rbp2(dC4) (lane 8) or Rbp2(dN4) (lane 5) had a marginal effect on ER. With the presence of both Rbp2 and CBP, the transcription activity of ER is increased dramatically (lane 4). When CBP was expressed with the increasing amounts of either Rbp2(dC4) (lanes 9 and 10) or Rbp2(dN4) (lanes 6 and 7), the transactivation activity of CBP on ER was reduced, indicating that the N-terminal and C-terminal domains of Rbp2 could also exert their dominant-negative effect on other NR coactivators and that Rbp2 could work together with other coactivators.

DISCUSSION

In this study, we have demonstrated that Rbp2 interacts with NRs and displays significant transcription activation activity toward them. The results also suggest that the presence of Rbp2 is required for the optimal transcription mediated by NRs. In addition, RB is necessary for the maximal potentiation of the transcription mediated by NRs. Molecular dissection of Rbp2 revealed that both the N terminus and C terminus of Rbp2 are necessary for its transcription activation activity. Deletion of either one of them resulted in a suppression of the transcription activity of Rbp2. These observations suggest that one possible function of Rbp2 is to act as a coregulator of NRs.

There are two LXXLL motifs (NR box 1 and NR box 2) present in Rbp2. As LXXL motifs have been implicated in interaction of several other proteins with NRs, its presence motivated us to investigate the role of Rbp2 in NR function. Our studies suggest that both LXXLL motifs are not important for Rbp2 to interact with NRs. The Phd program (39) shows that the secondary structure of NR box 2, but not NR box 1, is predicted to be α-helical. Mutation of either NR box 1, NR box 2, or both did not seem to affect the ability of Rbp2 to interact with NRs as well as their ability to potentiate the transcription mediated by NRs (data not shown). Heery et al. (39, 43) shows that the LXXLL motif is α-helical. They have defined a minimal “core” LXXLL motif as an 8-amino acid sequence spanning positions −2 to 6 relative to the primary conserved leucine residues, with a hydrophobic residue at position −1 relative to the first conserved leucine, and a nonhydrophobic residue at position +2 showing high affinity for steroid and retinoid receptors. Neither NR box 1 nor NR box 2 shows the above-mentioned characteristics, consistent with experiments demonstrating that the LXXLL motifs of Rbp2 are not involved in interaction with NRs. Wild type Rbp2 and all the NR box mutants of Rbp2 interact with ER equally well in the absence of ligand, whereas the conserved functional LXXLL motif has been showed to bind to NR only in the presence of ligand. These observations suggest that the LXXLL motifs of Rbp2 are not the NR interaction domain of Rbp2.

Since Rbp2 interacts with NRs in the absence of ligands and LXXLL motifs are not important for ER-Rbp2 interaction, this prompted us to map the region of both ER and Rbp2 responsible for their interaction. Different regions of ER were used to delineate the Rbp2 interaction domain. The DBD domain, but not A/B or LBD, was shown to interact with Rbp2. The recruitment of Rbp2 to ER complexed to ERE-conjugated beads clearly indicates that Rbp2 could form a ternary complex with NR bound to DNA. The hinge region, C-terminal to DBD, may also contribute to its interaction with Rbp2, as the DBD alone was not sufficient to achieve optimal interaction. Although most of the coactivators that were identified interact with the LBDs of NRs, there are proteins capable of recognizing DBDs of NRs. For example, PCAF (35), MBP1 of Drosophila (44), and SNURF of mammalian (45) all interact with the DBDs of NRs and act as cofactors of NRs.

By using ER-DBD fused to GST as the basis for affinity chromatography, we have established that the C-terminal 378 residues of Rbp2 are essential and sufficient for interacting with NRs. Rbp2-ER interaction occurs in a ligand-independent manner, but Rbp2 enhances the transcription mediated by NRs in a ligand- and AF2-dependent manner. Both the N terminus and C terminus of Rbp2 are shown to be critical for the transactivation. Deletion of either one of them resulted in an inhibition of the transcription mediated by ER. The C terminus is the region that interacts with NRs and is expected to be essential for the Rbp2’s enhancement of NR-dependent transcription. There is a DNA-binding domain termed ARID (for AT-rich interaction domain), first found in Drosophila dead ringer (dri) (46) and a PHD motif in the N terminus of Rbp2. The −100-residue ARID sequence is present in a series of proteins strongly implicated in the regulation of cell growth, development, and tissue-specific gene expression (41). Although ARID was originally found to bind preferentially to AT-rich sites, there are other proteins showing no sequence preference in its DNA binding activity (47). Our preliminary results suggest that Rbp2 binds to nonsequence-specific native DNA cellulose prepared from sheared thymus DNA. This demonstrates that the ARID family proteins may be involved in a wider range of DNA interaction.

PHD motifs are zinc finger-like motifs, speculated to mediate protein-protein interactions. These motifs are commonly found in transcription factors and are implicated in chromatin-mediated gene regulation (42). Thus, the N-terminal Rbp2 deletions were expected to have lost the ability to regulate some Rbp2-associated functions in cells. This is manifested when increasing amounts of the N-terminal domain of Rbp2 were coexpressed with wild type Rbp2 in the transcription assays. The more the mutant was expressed, the less transactivation activi-
ity of Rbp2 was observed. This N-terminal domain acted in a dominant-negative fashion by probably competing away some of the factors that are crucial for the transcription recruited by Rbp2. Although the detailed mechanism remains to be established, we currently favor the model that the N-terminal domain of Rbp2 is involved in interaction with factors that are important for transcriptional activities of NRs.

The DBD of NR does not possess transcription activation function like AF1 and AF2. When tethered to DBD of Gal4, Rbp2 failed to stimulate transcription from promoters containing Gal4-binding sites (data not shown), implying that the transactivation by Rbp2 is not mediated by Rbp2 alone. It probably involved other protein-protein interactions. Rbp2 is a large molecule with several PHD and RING finger motifs. It is thus possible that the transactivation by Rbp2 is mediated by other proteins that are either recruited by Rbp2 or associated with Rpb2. In this way, Rbp2 may function as a possible bridging factor participating in the coordination of activities of transcriptional signal from sequence-specific upstream factors and RNA polymerase II-basal transcription machinery. The C-terminal domain of Rbp2 also acted in a dominant-negative manner. TBP has been shown to interact the C terminus of Rbp2.
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(15). It is possible that the transactivation achieved by Rbp2 is through its ability to interact with NRs and TBP, via its C-terminal domain, and other factors via its N-terminal domain.

Since Rbp2 is an RB-binding protein, the observed effect of Rbp2 on NR might be dependent on RB, which has been shown to potentiate GR activity (48). We determined whether the loss of RB affected the activity of Rbp2 on the transcription mediated by NR. In the RB-negative cell line, C33A, Rbp2 enhances transcription mediated by ER. This suggests that RB may not be required for the basal transactivation of Rbp2 on ER. In the presence of RB, Rbp2 synergistically enhances the transcription mediated by ER, and this effect is suppressed by the presence of an RB inhibitory protein E7. This implies that RB is required for the optimal activity of Rbp2 on ER. Rbp2 was originally identified as an RB pocket domain-binding protein (12). Besides binding to the pocket domain of RB through its LXCXE motif (amino acids 1373–1377), Rbp2 also interacts with RB pocket domain through its non-T/E1A-binding site (amino acids 1547–1558) (15). RB has been shown to potentiate glucocorticoid receptor (GR)-mediated transcription through interaction of its pocket domain with the transcription activator hBRM (49), and the N-terminal domain of RB is essential for the potentiation of transcription by GR (49). hBRM was shown to interact with the ligand-binding domain of ER (50). Since RB interacts with Rbp2, NR (through hBRM), and potentiates NR-mediated transcription with Rbp2 or hBRM (48) synergistically, it is possible that RB, Rbp2, and NR could form a complex in activating the transcription by NR maximally.

In summary, we have provided evidence suggesting that one possible role of Rbp2 is to act as a coregulator of NR-mediated transcription. We showed that Rbp2 interacts with the DBDs of NRs and enhances transcription mediated by them in an AF-2-dependent manner. The C terminus and N terminus of RBp2 are both critical for its participation in enhancement of NR-mediated transcription. In addition, we have shown that RB is critical for the maximal activation of NR by Rbp2. These results imply a scenario in which Rbp2 acts as a bridging factor to recruit and/or coordinate multiple protein-protein interactions that are important for NR-mediated transcription.

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