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RIP-140 Interacts with Multiple Nuclear Receptors by Means of Two Distinct Sites

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We have characterized two distinct binding sites, called site 1 and site 2, in the nuclear protein RIP-140 which interact with the ligand binding domain of the estrogen receptor both in solution and when the receptor is bound to DNA. Both sites are capable of independently interacting with other nuclear receptors, including the thyroid hormone and retinoic acid receptors, but they are not identical since the interaction with retinoid X receptor is mediated primarily by site 1. The interaction is enhanced by agonists but not by antagonists, and the in vitro binding activities to a number of mutant receptors correlate with their abilities to stimulate transcription in vivo. When RIP-140 is fused to heterologous DNA binding domains, it is able to stimulate the transcription of reporter genes in both yeast and mammalian cells. Thus, RIP-140 is likely to function as a bridging protein between receptors and the basal transcription machinery and thereby stimulate the transcription of target genes.

The nuclear receptor superfamily of transcription factors binds to DNA and activates or represses the transcription of genes in higher organisms (31, 35). The activities of some of these receptors depend on the binding of hormonal ligands, including steroids, retinoids, and thyroid hormone, but the activating ligand has yet to be identified for the majority of them. Nevertheless, the modular structure of the entire superfamily seems to be conserved, since they all consist of three structural domains: an N-terminal domain containing an activation function, AF-1; a DNA binding domain; and a C-terminal ligand binding domain containing an additional activation function, AF-2 (6, 13). The activities of AF-1 and AF-2 depend on the promoter and cell type, and in some cases, both are required for full transcriptional stimulation (45). While the sequence of the N-terminal activation domain, AF-1, varies considerably in different nuclear receptors, that for AF-2 contains a highly conserved C-terminal amphipathic α-helix, which is essential for ligand-dependent transcriptional activity (4, 11, 12, 40).

The ability of nuclear receptors to stimulate transcription is likely to involve the recruitment of the basal transcription machinery into a preinitiation complex (16, 37). Although receptors bind directly with a number of basal transcription factors in vitro, including the TATA box-binding protein (41), TFIIB (3, 19), and human $TAF_{II}30$ (20), the interactions are unaffected by ligand binding or by mutations in the AF-2 amphipathic α-helix that abolish transcriptional activity, suggesting that receptors are likely to interact with alternative proteins. Furthermore, the observation that AF-2 activity can be inhibited by overexpressing the hormone binding domain in squelching experiments (43) suggests that AF-2 is likely to interact with target proteins that are distinct from basal transcription factors. Several candidate target proteins have been identified; RIP-140 and RIP-160 (8, 9), ERAP-140 and ERAP-160 (17), TIF-1 (28), a number of isoforms of SRC-1 (22, 34), and TRIP1/SUG-1 (29, 46). However, although SRC-1 has been shown to stimulate the transcriptional activity of nuclear receptors, the physiological roles of receptor interacting proteins are still unclear.

Previous work on RIP-140 has established that it interacts with the estrogen receptor (ER) in the presence of estrogen but not antiestrogens and that its in vitro binding with mutant receptors correlates with their transcriptional activities (9). In this study, we demonstrate that RIP-140 interacts with other nuclear receptors, including the retinoic acid receptors (RARs), retinoid X receptors (RXRs), and thyroid hormone receptors (TRs), by means of two distinct regions whose properties are similar but not identical. In common with the ER, the binding of RIP-140 to these receptors is enhanced in the presence of ligand and reduced by the presence of mutations which inhibit their transcriptional activities. Furthermore, RIP-140 is able to stimulate the transcription of reporter genes when it is tethered to a DNA binding domain, suggesting that it is likely to function as a bridging protein between receptors and the basal transcription machinery.

MATERIALS AND METHODS

Construction of recombinant vectors. A cDNA clone encoding full-length RIP-140 in pBluescript, pBRIP140, and a number of N-terminal deletion mutants, isolated from the ZR 75-1 human breast cancer cDNA expression library, have been described previously (9). The N-terminal deletion mutants RIP 301-1158, RIP 393-1158, RIP 600-1158, and RIP 753-1158 were transferred into pBluescript. The C-terminal deletion mutants RIP 1-1055, RIP 1-963, RIP 1-863, RIP 1-733, RIP 1-581, and RIP 1-439 were generated by exonuclease III digestion of pBRIP140. Digestion was carried out after insertion of an oligonucleotide, containing stop codons in all three reading frames, into the 3′ untranslated region of pBRIP140 between *Bgl*II and *Kpn*I sites. The DNA sequence of the 3′ end of each clone was then determined to establish the C-terminal endpoints of the RIP proteins. Two regions of RIP-140, positions 27 to 439 and 753 to 1158, called sites 1 and 2, were fused to glutathione *S*-transferase (GST) by inserting an *Nsi*I-*Spe*I-digested fragment of RIP 27-439 and a *Bam*HI-*Bgl*II digested fragment of RIP 753-1158 into the *Bam*HI site of pGEX2TK (Pharmacia) to generate GST-RIP site 1 and GST-RIP site 2 fusion proteins, respectively. A fusion protein containing full-length RIP-140 and the DNA binding domain of the B-cell-specific activator protein (BSAP) was generated by inserting a *Spe*I fragment of pRIP140 into *Eco*RI-*Xba*I-filled pBSAP vector derived from pBSAP-CREB described elsewhere (24). The reporter, pBS4, contains four BSAP elements in front of the thymidine kinase promoter and the chloramphenicol

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acetyltransferase (CAT) gene (24). Construction of GST fusion proteins containing the hormone binding domains of receptors has been described elsewhere as follows: GST-ER (8), GST-TRβ (44), GST-RARβ1 (15), and GST-RXRα (32). Expression vectors for in vitro translation of receptors have been described elsewhere: human RARα (48), mouse RARα E412P (12), and transcriptionally defective ERs E-546A and M-547A/L-548A (11). The pBL1-RIP140 multicopy yeast expression plasmid, containing RIP-140 fused in frame to the DNA binding domain of the ER, was constructed by first introducing a *Sac*II site at the 5′ end and a *Bgl*II site at the 3′ end of the coding sequence of RIP-140 cDNA by PCR amplification. The PCR fragment was then digested with *Sac*II and *Bgl*II, gel purified, and cloned into *Sac*II-*Bam*HI-digested vector pBL1 (27).

In vitro protein-protein interaction assays. (i) GST probes. The expression and purification of GST fusion proteins were carried out as previously described (8, 21). Fusion proteins were then purified on glutathione-Sepharose beads (Pharmacia) and either radiolabeled with ^{32}P by using protein kinase A (Sigma) for far-western blotting (8) or used directly for GST pull-down experiments (41).

(ii) Far-western blotting. In vitro-translated RIP-140 proteins were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) on a 10% gel and electroblotted onto nitrocellulose in transfer buffer (25 mM Tris [pH 8.3], 192 mM glycine, 0.01% SDS). After denaturation-renaturation, the blots were incubated with ^{32}P-labeled GST-ER probe as previously described (8) in the presence of 10^{-6} M 17β-estradiol. The filters were then washed, dried, and exposed for autoradiography.

(iii) GST pull-down. Recombinant RIP-140 cDNAs in pBluescript were transcribed and translated in rabbit reticulocyte lysates in the presence of [^{35}S] methionine (Promega) as instructed by the manufacturer. ^{35}S-labeled RIP translation products were then incubated with GST fusion proteins loaded on glutathione-Sepharose beads in the presence of the appropriate ligands in buffer containing 0.2 M NaCl or as otherwise indicated in the figure legends as previously described (8). After washing the beads, samples were analyzed by SDS-PAGE (10% gel), and the amounts of ^{35}S-labeled RIP were amplified, detected by fluorography, and quantified with a PhosphorImager (Molecular Dynamics).

(iv) DNA binding assays. The ability of DNA-bound ER to interact with RIP-140 was determined by using a modification of the method described by Kurokawa et al. (26), employing similar incubation and washing conditions. Oligonucleotides containing the estrogen response element (ERE) from the vitellogenin A2 gene were synthesized, and one was biotinylated at its 5′ end. ER was expressed by using a recombinant baculovirus vector in insect cells, and whole cell extracts were prepared (14). Purified double-stranded oligonucleotide (1 μg) was incubated with 2 pmol of ER in the presence or absence of 17β-estradiol and then immobilized with streptavidin MagneSphere Paramagnetic beads (Promega). After 10 min, the beads were washed and incubated with [^{35}S] methionine-labeled RIP-140 site 1 or site 2 for 1 h. After washing, the radiolabeled proteins were eluted, separated by SDS-PAGE (6% gel), and detected by fluorography.

Cell culture and transient-transfection experiments. JEG3 cells were routinely maintained in Dulbecco's modified Eagle's medium containing 10% (vol/vol) fetal calf serum (GIBCO). Cells were transiently transfected in 24-well plates (Falcon) by using calcium phosphate coprecipitation as previously described (11). The transfected DNA included a pJ7lacZ internal control plasmid (0.15 μg) and the reporter plasmid pBS4 (0.5 μg), in the presence or absence of either BSAP, BSAP-CREB, or BSAP-RIP (0.1 μg). After 48 h, the cells were harvested and extracts were assayed for β-galactosidase (Galacto-Light; Tropix) and CAT (42) activities. β-Galactosidase activity was used to correct for differences in transfection efficiency.

Yeast reporter assays. The ability of DBD–RIP-140 (a fusion of RIP-140 to the DNA binding domain of ER) to activate transcription in yeast cells was analyzed in *Saccharomyces cerevisiae* W303-1B (*HML*α *MAT*α *HMR***a** *his3-11,15 trp1-1 ade2-1 can1-100 leu2-3,112 ura3*). W303-1B was cotransformed with the centromeric plasmid pLRΔ21-U3ERE, which contains a *lacZ* reporter gene driven by a *URA3*-derived promoter containing three EREs (33) and either pBL1-RIP140 or pBL1 vector as a negative control. Transformants containing both plasmids were grown to late log phase in 15 ml of selective medium (yeast nitrogen base containing 1% glucose and appropriate supplements). The preparation of cell-free extracts and β-galactosidase assays were performed as previously described (33).

RESULTS

RIP-140 contains two distinct receptor interaction sites. Previous work has demonstrated that the nuclear protein RIP-140 interacts with the hormone binding domain of ER in the presence of 17β-estradiol but not when mutations that disrupt transcriptional activity were introduced into the AF-2 amphipathic α-helix (9). To determine the site(s) in RIP-140 necessary to interact with the ER, we generated and expressed a series of N-terminal and C-terminal deletion mutants by in vitro transcription-translation and tested their abilities to bind the receptor. In addition to full-length translation products (indicated with solid arrowheads in Fig. 1 and 2), RIP-140 mRNA generated truncated fragments which were probably derived from initiation at internal ATG codons as discussed previously (9). For example, the product of approximately 69 kDa (indicated with open arrowheads in Fig. 1A) is probably initiated at Met-639, since it is apparent when RIP 600-1158 but not RIP 753-1158 was translated. Some initiation at this site is also apparent when C-terminal deletion mutants are analyzed (open arrowheads in Fig. 1B). The interactions between these RIP fragments and the receptor were then investigated by using a fusion protein, GST-ER, consisting of GST, the recognition motif for cyclic AMP-dependent protein kinase, and the hormone binding domain of ER containing the activation domain AF-2.

Initially, we used far-western blotting using ^{32}P-labeled GST-ER as a probe to monitor direct interactions between receptor and the RIP fragments. Analysis of a series of N-terminal deletion mutants indicated that RIP 301-1158, RIP 393-1158, RIP 600-1158, and RIP 753-1158 (Fig. 1A, closed arrowheads) and the fragment presumably initiated at residue 639 (Fig. 1A, open arrowheads) were all detected with the GST-ER probe, indicating that residues 753 to 1158 were sufficient to interact with the hormone binding domain of the receptor. The interaction was dependent on the presence of estradiol, since negligible binding of the probe was detected in the absence of hormone (data not shown). Surprisingly, a series of C-terminal deletion mutants lacking these residues (RIP 1-733, RIP 1-581, and RIP 1-439) were also able to bind the probe (Fig. 1B). Although it was apparent that the shorter RIP fragments bound less probe, control experiments indicated that less of these proteins were transferred onto the blots (data not shown). An interaction was also detected with fragments presumably initiating at residue 639 (Fig. 1, open arrowheads); the weak interaction of the shorter fragments in Fig. 1B reflects their low levels of expression. These findings indicate that RIP-140 contains two sites capable of independently interacting with the receptor, site 1 at the N terminus between residues 1 and 439, and site 2 near the C terminus between residues 753 and 1158.

To confirm that the individual sites are capable of interacting with the receptor, we also examined the ability of GST-ER to retain [^{35}S]methionine-labeled RIP mutants in GST pull-down experiments. In contrast to far-western blotting, this method may also detect indirect interactions mediated by endogenous factors present in the extracts. Binding to GST alone was negligible, and although a small amount of ^{35}S-labeled RIP-140 was retained by GST-ER in the absence of hormone, the interaction was greatly enhanced in the presence of 17β-estradiol (Fig. 2A; compare tracks 3 and 4). Similarly, the C-terminal truncation mutants RIP 1-733 and RIP 1-439, containing site 1, and the N-terminal deletion mutants RIP 393-1158 and RIP 753-1158, containing site 2, were retained by GST-ER, and all of these interactions were enhanced by estradiol (Fig. 2A). These data are consistent with our conclusions from far-western blotting experiments and confirm that two distinct regions of RIP-140 can interact with ER. Interestingly, when we analyzed RIP 1-439 or RIP 753-1158, we observed that a number of lower-molecular-weight proteins were also retained by GST-ER in the presence of estradiol, suggesting that shorter sequences were sufficient for binding to ER.

To determine the boundaries of the two sites of interaction more precisely, we tested several shorter truncation mutants of RIP-140 in the GST pull-down assay (Fig. 2B). We found that GST-ER strongly retained RIP 1-241 in an estrogen-inducible manner but RIP 1-176 showed greatly reduced binding activity (Fig. 2B; compare tracks 24 and 28 with their respective input levels). In addition, construct RIP 27-439 also showed strong

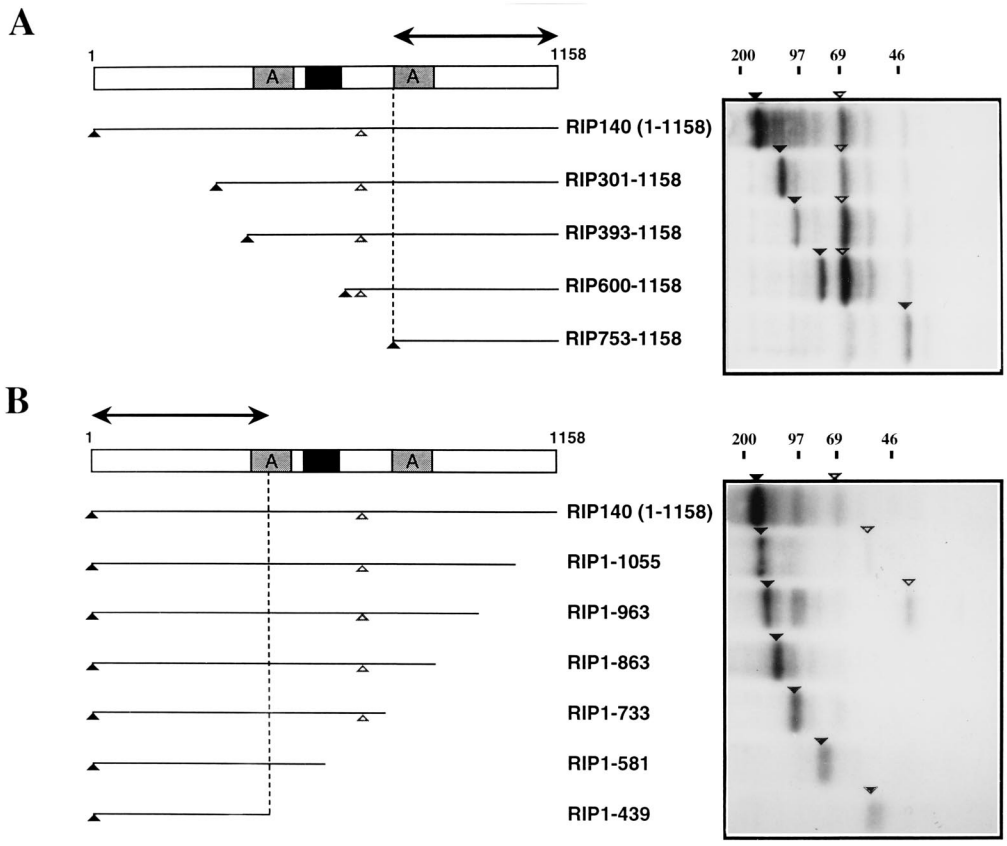

FIG. 1. Mapping of sites in RIP-140 which interact with ER. The interactions of N-terminal (A) and C-terminal (B) deletion mutants of RIP-140 with the hormone binding domain of ER were determined by far-western blotting analysis. Schematic representations of the wild-type and mutant receptors show the positions of two sites of interaction, called site 1 and site 2, two acidic regions (labeled A), and a serine/threonine-rich region (solid box). The RIP proteins were translated in vitro, separated on a 10% polyacrylamide gel, and transferred onto filters. After renaturation, the proteins were tested for their interactions with ^{32}P-labeled GST-ER probe in the presence of 10^{-6} M 17β-estradiol; after washing, the blots were exposed to autoradiography. The filled arrowheads show the positions of RIP proteins of expected sizes, while the open arrowheads indicate truncated products which are probably initiated at Met-639. The positions of molecular mass markers are shown in kilodaltons above each autoradiograph.

estrogen-inducible binding to GST-ER (Fig. 3). Taking these results together, we conclude that the minimal sequences required for the interaction of site 1 with ER in this assay reside within the sequence from residues 27 to 241. Note, however, that although the binding of RIP 1-176 to GST-ER was reduced to 5 to 10% of that of RIP 1-241, the residual binding was enhanced by ligand (Fig. 2B; compare tracks 27 and 28). This result may indicate that this region contains a partial site 1 with reduced affinity for ER. In the case of site 2, we found that GST-ER retained RIP 753-981 in an estrogen-inducible manner but RIP 854-1158 showed greatly reduced binding activity (Fig. 2B; compare tracks 32 and 36). Smaller deletion mutants were poorly resolved and not retained by GST-ER (data not shown). Thus, we conclude that residues 27 to 241 and 753 to 981 are required for optimum binding of ER to site 1 and site 2, respectively. Comparison of their amino acid sequences with the sequence alignment program AMPS (5) did not reveal any significant homology between the two sites. The prediction of secondary structures by using the structural analysis program PHD (39) suggests that site 1 is mostly α-helical whereas site 2 contains both α-helical and β-strand structures. Although we have previously noted the presence of two acidic regions in RIP-140 (9) (labeled A in Fig. 1), they do not seem to be necessary for the interaction, at least in the case of site 1.

We next compared sites 1 and 2 by analyzing the interaction of GST-site 1 and GST-site 2 with the wild-type ER in the presence of different ligands. In contrast to 17β-estradiol, the antiestrogen 4-hydroxytamoxifen or ICI 182780 failed to stimulate the interaction of [^{35}S]methionine-labeled receptor with either site (Fig. 3; compare tracks 5 and 6 with track 3). However, the two sites do differ slightly in that the interaction observed in the absence of ligand was higher with site 1 than with site 2 (Fig. 3; compare tracks 3 and 7). We then investigated their interaction with transcriptionally defective mutant receptors, two of which are presented in Fig. 3; E-546A, which has reduced ligand-dependent transcriptional activity, and M-547A/L-548A, which has negligible activity (11). The binding of ^{35}S-labeled E-546A was approximately 25% of that of the wild-type receptor, while that of M-547A/L-548A was less than 5%. Therefore the binding activities of the mutant receptors to both sites correlate well with their transcriptional activities. As with the wild-type receptor, there was a basal interaction observed with both mutant receptors which was more apparent with site 1 (Fig. 3; compare tracks 3 and 7).

Finally, we investigated whether the two sites can each interact with the ER bound to DNA. To test this, we analyzed the ability of the receptor, bound to an ERE to retain [^{35}S]methionine-labeled RIP-140, site 1, or site 2 in the presence and absence of 17β-estradiol (Fig. 4). Site 1 and site 2 were each observed to interact with DNA-bound receptor, and the inter-

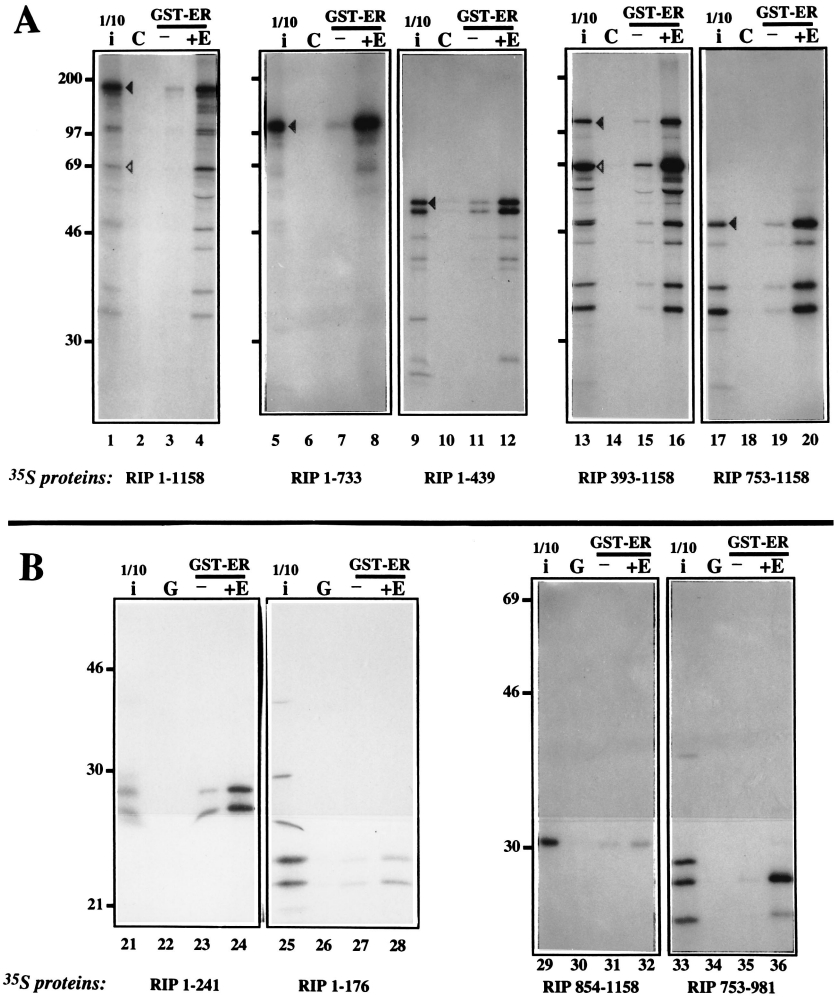

FIG. 2. Mapping of sites in RIP-140 which interact with ER by GST pull-down experiments. RIP proteins, radiolabeled with [^{35}S]methionine, were translated in vitro and tested for the ability to bind to GST alone (G) and fused to the hormone binding domain of ER (GST-ER) in the absence (−) or presence (+E) of 10^{-6} M 17β-estradiol. In each panel, 1/10 i represents 1/10 of the amount of ^{35}S-labeled RIP proteins that were used in the binding assays. After washing of the GST beads, the proteins were eluted, separated on 10% polyacrylamide gels, and detected by fluorography. The filled arrowheads show the positions of RIP proteins of expected sizes, while the open arrowheads indicate truncated products which are probably initiated at Met-639. Positions of molecular mass markers are shown in kilodaltons on the left. Lanes C, control.

action, particularly that of site 2 (Fig. 4; compare tracks 10 with tracks 11 and 12), was enhanced by hormone.

RIP-140 interacts with other nuclear receptors. We next investigated the ability of RIP-140 to interact with other nuclear receptors by testing the ability of GST fusion proteins containing the hormone binding domain of human TRβ, human RARβ, and human RXRα to retain [^{35}S]methionine-labeled RIP-140. In addition to analyzing full-length RIP-140, we compared the binding properties of site 1 and site 2 (Fig. 5). A ligand-independent interaction between RIP-140 and each receptor is detectable, mediated predominantly by site 1, but binding to both sites 1 and 2 is markedly increased in the presence of their respective ligands. Moreover, since the amount of site 1 or site 2 retained was similar to that of the full-length RIP-140, containing both sites, it seems likely that they both interact with these receptors independently. However, while ER, TRβ, and RARβ interact with both sites, RXRα binds preferentially to site 1 (Fig. 5). Thus, the binding properties of sites 1 and 2 are distinct, consistent with their lack of sequence homology.

The interaction of the two sites with RARα was further explored by using selective ligands (Fig. 6). Both RAR-specific (LG100272 and RO13-7410) and RARα-selective (RO40-6055) agonists enhance the interaction similarly with both sites 1 and 2, whereas the RARα-selective antagonist RO41-5253 (2) is without effect. We then analyzed the interaction of RIP-140 with the transcriptionally defective mutant of RARα, E-412P (12). Neither site was able to interact with E-412P irrespective of the ligand bound. We also failed to detect an interaction of RIP-140 with transcriptionally defective mutants of TR (data not shown).

We next tested the effects of increasing salt concentrations on the interaction to assess the relative strength of binding of RIP-140 with ER, RAR, and RXR (Fig. 7). The strongest interaction was observed with ER, since it was maintained at concentrations as high as 0.5 M NaCl and was still appreciable at a concentration of 1.0 M. Moreover, the effects of increasing salt concentrations were identical when sites 1 and 2 were analyzed individually. The interaction of RIP-140 with RAR and RXR was less stable than that with ER, since the binding

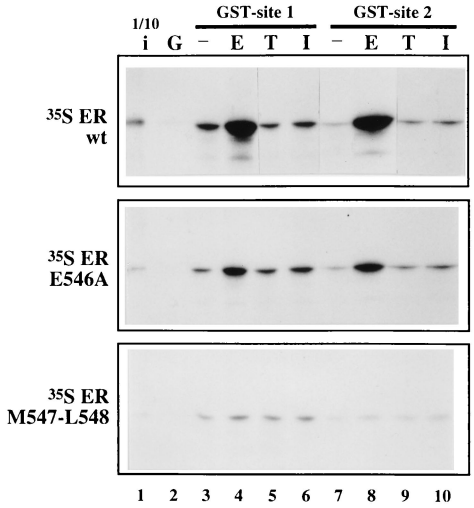

FIG. 3. Binding activities of ERs with sites 1 and 2 in RIP-140 correlate with their transcriptional activities. The wild-type ER (ER wt) and the transcriptionally defective mutants E-546A and M-547A/L-548A were radiolabeled with [^{35}S]methionine by in vitro translation and tested for their interactions with GST alone (G) and GST-site 1 and GST-site 2 in pull-down experiments. The receptors were incubated with GST-site 1 and GST-site 2 with no hormone (−), 10^{-6} M 17β-estradiol (E), 10^{-6} M 4-hydroxytamoxifen (T), and 10^{-6} M ICI 182,780 (I). 1/10 i represents 1/10 of the amount of ^{35}S-labeled RIP proteins that were used in the binding assays.

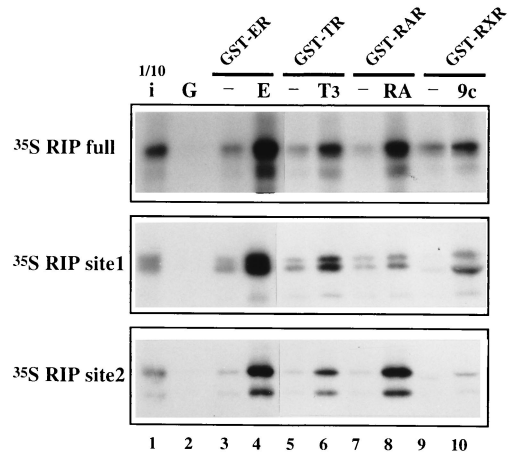

FIG. 5. RIP-140 interacts with retinoid and thyroid hormone receptors. The ability of full-length RIP-140 and sites 1 and 2 to interact with GST-ER, GST-TR, GST-RAR, and GST-RXR was tested in GST pull-down experiments. The interaction was analyzed in the absence (−) or presence of 10^{-6} M their respective ligands, 17β-estradiol (E), 3,5,3′-triiodo-L-thyronine (T3), all-*trans*-retinoic acid (RA), and 9 *cis*-retinoic acid (9c). 1/10 i represents 1/10 of the amount of ^{35}S-labeled RIP proteins that were used in the binding assays, and G represents the amount retained by GST alone.

was progressively reduced when the NaCl concentration was increased above 100 mM. However, as mentioned above, sites 1 and 2 did not bind these receptors equivalently since the binding detected in the absence of ligand was greater with site 1, especially at 100 mM NaCl, and this probably accounts for the interaction of full-length RIP-140 observed in the absence of ligand.

RIP-140 activates transcription of reporter genes when it is fused to a DNA binding domain. We tested whether RIP-140 was able to stimulate the transcriptional activities of reporter genes when it was fused to the DNA binding domain of BSAP, which binds to DNA as a monomer (1). The ability of BSAP-RIP to stimulate the activity of a reporter containing four BSAP binding sites was analyzed in human JEG3 cells. As a positive control, we used BSAP-CREB, which functions as a constitutive activator in these cells (24). As shown in Fig. 8A, BSAP-RIP activates the reporter 8- to 10-fold, similar to the result for BSAP-CREB. We also analyzed the activity of RIP-140 in yeast cells by fusing it to the DNA binding domain of ER (DBD–RIP-140). It was able to stimulate transcription of a β-galactosidase reporter gene approximately 20-fold in yeast cells (Fig. 8B). Thus, when RIP-140 is recruited to the vicinity of a promoter, it is capable of stimulating the rate of transcriptional initiation.

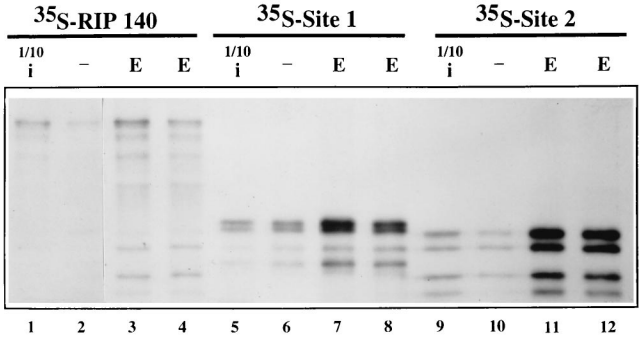

FIG. 4. Sites 1 and 2 can each interact with ERs bound to DNA. The ER bound to an ERE was tested for its ability to interact with [^{35}S]methionine-labeled RIP-140, site 1, and site 2 in the absence (−) or presence of 10^{-6} M 17β-estradiol (E). 1/10 i represents 1/10 of the amount of ^{35}S-labeled RIP proteins that were used in the binding assays.

DISCUSSION

Nuclear receptors modulate target gene transcription by means of several distinct mechanisms, the best studied of which is their function as ligand-dependent transcriptional ac-

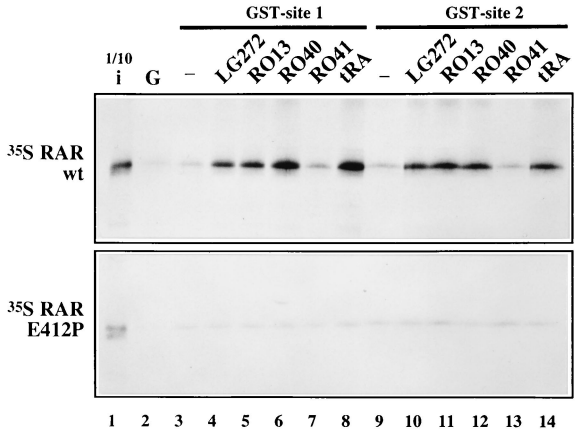

FIG. 6. Interactions of both sites 1 and 2 in RIP-140 with RARs correlate with their transcriptional activities. The wild-type RARα (RAR wt) and the transcriptionally defective mutant E-412P were radiolabeled with [^{35}S]methionine by in vitro translation and tested for their interactions with GST alone (G) and GST-site 1 and GST-site 2 in pull-down experiments. The receptors were incubated with no hormone (−), the RAR-specific agonists LG100272 (LG272) and RO 13-7410 (RO13), the RARα-specific agonist RO 40-6055 (RO40), and the RARα-specific antagonist RO 41-5253 (RO41) at a concentration of 10^{-5} M. 1/10 i represents 1/10 of the amount of ^{35}S-labeled RIP proteins that were used in the binding assays. tRA, all-*trans*-retinoic acid.

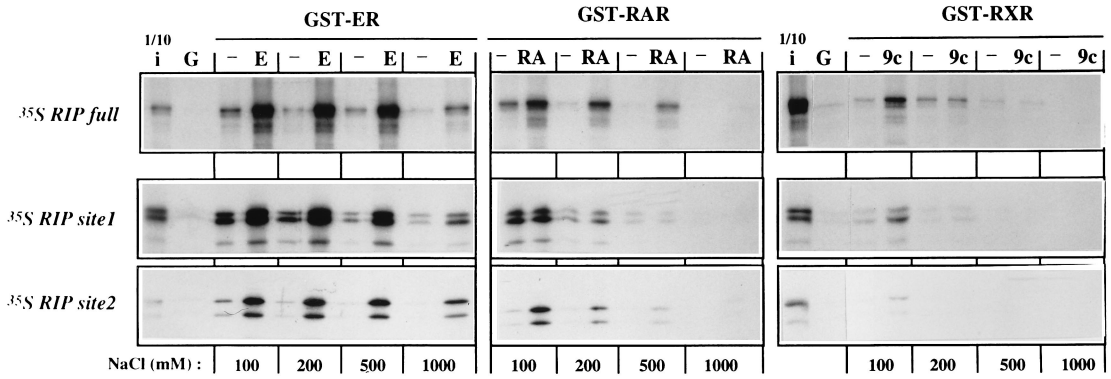

FIG. 7. Effects of increasing salt concentrations on the interactions between RIP-140 and the receptors for estrogen and retinoids. The stability of the interaction of full-length RIP-140 and sites 1 and 2 with GST-ER, GST-RAR, and GST-RXR was tested at increasing salt concentrations from 100 mM to 1.0 M. The interactions were analyzed in the absence (−) or presence of 10^{-6} M of their respective ligands, 17β-estradiol (E), all *trans*-retinoic acid (RA), and 9 *cis*-retinoic acid (9c). 1/10 i represents 1/10 of the amount of ^{35}S-labeled RIP proteins that were used in the binding assays, and G represents the amount retained by GST alone.

tivators. Transcriptional interference/squelching experiments have provided evidence that the nuclear receptor activation functions, AF-1 and AF-2, contact the basal transcription machinery via intermediary factors, also known as coactivators or bridging proteins, although there may also be direct interactions between receptors and components of the basal machinery. Given their ability to bind to the ligand binding domains of nuclear receptors in the presence of ligand, RIP-140, RIP-160 (8, 9), ERAP 140, ERAP 160 (17), TIF-1 (28), TRIP1/SUG-1 (29, 46), and SRC-1 isoforms (22, 34) are all potentially involved in mediating transcription activation by AF2. The lack of sequence similarity among these proteins suggests that they may be functionally as well as structurally diverse.

In this report, we have shown that RIP-140 contains two distinct sites (site 1 and site 2), capable of interacting with the ligand binding domain of ER in a ligand-dependent manner, both in solution or following binding of ER to DNA. In this respect, RIP-140 differs from the proteins SUG1 and TIF1, in which single sites of interaction were identified (28, 29, 46). The interaction of both site 1 and site 2 with receptors is dependent on an intact AF-2 amphipathic α-helix, (helix 12), which has been found to be essential for AF-2 function in a number of nuclear receptors (4, 11, 12, 40). The structures of the ligand binding domains of RARγ (38) and TRα (47), crystallized in the presence of their respective ligands, revealed that helix 12 was aligned over the ligand binding pocket, in contrast to its position in unliganded RXRα, in which it protrudes away from the ligand binding domain (7). In both of the liganded receptors, hydrophobic residues within the α-helix 12 face toward the pocket, perhaps contacting the ligand, while negatively charged residues are exposed on the protein surface. Thus, as suggested by Renaud et al. (38), realignment of helix 12 over the ligand binding pocket when the receptor binds ligand may generate a novel surface for interaction with coactivators or bridging proteins. In ER, the integrity of this surface seems to be absolutely dependent on the hydrophobic residues in helix 12, since their mutation abolishes AF-2 activity and completely disrupts the interaction of ER with sites 1 and 2 of RIP-140 (Fig. 3), with TIF1 (28), and with SUG1 (46). Our results also indicate that charged residues are important for optimum RIP-140 binding and AF-2 activity and may form part of the interaction surface itself.

We have shown here that in addition to binding ER, RIP-140 interacts with RAR, RXR, and TR in a ligand-dependent manner. In the case of RAR, synthetic molecules which behave as agonists for transcription activation also stimulate interaction between RAR and RIP-140, whereas antagonists do not. Similar results were previously reported for antagonists of ER (9). SUG1 and TIF1 show differential binding to a panel of nuclear receptors (46), with a negligible interaction between TIF1 and TR and only weak binding between SUG1 and RXR. Although RIP-140 interacts with RAR, RXR, and TR in our experiments, examination of the individual binding properties of site 1 and site 2 revealed that the interaction of RIP-140 with RXR probably occurs predominantly through site 1. The presence of two distinct receptor binding sites in RIP-140 raises the possibility that one molecule of RIP-140 is sufficient to bind to both receptor partners in an ER homodimer. Moreover, the different affinities of site 1 and site 2 for RXR might also provide a mechanism for binding of one molecule of RIP-140 to both partners in RAR-RXR and TR-RXR heterodimers.

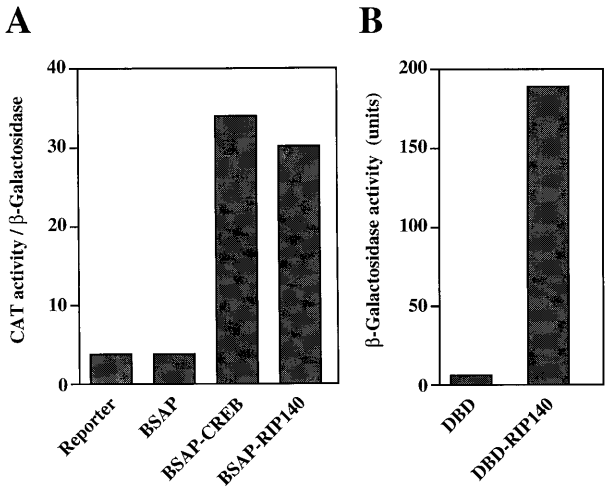

FIG. 8. RIP-140 functions as a transcriptional activator when tethered to a DNA binding domain. (A) The ability of BSAP–RIP-140 to stimulate the transcription of a BSAP reporter, pBS4, was tested in transiently transfected JEG3 cells. As controls, the effects of the BSAP DNA binding domain alone and BSAP fused to CREB were tested. β-Galactosidase activity was used to correct for differences in transfection efficiency. (B) The ability of DBD–RIP-140 to activate transcription was analyzed in *S. cerevisiae* W303-1B. The reporter pLRΔ21-U3ERE contains a *lacZ* reporter gene containing three EREs (33). The DNA binding domain alone was used as a negative control.

However, the stoichiometry of binding of RIP-140 or other proteins to nuclear receptors has not been addressed, and it is equally possible that different combinations of coactivators bind to homo- and heterodimers.

Ligand-dependent activation by the RARs and TR has recently been shown to involve the dissociation of corepressors such as N-CoR (18) and SMRT (10) prior to the recruitment of coactivators. The activity of RAR-RXR heterodimers is modulated by the spacing of the half-sites such that RAR-specific ligands activate the heterodimer on DR-5 elements when RAR occupies the downstream half-site but not on DR-1 elements when it occupies the upstream half-site (25, 36, 49). Interestingly, it appears that putative coactivators such as p140 and p160 are recruited to RAR-RXR heterodimers irrespective of the binding site but N-CoR is displaced only when it occupies a DR-5 element (26).

The properties of RIP-140 outlined above suggest that it plays a role in transcription activation by nuclear receptors. However, the activation of ER (9), RAR, or TR (data not shown) by RIP-140 was never greater than twofold in transiently transfected cells. This modest activation might indicate that the levels of endogenous RIP-140, whose expression is induced by retinoids and estradiol (our unpublished results), or other coactivators, are already sufficient for maximum AF-2 activity in the cell lines tested. Indeed, consistent with this hypothesis, there was strong squelching of AF-2 activity (reference 9 and data not shown) as the amount of transfected RIP-140 expression vector was increased. Alternatively, it is possible that inappropriate promoter contexts or cell types were used in our experiments or that additional proteins that work in concert with RIP-140 would also need to be overexpressed for a significant increase of AF-2 activity to be observed. Note that in similar experiments, neither TIF1 (28) nor mouse SUG1 (46) was found to increase the transcriptional activity of nuclear receptors, despite indications that at least in yeast cells, SUG1 may be associated with the holoenzyme complex (23). To date, only SRC-1 has been shown to significantly enhance AF-2 activity in transient-transfection experiments (34).

One predicted property of a coactivator or bridging protein is that when fused to a heterologous DNA binding domain, it should be able to activate transcription of an appropriate reporter gene by recruiting the basal transcription machinery to the promoter of that gene. We have shown here that fusion of RIP-140 to different DNA binding domains results in the transcriptional activation of reporter genes in both mammalian and yeast cells. The level of activation achieved is comparable to that of the strong activation domain of the CREB protein. These results provide further support for our proposal that RIP-140 acts as a bridging protein between the ligand binding domains of some nuclear receptors and the basal transcription machinery.

At present, it is possible only to speculate as to why there are so many proteins capable of interacting with the ligand binding domain of receptors. It is possible that while some may function as transcriptional coactivators, others may have additional functions, such as in chromatin remodeling. In addition, the recruitment of coactivators may vary depending on the DNA binding site which may function as an allosteric effector (30) or on the mode of receptor activation, namely, by binding its cognate ligand or by a ligand-independent pathway. Further insights into the role of the different receptor-interacting proteins may become evident when dominant negative versions are analyzed or when mice lacking the genes are generated.

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