

## ERAP140, a Conserved Tissue-Specific Nuclear Receptor Coactivator

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**We report here the identification and characterization of a novel nuclear receptor coactivator, ERAP140. ERAP140 was isolated in a screen for ER $\alpha$ -interacting proteins using the ER $\alpha$  ligand binding domain as a probe. The ERAP140 protein shares no sequence and has little structural homology with other nuclear receptor cofactors. However, homologues of ERAP140 have been identified in mouse, *Drosophila*, and *Caenorhabditis elegans*. The expression of ERAP140 is cell and tissue type specific and is most abundant in the brain, where its expression is restricted to neurons. In addition to interacting with ER $\alpha$ , ERAP140 also binds ER $\beta$ , TR $\beta$ , PPAR $\gamma$ , and RAR $\alpha$ . ERAP140 interacts with ER $\alpha$  via a noncanonical interaction motif. The ER $\alpha$ -ERAP140 association can be competed by coactivator NR boxes, indicating ERAP140 binds ER $\alpha$  on a surface similar to that of other coactivators. ERAP140 can enhance the transcriptional activities of nuclear receptors with which it interacts. In vivo, ERAP140 is recruited by estrogen-bound ER $\alpha$  to the promoter region of endogenous ER $\alpha$  target genes. Furthermore, the E<sub>2</sub>-induced recruitment of ERAP140 to the promoter follows a cyclic pattern similar to that of other coactivators. Our results suggest that ERAP140 represents a distinct class of nuclear receptor coactivators that mediates receptor signaling in specific target tissues.**

Estrogen plays a central role in the control of sexual behavior and reproductive functions, embryonic and fetal development, and cardiovascular and neuronal physiology (2, 36). Estrogenic effects have been linked to the pathophysiology of a variety of human diseases, including breast and endometrial cancers, cardiovascular disease, and osteoporosis (2, 23). The diverse biological effects of estrogens are mediated by two estrogen receptors, ER $\alpha$  and ER $\beta$ , which are members of the nuclear receptor superfamily (33). Functional domains of ER contain a central DNA binding domain (DBD) and a carboxyl-terminal ligand binding domain (LBD). Transcriptional activation by ER is mediated by two separate activation functions (AFs): AF-1 in the N terminus, which is regulated by growth factors through the MAP kinase pathway, and AF-2 in the LBD, which is essential in mediating ligand-dependent transactivation. Upon ligand binding, ERs form homodimers and bind specific consensus DNA sequences located in the promoter regions, known as ER response elements (EREs). ERs then interact with other transcription factors to ultimately regulate transcription of target genes.

In the past few years, research interests have been directed towards understanding the basis of the pleiotropic effects of estrogen and, in particular, how ER cooperates with its coregulatory factors to carry out these effects. In addition to binding directly to its response elements, ER can interact with components of the basal transcription machinery. By targeting the basal transcription factors, ER facilitates the formation of a stable preinitiation complex, thus increasing the transcription initiation rate of RNA synthesis (25, 26). Further, ER can modulate the chromatin state by associating with different classes of coregulatory factors that have either a coactivator or corepressor function. To date, more than 20 coactivators or

coactivator complexes have been implicated in potentiating transcriptional activation by nuclear receptors. In general, these coactivators can be divided into five families: (i) the p160 family of proteins, including SRC-1/NCoA-1 (21, 39), TIF2/mGRIP1/NCoA-2 (24, 47), and p/CIP/ACTR/AIB1/RAC3 (1, 10, 32, 46); (ii) the non-p160 members, including ARA<sub>70</sub>, RIP140, TIF1, and Trip/SUG1 (8, 31, 49, 52); (iii) the cointegrators CBP/p300 and its associating protein p/CAF (9, 51); (iv) the distinct DRIP/TRAP/ARC complex, which serves as a direct bridge with general transcription complexes (18, 35, 40); and (v) the ATP-binding regulators, such as hBrm and BRG-1 (28, 34).

A critical aspect of gene activation by these coactivators involves chromatin remodeling. Members of the p160 family, as well as the CBP/p300 and p/CAF proteins, possess intrinsic histone acetyltransferase activity (37, 43) and therefore enhance gene activation by modifying chromatin organization. In addition to having histones as substrates, CBP/p300 acetylation has been directly linked with transcriptional activation. Studies by Chen et al. demonstrate that upon ligand binding, ER $\alpha$  recruits the p160 coactivator AIB1/ACTR, as well as CBP/p300, to the target gene. CBP/p300 subsequently acetylates the lysine residues in AIB1/ACTR, which leads to the disruption of coactivator-receptor binding. This in turn results in dissociation of the coactivator complex from the receptor and the target gene promoter. Such transient association of p160 coactivators and CBP/p300 with the promoter is consistent with the rapid attenuation of target gene expression upon hormone stimulation (11). The effects of acetylation are complemented by structural alteration of chromatin, which is accomplished by distinct ATP-dependent chromatin-remodeling complexes, including the SWI/SNF and the ISWI-based families (29). The mammalian homologues of yeast SWI2/SNF2, hBrm and BRG-1, have been demonstrated to interact with several nuclear receptors and enhance transcriptional activation by ER $\alpha$  and retinoic acid receptor (RAR) (12). Given the abundance of coactivators, a present research focus is to determine the

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sequential events of receptor-coactivator interactions following ligand stimulation. We have proposed a dynamic model of a cyclic assembly of ER $\alpha$  transcription complexes upon estradiol treatment and provided evidence for functional differences among coactivators in ER $\alpha$ -mediated transcription (41). Our results support a general model of receptor activation: ligand binding first recruits chromatin remodeling complexes to facilitate basal transcription machinery assembly, CBP acetylation leads to release of the p160 coactivator along with the receptor, and finally, CBP dissociates, and the cycle is repeated.

Specific ligand binding could also determine the biological activity of the receptor by influencing selective recruitment of the coactivator complexes. Structural and mutational analyses have indicated that binding of agonists to ER $\alpha$  induces a conformational change involving the repositioning of helix 12 in the most C-terminal of the LBDs, which creates a contact surface for coactivator interaction. The coactivators interact with agonist-bound ER $\alpha$  through multiple LXXLL signature motifs (L denotes a leucine, and X denotes any amino acid), also known as NR boxes (16, 22, 46). In contrast, ER $\alpha$  LBD bound by its antagonists, such as tamoxifen and raloxifene, adopts a configuration in which the positioning of helix 12 mimics intramolecular interaction with the AF-2 surface and occludes the coactivator association (7, 42).

We report here the identification of a novel protein, ERAP140, that associates with ER $\alpha$  in an agonist- but not antagonist-dependent manner. Interaction of ERAP140 with ER $\alpha$  is mediated through a motif located in the central region of the protein. In addition to binding ER $\alpha$ , ERAP140 exhibits binding specificity to a variety of nuclear receptors in response to their cognate ligands. ERAP140 enhances transactivation by these receptors on their response elements *in vitro* and possesses transcriptional activity when tethered to DNA. Using the chromatin immunoprecipitation (ChIP) assay, we are able to demonstrate that ERAP140 is recruited to ER $\alpha$  target gene promoters following estradiol treatment in a dynamic fashion, similar to other coactivators. The ERAP140 protein does not have any sequence or structural similarity to previously identified nuclear receptor coactivators; however, ERAP140 homologues have been found in both vertebrates and invertebrates, including human, mouse, *Drosophila*, and *Caenorhabditis elegans*. Moreover, unlike most reported cofactors, ERAP140 exhibits both cell- and tissue-specific expression. Thus, ERAP140 may represent a distinct class of receptor coactivators.

#### MATERIALS AND METHODS

**cDNA library screening.** The glutathione *S*-transferase (GST)-ER LBD protein was labeled with [ $\gamma$ -<sup>32</sup>P]ATP and used to screen a  $\lambda$ gt11 Akata Burkitt's lymphoma cell cDNA expression library following a standard far-Western protocol (27). The filters were screened with ER LBD probes that had been preincubated with estradiol. Plaques expressing induced LBD-bound proteins were isolated and replated. In the secondary screen, the filter was cut in half, and the halves were probed in parallel in the presence or absence of estradiol. Plaques from the secondary plates that exhibited estradiol-dependent LBD binding were picked, replated for isolation, and purified, and the cDNA inserts were recovered.

**Plasmids and antibodies.** The full-length ERAP140 was cloned in the expression vector pcDNA3.1(+) (Clontech). Various GST fusions were constructed by PCR using primers containing *Bam*HI at the 5' end and *Xho*I at the 3' end; the PCR fragments were inserted into *Bam*HI-*Xho*I sites of pGEX-4T-1 (Pharmacia Biotech). GAL4 DBD-ERAP140 was constructed by PCR amplifying

full-length ERAP140 using primers containing *Nhe*I sites at both ends and ligating it into *Nhe*I-digested and dephosphorylated GAL4 DBD (kindly provided by William Sellers, Dana-Farber Cancer Institute). To make the T7-tagged constructs, ERAP140 was cloned into *Bam*HI-*Xho*I sites of the pcDNA3.1/T7 vector (kindly provided by William Sellers); the two mutant constructs of ERAP140 were made following the site-directed mutagenesis protocol (Stratagene), using the wild-type ERAP140 as a template. The anti-ERAP140 polyclonal antibody was raised in rabbit against a GST- $\lambda$ 7 fusion protein. The AIB1 polyclonal antibody was developed against GST-AIB1 (amino acids 695 to 933). The T7 tag monoclonal antibody was purchased from Novagen,  $\alpha$ ER $\alpha$  was purchased from Neomarkers, and  $\alpha$ Pol II was purchased from BabCo.

**Cell lines and culture conditions.** The MCF-7, MDA231, BT20, ECC1, Ishikawa, HeLa, U2OS, and HepG2 cells were cultured in Dulbecco's modified Eagle medium (Cellgro) supplemented with 10% fetal calf serum (Cellgro), L-glutamine (GIBCO BRL), and penicillin-streptomycin (GIBCO BRL) at 37°C in a 5% CO<sub>2</sub> humidified chamber. LnCaP cells were cultured in RPMI medium with supplements.

**Northern and Western analyses.** For Northern analysis, total RNA was isolated using the TRIzol reagent (GIBCO BRL), electrophoresed on a 1% formaldehyde agarose gel, and transferred onto Zeta probe membranes (Bio-Rad). The membranes were hybridized with a <sup>32</sup>P-labeled ERAP140 probe, and signals were detected by autoradiography. For Western analysis, nuclear extracts were prepared by the method described by Dignam et al. (15). Proteins were resolved on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane (Fisher Scientific); proteins that reacted with the antibodies were detected using SuperSignal chemiluminescent substrate (Pierce).

**In situ hybridization.** Nonradioactive *in situ* hybridization was performed as previously described (6). Digoxigenin (DIG)-labeled sense and antisense ribonucleotide probes were generated from pBS/SK<sup>+</sup>-ERAP140 containing 427 bases from a mouse-expressed sequence tag sequence. Frozen sections (10  $\mu$ m thick) were cut in a cryostat and captured on Superfrost plus microscope slides (Fisher Scientific). Alternatively, paraffin sections were deparaffinized and treated with proteinase K (0.05% in 0.1 M TBST containing 2 mM CaCl<sub>2</sub>) for 20 min at 37°C. Both types of sections were then fixed, acetylated, and hybridized at 68°C over 3 nights with the probe (approximate concentration, 100 ng/ml). The sections were rinsed several times in 100 mM Tris-150 mM NaCl-20 mM EDTA (pH 9.5) and coverslipped with glycerol gelatin (Sigma). Hybridized probe was visualized using alkaline phosphatase-conjugated anti-DIG Fab fragments (Roche) and 5-bromo-4-chloro-3-indolyl-phosphate-Nitro Blue Tetrazolium substrate (Kierkegaard and Perry Laboratories).

**In vitro GST binding assay.** Immobilized GST fusion proteins were preincubated at 4°C for 1 h with or without ligand in the binding buffer (20 mM HEPES-KOH [pH 7.9], 180 mM KCl, 0.2 mM EDTA, 0.05% NP-40, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol) containing 1 mg of bovine serum albumin/ml. The fusion proteins were then incubated with 200,000 cpm of <sup>35</sup>S-labeled *in vitro*-translated proteins (TNT Coupled Reticulocyte Lysate System; Promega) in the binding buffer at 4°C for 1 h in the presence or absence of ligand. The Sepharose beads were then washed three times with the same binding buffer, resuspended in 20  $\mu$ l of 2 $\times$  SDS sample buffer, and boiled, and the eluted proteins were analyzed by electrophoresis.

**Transient transfection.** HepG2 cells were seeded at 40,000 per well in six-well plates and grown under estrogen-free conditions overnight before transfection. The cells were transfected with 100 ng of pCMX- $\beta$ Gal, 100 ng of receptors, 100 ng of DNA response elements, and with or without 100 ng of pcDNA3.1-ERAP140 (see Results); salmon sperm DNA was used to make up the DNA quantity. The transfections were carried out using PolyFect (Qiagen) according to the manufacturer's protocol. At 20 h posttransfection, the cells were treated with specific ligands or vehicle (ethanol) and were harvested after 24 h of incubation. The cells were lysed in reporter lysis buffer (Promega) and assayed for luciferase and  $\beta$ -galactosidase activities. Similar transfection studies were conducted in Cos-1 cells cotransfected with GAL4 DBD, the GAL4 DBD-ERAP140 fusion gene, and the (GAL4 X 5)SV40-Luc reporter.

**ChIP.** MCF-7 cells were plated on 10-cm-diameter culture dishes and transfected with pcDNA3.1/T7-ERAP140 wild type or mutants by the method described above; the cells were cultured under estrogen-free conditions. On the third day posttransfection, the cells were treated with 100 nM 17  $\beta$ -estradiol (E<sub>2</sub>) for various periods as indicated below. Following treatment, ChIP was performed as previously described (41). The PCR primers used were as follows: pS2 sense, 5'-GGCCATCTCTCACTATGAATCACTTCTGC-3', and pS2 antisense, 5'-GGCAGGCTCTGTTTGCTAAAGAGCG-3'.

**Nucleotide sequence accession number.** The GenBank accession number for the full-length ERAP140 cDNA sequence is AF493978.

## RESULTS

**Cloning of a novel ER-associated protein, ERAP140.** The estrogen dependence of the ER $\alpha$  AF-2 activation and the elucidation of ligand-induced conformational changes in the C terminus of ER $\alpha$  suggest that these ligand-specific structures may be regulatory. Supporting this concept, studies to find proteins that interact with the LBD of ER or other nuclear receptors in the presence of agonistic ligands, but not antagonists, have led to the identification of a spectrum of putative coactivators. Given the essential role of LBD in mediating hormone-dependent ER $\alpha$  interactions with other cellular factors, we used a [ $\gamma$ - $^{32}$ P]ATP-labeled GST-ER $\alpha$  LBD fusion protein as a probe in a far-Western protocol to screen for novel ER-associated factors from a lymphoma cell line (Akata) cDNA expression library. The filters were hybridized with LBD probes that had been preincubated with E $_2$ . Positive clones were selected based on binding of the  $^{32}$ P-labeled LBD probe, subsequently isolated, and sequenced. One positive clone,  $\lambda$ 7, contained a 1.4-kb sequence including a continuous open reading frame. This partial cDNA was further used as a probe for the complete cDNA sequence. As a result, two overlapping cDNAs of 3.3 and 4.5 kb were isolated. A contiguous sequence comprising 5.5 kb tailed by a polyadenylate track and containing an open reading frame of  $\sim$ 3 kb was obtained. The cDNA encodes a protein of 942 amino acids with a predicted molecular mass of  $\sim$ 110 kDa. One of the cDNAs contains two inserted sequences that encode 38 and 11 amino acids in frame, which represent splice variants (Fig. 1A). When the full-length cDNA sequence was inserted into an expression vector and examined by *in vitro* translation, a band migrating with an apparent mass of 140 kDa was observed. The significantly higher mass than predicted may be due to aberrant electrophoretic mobility imparted by the highly charged amino acid content (40%) of the protein sequence.

To verify that the cloned cDNA encodes a protein that interacts with ER $\alpha$  *in vivo*, we incubated MCF-7 whole-cell extracts with GST-ER LBD in the absence or presence of estradiol, and the bound proteins were eluted and analyzed by Western assay with a polyclonal antibody raised against the  $\lambda$ 7 fragment. As a result, the antibody detected a protein of 140 kDa which complexed with GST-ER LBD in the presence of estradiol but not in the absence of ligand (Fig. 2A). This suggests that the isolated clone encodes a protein that specifically binds to liganded ER $\alpha$ ; thus, we named it ERAP140 for ER-associated protein of 140 kDa. We further investigated whether the association between ERAP140 and ER LBD requires the LBD/AF-2 domain. As demonstrated in a GST pull-down assay, the  $\lambda$ 7 fragment of ERAP140 failed to interact with ER $\alpha$  with its AF-2 domain deleted (ER $\alpha$   $\Delta$ AF-2) in the presence of estradiol (Fig. 2B), indicating that ERAP140-ER $\alpha$  interaction is dependent on a correct and active conformation of the liganded ER $\alpha$ .

Computerized structural analysis revealed little about the function of ERAP140, other than a bipartite nuclear localization signal located at the extreme N terminus and several putative phosphorylation sites. The ERAP140 protein shares no sequence and little structural homology with other nuclear receptor cofactors, it is distinct from another cofactor with similar mass, RIP140. The protein has a region with abundant

basic residues in its N terminus, an acidic central area, and a small cysteine- and histidine-rich region in the C terminus (Fig. 1B). A search of the human genome sequence indicates that ERAP140 is localized on chromosome 6 q22.33, spanning a region of approximately 150 kb and consisting of 15 exons (Fig. 1C). A search in the GenBank database identified several ERAP140 homologues in a variety of species (Fig. 1D). The *Drosophila* homologue, L82, is involved in *Drosophila* development in response to the insect steroid hormone ecdysone (44). The mouse C7 protein is encoded by a gene that is up-regulated upon cell attachment to extracellular matrix proteins (17). Sequence alignment indicates that there are three domains within ERAP140 that display the highest identities to its homologues, two of which are located in the N terminus and the third in the C terminus. In addition to mouse, *Drosophila*, and *C. elegans*, domain III is also conserved in yeast and in a human oxidation resistance gene, hOXR1, indicating that a novel functional motif might be present in this domain.

**ERAP140 expression is cell and tissue type specific.** To establish the ERAP140 expression pattern, we first examined its mRNA expression in different tumor cell lines by Northern blotting analysis. Total RNAs were isolated from tumor cells, blotted, and hybridized with an ERAP140-specific probe. The ERAP140 transcript had a size of approximately 5.5 kb and was detected in all tumor lines examined. The highest expression was found in an ER-negative breast cancer cell line, MDA231; relatively high expression was also observed in an ER-positive breast cancer cell line, MCF-7, and in the cervical carcinoma HeLa cells (Fig. 3A). We next investigated whether ERAP140 expression was regulated by estradiol. Cells were grown under hormone-free conditions for 3 days and then treated with or without a saturating level of E $_2$  for 6 h, and total RNA was collected and analyzed by Northern blotting. Consistent with Fig. 3A, the basal level of ERAP140 mRNA expression varied among different tumor lines; moreover, ERAP140 expression was not affected by treatment with E $_2$  (Fig. 3B). We also examined the expression profile of ERAP140 in human and mouse tissues. Northern analysis of a panel of 12 human tissues demonstrated that ERAP140 mRNA expression was not detected in bone marrow or thyroid gland tissue and was detected at low levels in mammary gland, ovary, uterus, prostate, stomach, bladder, spinal cord, and pancreas tissue (Fig. 3C). Expression of ERAP140 was most abundant in the brain, indicating that ERAP140 may have specific functions in that organ.

To determine the expression pattern of ERAP140 in mouse tissues, we obtained a mouse-expressed sequence tag sequence that has 89% identity to the human ERAP140 and corresponds to nucleotides 2679 to 3153 in the human gene. This mouse fragment was used as a probe in the Northern analysis of mouse tissues. In addition to the abundant expression in the brain, high expression of mouse ERAP140 was also observed in the kidney. Its expression was not detected in liver, intestines, or muscle and was detected at very low levels in mammary gland, lungs, and testis (data not shown). The tissue-specific expression of the mouse ERAP140 was further investigated in detail by *in situ* hybridization assays. In mouse brain, the expression of ERAP140 mRNA was exclusively found in neurons: it was detected in neurons of the cerebral cortex, thalamus, hypothalamus, hippocampus, cerebellum,

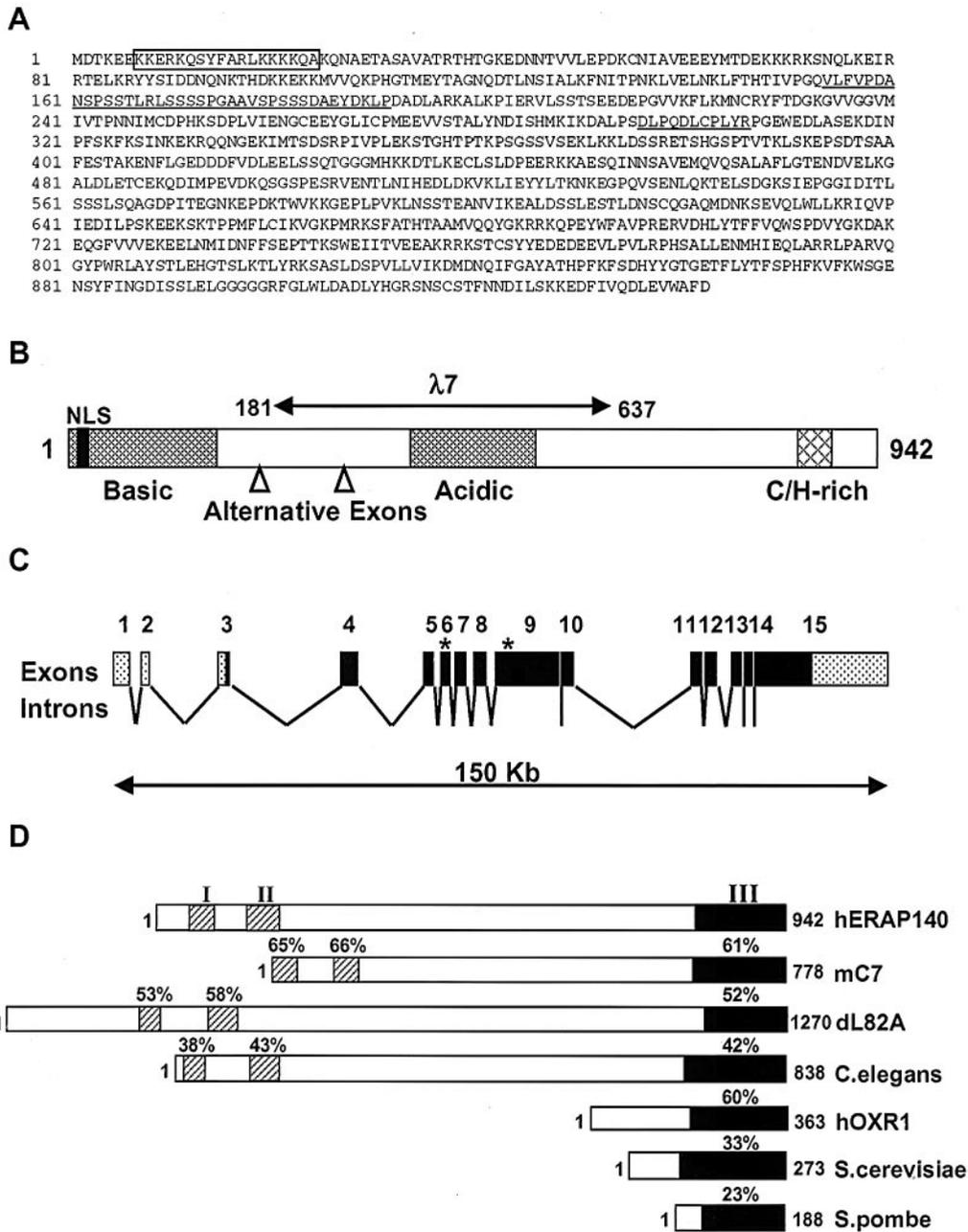


FIG. 1. Identification of a novel ER-associated protein, ERAP140. (A) Amino acid sequence of ERAP140. Full-length ERAP140 cDNA encodes a protein of 942 amino acids with an approximate mass of 140 kDa. The bipartite nuclear localization signal located in the extreme N terminus is indicated by the box. Two inserted sequences (underlined) likely represent splice variants. (B) Schematic diagram of the ERAP140 protein structure. Regions rich in basic, acidic, or cysteine and histidine residues are labeled, and the positions of two splice variants are indicated ( $\Delta$ ). NLS, nuclear localization signal. (C) ERAP140 genomic DNA consists of 15 exons and spans 150 kb; its coding region is represented by the solid boxes, and the noncoding region is represented by the stippled boxes. The asterisks indicate the locations of splice variants. (D) ERAP140 has sequence homology with proteins identified in various species, ranging from human to yeast. The three most conserved domains in ERAP140 are shown, and the percentage of identity in each protein relative to ERAP140 is indicated above the homologous domain.

striatum, and choroid plexus (Fig. 4A to I and data not shown). In situ hybridization in the kidney exhibits significant expression of ERAP140 mRNA in the cortex but not in the medulla (Fig. 4K and L). These results suggest that ERAP140 mRNA is specifically expressed in distinct brain and kidney regions and, even more, in particular cell types.

In addition to mRNA, the levels of ERAP140 were also examined in a group of tumor cells. In accordance with the Northern analysis results, ERAP140 protein was expressed at different levels among tumor lines and was highest in MDA231, MCF-7, and HeLa cells (Fig. 3D). The only exception was the breast cancer cells, BT20, which had a low level of

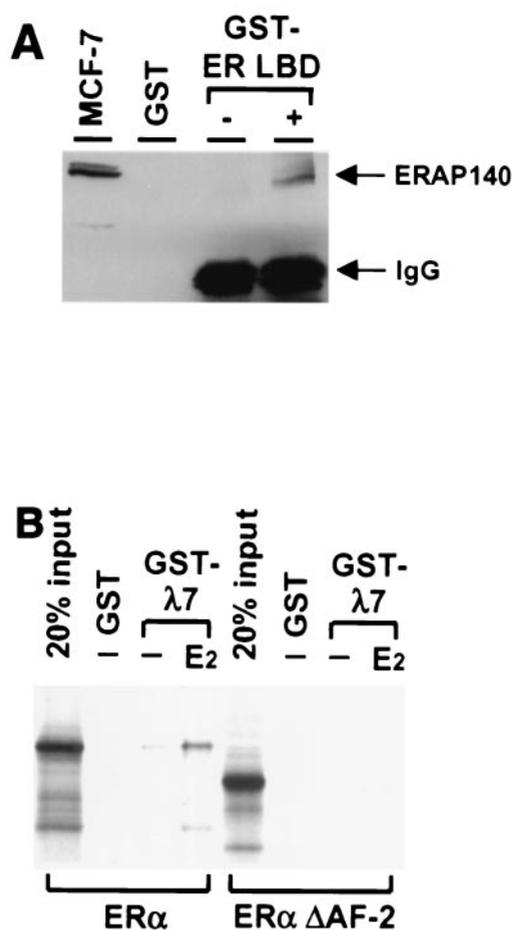


FIG. 2. ERAP140 interacts with ER $\alpha$  LBD in the presence of estradiol in vivo, and the interaction requires the ER $\alpha$  LBD/AF-2 domain. (A) Whole-cell extracts were prepared from MCF-7 cells and incubated with GST-ER LBD in the absence (-) or presence (+) of 1  $\mu$ M E<sub>2</sub>. The bound proteins were eluted, electrophoresed on SDS-PAGE, transferred to a nitrocellulose membrane, and analyzed by Western assay with a polyclonal antibody raised against the  $\lambda$ 7 region of ERAP140 (amino acids 181 to 637). GST alone was included in the gel as a positive control, and 25  $\mu$ g of MCF-7 whole-cell extracts was included in the gel as a negative control. (B) GST- $\lambda$ 7 fusion protein was incubated with [<sup>35</sup>S]methionine-labeled in vitro-translated ER $\alpha$  or ER $\alpha$   $\Delta$ AF-2 in the absence (-) or presence of 1  $\mu$ M E<sub>2</sub>. Twenty percent of the receptor used in the binding reaction is shown as input. The bound protein was eluted, resolved on SDS-PAGE, and visualized by autoradiography.

mRNA but a relatively high expression of the protein. The ERAP140 transcript in these cells may have a shorter half-life, resulting in detection of a low signal by Northern blotting. We consistently detected multiple bands corresponding to the ERAP140 protein, possibly due to the splice variants or post-translational modifications. Overall, ERAP140 expression displays a cell- and tissue-specific pattern.

**ERAP140 interacts with a variety of nuclear receptors in a ligand-inducible manner.** ERAP140 was cloned for its ligand-induced association with ER $\alpha$ . We therefore asked whether ERAP140 is capable of interacting with other members of the nuclear receptor family. A GST pull-down assay was employed

to investigate the in vitro interactions between ERAP140 and various nuclear receptors. The  $\lambda$ 7 fragment of ERAP140 was fused to the GST protein, immobilized on the glutathione-Sepharose column, and incubated with <sup>35</sup>S-labeled in vitro-translated nuclear receptors in the absence or presence of 1  $\mu$ M concentrations of their respective ligands. GST alone was included as a negative control (Fig. 5). ERAP140 showed a low level of ligand-independent constitutive binding to ER $\alpha$ , ER $\beta$ 1, ER $\beta$ 2, TR $\beta$ , PPAR $\gamma$ , and RAR $\alpha$ . Binding was further induced by the presence of ligands. In contrast, ERAP140 did not interact with RXR $\alpha$  in the absence or presence of the ligand. Thus, although ERAP140 can interact with a wide spectrum of nuclear receptors, it does display a certain degree of receptor selectivity.

**ERAP140 interacts with ER $\alpha$  via a noncanonical interaction motif.** Nearly all nuclear receptor coactivators that have been identified possess one or several LXXLL signature motifs (NR boxes) that mediate the receptor-coactivator interaction. However, the ERAP140 protein sequence lacks any apparent LXXLL motifs. To determine the domain of ERAP140 that is required for the functional interaction with ER $\alpha$ , we performed deletional and mutational analyses.  $\lambda$ 7 was the partial cDNA clone initially isolated from the far-Western screen, indicating that the ER $\alpha$  interaction domain in ERAP140 resides between amino acids 181 and 637. To further define the ER $\alpha$  interaction region, we first performed exonuclease digestion and generated serial deletion mutants, which we tested for estradiol-dependent binding to GST-ER LBD in GST pull-down assays. The experiments revealed that a central domain from amino acids 395 to 637 retained the ER $\alpha$  association (data not shown). This domain was then further dissected into smaller fragments and fused to GST. The fusion proteins were incubated with <sup>35</sup>S-labeled in vitro-translated ER $\alpha$  in the absence or presence of either the ER agonist E<sub>2</sub> or the antagonist tamoxifen. The level of ER $\alpha$  binding was compared to that of the  $\lambda$ 7 fragment. As shown in Fig. 6A, ERAP 489-637 but not ERAP 395-488 retained agonist-induced ER $\alpha$  binding, and within this region, ERAP 489-559 contained ER $\alpha$  binding activity comparable to those of  $\lambda$ 7 and ERAP 489-637.

Although the sequence between residues 489 and 559 does not possess any LXXLL, computer analyses of the secondary structure of this sequence predicted a short central  $\alpha$ -helical domain (Fig. 6B), which is a common structural feature mediating protein-protein interactions. This domain contains several leucine (L) and isoleucine (I) residues that have been demonstrated to be essential in maintaining the integrity of helical structure. To determine whether these residues are directly involved in ER $\alpha$  interaction, we made two mutant ERAP 489-559 fragments with various leucines or isoleucines mutated to alanines (A). These two mutant fragments were then assayed for their ER $\alpha$  binding abilities (Fig. 6B). E<sub>2</sub>-induced ER $\alpha$  binding was not impaired by mutations in Mut A. On the other hand, the interaction was completely abolished by mutations in the Mut B construct. These findings indicate the leucine residue 523 and/or the isoleucine 524 are critical for ER $\alpha$  interaction. L523 and I524 lie in the center of the predicted  $\alpha$ -helix, and their mutations would likely perturb the helical protein configuration.

**ERAP140 binds ER $\alpha$  on a surface, similar to the p160 co-**

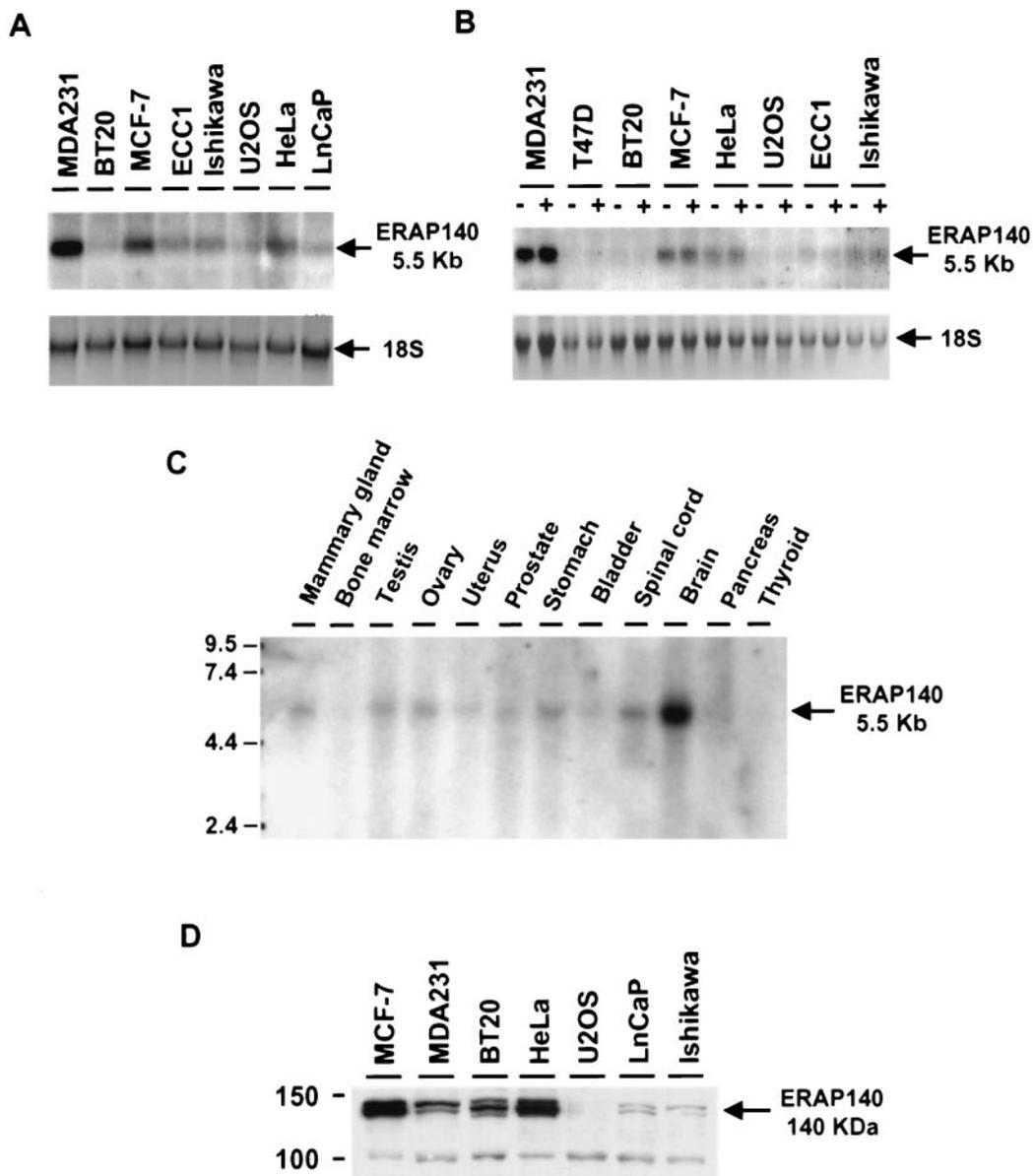


FIG. 3. ERAP140 mRNA and protein are expressed at different levels in various tumor cell lines and human tissues. (A) ERAP140 mRNA levels vary among different tumor lines. Cells were cultured under estrogen-free conditions for 3 days prior to being harvested. Total RNA was prepared from each cell type and analyzed by Northern blotting; 18S was included as a loading control. (B) ERAP140 mRNA level is not regulated by estradiol. Cells were cultured under estrogen-free conditions for 3 days, followed by treatment with (+) or without (-) 1  $\mu$ M E<sub>2</sub> for 6 h. Total RNA was then collected and analyzed by Northern blotting. (C) ERAP140 mRNA is expressed at low levels in most of the human tissues examined, with the exception of the brain, where it is highly expressed. Northern analysis was performed on a blot containing 1  $\mu$ g of poly(A)<sup>+</sup> RNA per lane from 12 different human tissues (Clontech). (D) ERAP140 protein is expressed at different levels in a variety of tumor lines. Twenty micrograms from each cell line was resolved on SDS-PAGE and analyzed by Western blotting using the polyclonal antibody raised against the  $\lambda$ 7 fragment.

**activator.** Even though ERAP140 lacks the NR-binding signature motif, LXXLL, its ligand-inducible interaction with ER, as well as with other nuclear receptors, strongly indicates that it might bind in a fashion similar to that of other coactivators. In an effort to gain insights into the binding mode of ERAP140 to ER $\alpha$  in comparison with other coactivators, we first assessed whether the ER $\alpha$  interaction domain within ERAP140 could compete out ER $\alpha$  binding of the p160 coactivator, mGRIP1.

The interaction between GST-ER LBD and mGRIP1 was examined in the absence or presence of increasing concentrations of purified fragment ERAP 489-559 or 489-559 Mut B (Fig. 7A). The ER $\alpha$ -mGRIP1 interaction was greatly inhibited when a 100 nM concentration of the wild-type ERAP 489-559 peptide was added; the interaction was completely abrogated by 1  $\mu$ M ERAP 489-559. On the other hand, the Mut B that impaired ER $\alpha$  binding could not compete with mGRIP1 for

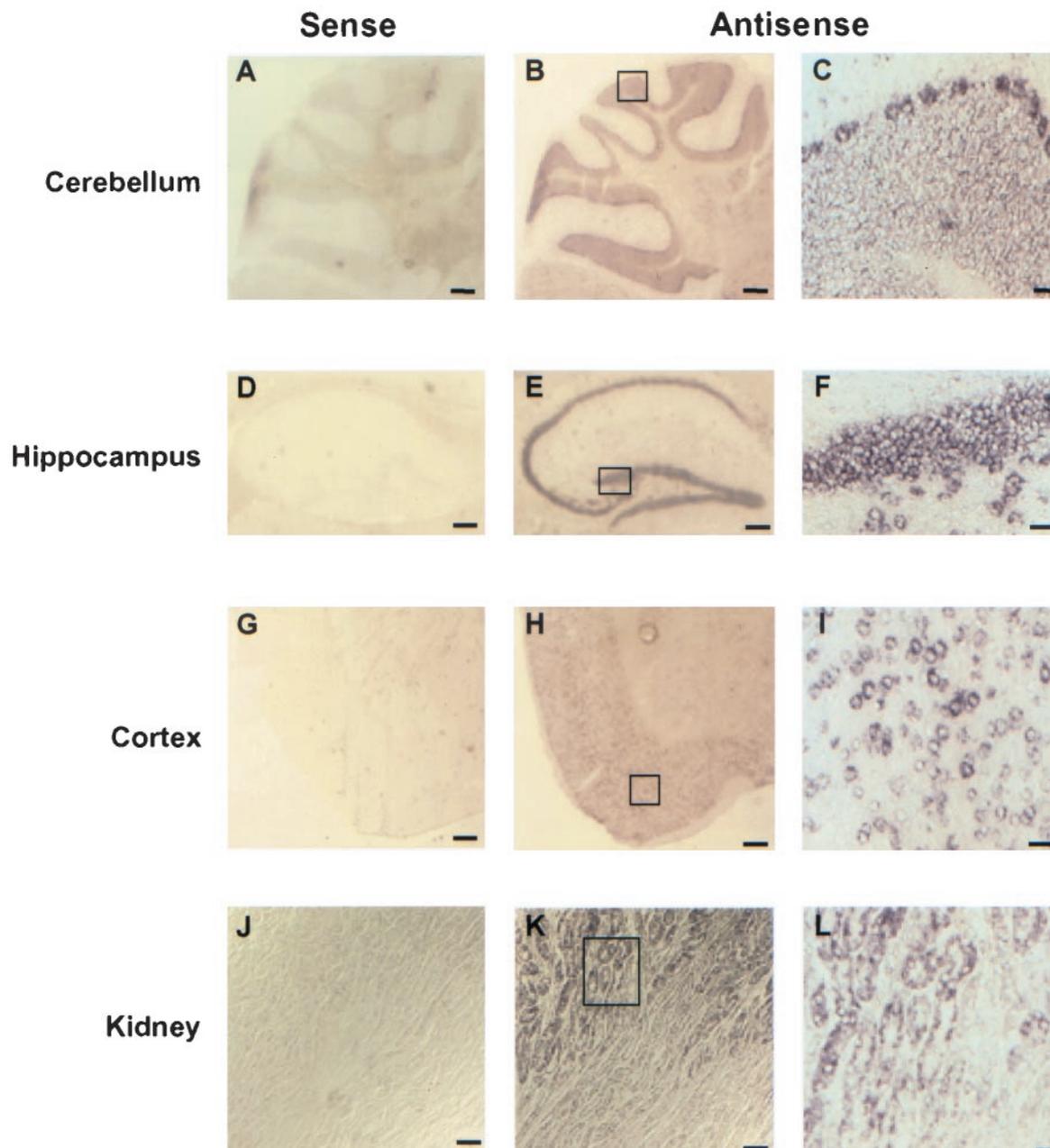


FIG. 4. ERAP140 is specifically expressed in the mouse renal cortex and in neurons in the brain by in situ hybridization. The areas indicated within the boxes in panels B, E, H, and K were enlarged for better visualization in panels C, F, I, and L. DIG-labeled antisense RNA was used to detect ERAP140 mRNA in a sagittal section of an adult mouse brain; the neuron-specific localization of ERAP140 mRNA is demonstrated in cerebellum (B and C), hippocampus (E and F), and cerebral cortex (H and I). In the cerebellum, ERAP140 mRNA was found in all three layers, including the molecular layer, the Purkinje cell layer, and the granular layer. ERAP140 mRNA in the hippocampus was localized in the pyramidal cell layer, and in the cerebral cortex, it was localized in all neuronal cells. In the kidney, ERAP140 mRNA was found in the cortex but not in the medulla (K and L). Sense RNA was used as a negative control (A, D, G, and J). Bars: A and B, 500  $\mu\text{m}$ ; C, 50  $\mu\text{m}$ ; D, E, G, and H, 300  $\mu\text{m}$ ; F and I, 30  $\mu\text{m}$ ; J and K, 120  $\mu\text{m}$ ; L, 12  $\mu\text{m}$ .

binding to ER $\alpha$ . The 489-559 Mut A construct, which retains ER $\alpha$  interaction, could inhibit mGRIP1 binding to ER $\alpha$  as efficiently as the wild type (data not shown). These observations confirm that the ER $\alpha$  binding domain is located between amino acids 489 and 559 in ERAP140. Further, although the binding domain does not contain the LXXLL motif, it appears to interact with ER $\alpha$  through a binding surface similar to that

of other coactivators. This notion is supported by our finding that ER $\alpha$ -ERAP140 association requires an intact AF-2 domain of ER $\alpha$  (Fig. 2B). We then performed the reciprocal experiment, in which the interaction between ER $\alpha$  and ERAP140 was analyzed in the absence or presence of peptides containing the sequences of NR boxes taken from mGRIP1 (Fig. 7B). mGRIP1 NR consists of two NR boxes, whereas

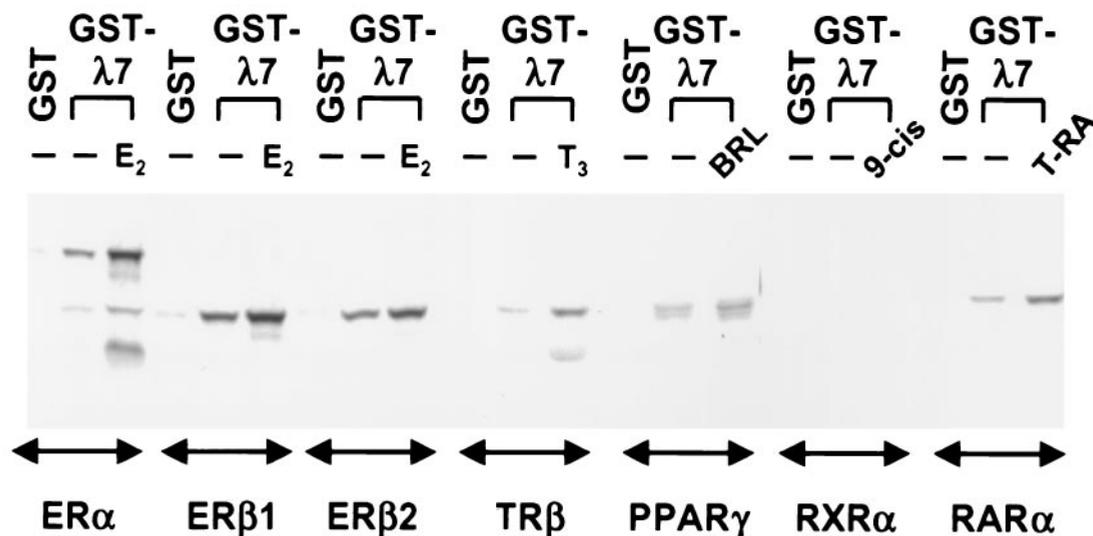


FIG. 5. ERAP140 interacts with a specific subset of nuclear receptors in vitro. GST- $\lambda$ 7 fusion protein was incubated with [ $^{35}$ S]methionine-labeled in vitro-translated receptors in the absence (–) or presence of a 1  $\mu$ M concentration of their respective ligands. GST alone was included as a negative control.

mGRIP NR2 contains only the second NR box (16). In the sequence of mGRIP mutNR2, mutations of leucine residues to alanines have been shown to lead to loss of NR binding.  $^{35}$ S-labeled in vitro-translated ERAP140 was incubated with GST-ER LBD in the absence or presence of  $E_2$ , and increasing amounts of peptides were then added to the reaction. mGRIP NR, starting at 10 nM concentration, inhibited ERAP140 binding to ER $\alpha$  by nearly half and completely abolished the binding at 100 nM. Compared to mGRIP NR, mGRIP NR2 was less potent in inhibiting binding of ERAP140 to ER $\alpha$ : the inhibition was observed starting at 100 nM and was complete at 1  $\mu$ M. This is consistent with the fact that mGRIP NR2 contains only one of the two NR boxes. In contrast, mGRIP mutNR2 has lost the ability to compete with the binding of ERAP140 to ER $\alpha$ . These results are consistent with our hypothesis that ERAP140 binds to an ER $\alpha$  surface similar to that of other coactivators. In addition, the comparable concentrations at which ERAP 489-559 and mGRIP NR2 inhibited ER $\alpha$  binding suggests that domain 489 to 559 contains one receptor binding motif.

**ERAP140 enhances the transcriptional activities of several nuclear receptors.** We have demonstrated that in addition to ER $\alpha$ , ERAP140 associates with various other nuclear receptors in a ligand-induced fashion. The ER-ERAP140 association is mediated via a novel motif located in the center of ERAP140 that can be competed with LXXLL-containing NR boxes found in many coactivators. To further test the hypothesis that ERAP140 functions as a nuclear receptor coactivator, we examined the effect of ERAP140 on receptor-mediated transcriptional activity. Transient-cotransfection experiments were conducted in a hepatocarcinoma cell line, HepG2, which expresses low levels of ERAP140 (data not shown). The transactivation level of a luciferase reporter gene in the absence or presence of specific ligand was measured and is represented in Fig. 8A. On an ERE-containing reporter,  $E_2$  treatment led to

a 2.5-fold activation, which was further increased by more than 50% by the addition of ERAP140. ERAP140 coactivation was seen with several other receptors with which it interacts, including TR $\beta$ , PPAR $\gamma$ , and RAR $\alpha$ . No activity of ERAP140 was seen with RXR $\alpha$ , with which it does not interact in vitro (data not shown). Therefore, ERAP140 can function as a coactivator for ER $\alpha$ , as well as several other nuclear receptors.

We then investigated whether ERAP140 itself could act as an activator when tethered to DNA. For this, we fused the ERAP140 cDNA to the GAL4 DBD and transfected various amounts of this expression plasmid into Cos-1 cells. The activity level on a (GAL4 X 5)SV40-Luc reporter was measured by luciferase assay (Fig. 8B). In comparison to GAL4 DBD, GAL4 DBD-ERAP140 activated the GAL4 reporter transcription in a dose-dependent fashion. Thus, ERAP140 exhibits transcriptional activity when tethered to a target gene promoter.

**ERAP140 functions on the promoter of an estrogen target gene in a fashion similar to those of other NR coactivators.** We have shown above that ERAP140 possesses transcriptional activity when recruited to a promoter and enhances ligand-dependent receptor transactivation on its response element in vitro. To address the question of how ERAP140 may function on an endogenous promoter of the receptor target gene under biologically relevant conditions, we performed CHIP assays. CHIP permits direct detection of proteins at their in vivo binding sites; in particular, proteins that are not bound directly to DNA or that depend on other proteins for binding can be analyzed by this method, thus allowing examination of dynamic assembly of protein complexes on the promoter. We first analyzed the recruitment of ERAP140 to one of the estrogen target genes, pS2, in comparison to ER $\alpha$  and the p160 coactivator, AIB1. To circumvent the poor immunoprecipitation quality of an anti-ERAP140 antibody, we made a T7-tagged ERAP140 construct and transiently expressed it in MCF-7

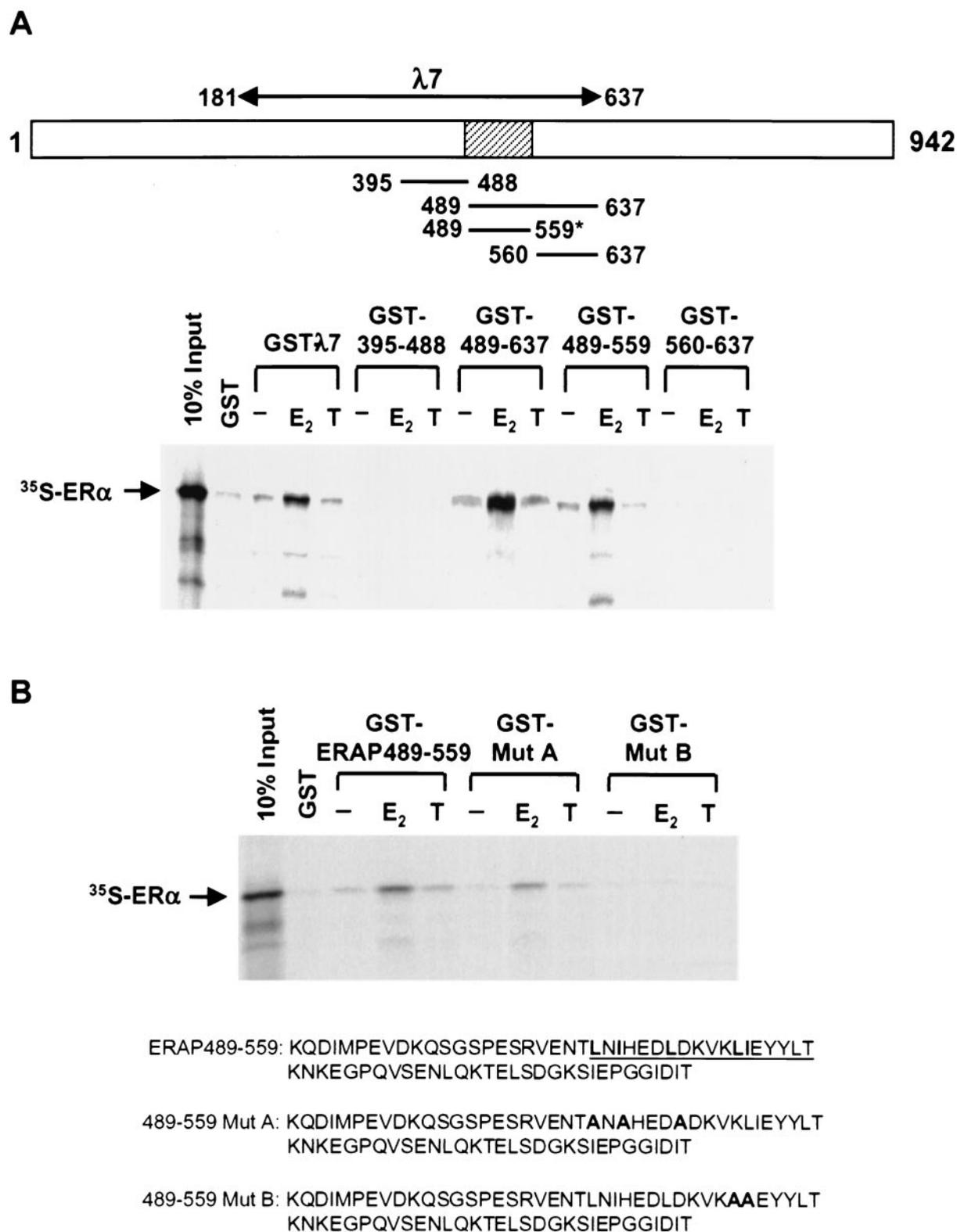
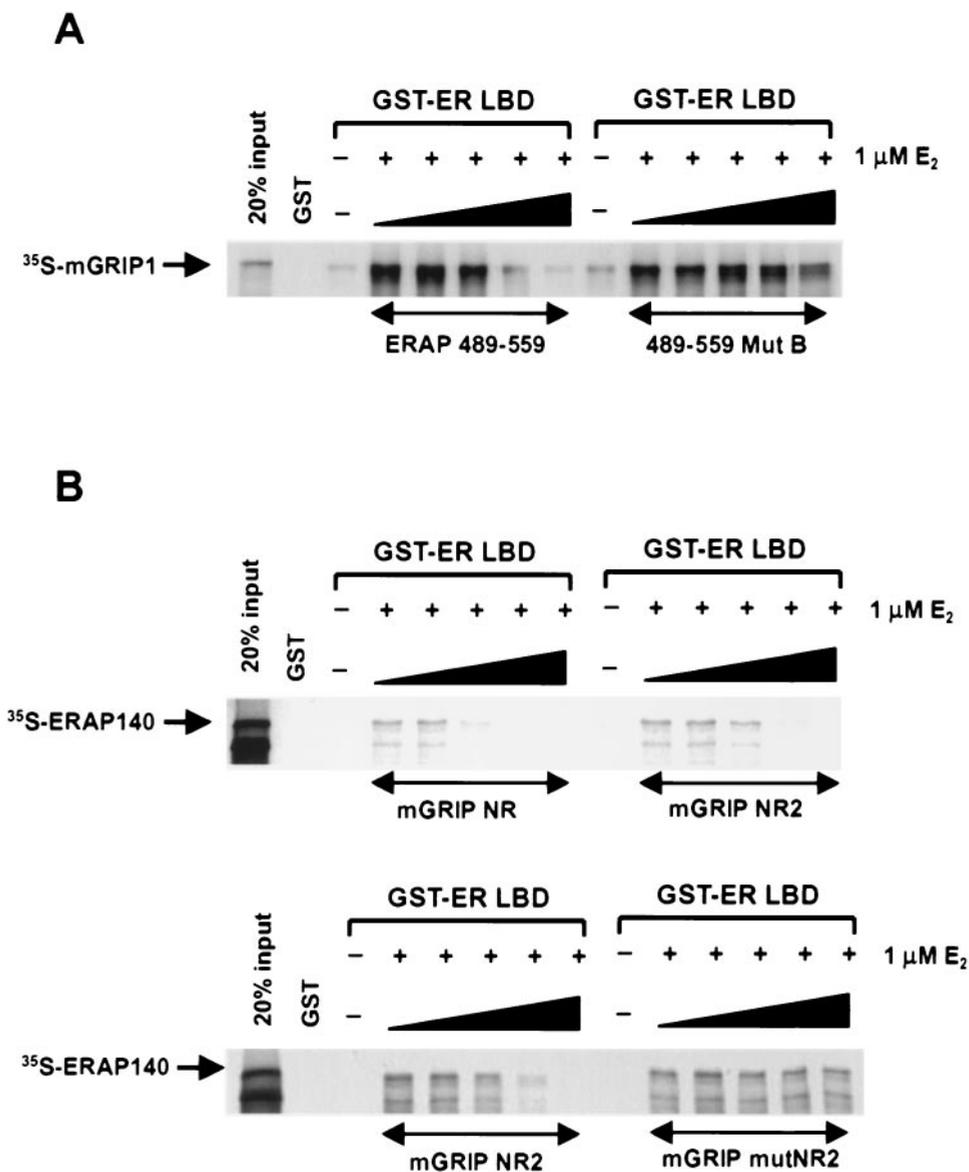


FIG. 6. The ER $\alpha$  interaction domain maps to a central region in ERAP140 between amino acids 489 and 559 in which the L523 and I524 residues are essential for ER $\alpha$  binding. (A)  $\lambda 7$  and different fragments of ERAP140 are indicated in the schematic diagram. The fragments were fused to GST and incubated with <sup>35</sup>S-labeled ER $\alpha$  in the absence (–) or presence of 1  $\mu$ M E<sub>2</sub> or an ER antagonist, tamoxifen (T). Ten percent of the ER $\alpha$  used in the binding reaction was included as input. (B) Specific leucine and isoleucine residues within fragment ERAP 489-559 were mutated to alanines (boldface), and the mutants were examined for their ER $\alpha$  binding ability in the absence (–) or presence of 1  $\mu$ M E<sub>2</sub> or T. The underlined sequence within ERAP 489-559 was predicted to be an  $\alpha$ -helical structure by computer analysis.



mGRIP NR: SKGQTK**LLQLL**TTKSDQMEPSPLASSLSDTNKDSTGSLPGSGSTHGTSLSKEKHK**LHRL**LQDSS

mGRIP NR2: KHK**LHRL**LQDSS

mGRIP mutNR2: KHK**LHRAA**LQDSS

FIG. 7. ERAP140 interacts with ER $\alpha$  through a binding surface similar to that of the p160 coactivator, mGRIP1. (A)  $^{35}\text{S}$ -labeled in vitro translated mGRIP1 was incubated with GST-ER LBD in the absence (-) or presence (+) of 1  $\mu\text{M}$  E $_2$ . Increasing concentrations (1, 10, and 100 nM and 1  $\mu\text{M}$ ) of purified wild-type ERAP 489-559 fragment or 489-559 Mut B was added to the reaction. (B) The interaction between GST-ER LBD and  $^{35}\text{S}$ -ERAP140 was examined in the absence (-) or presence (+) of 1  $\mu\text{M}$  E $_2$ , with increasing doses of peptides added. The sequences of the peptides are indicated at the bottom; the NR boxes are in boldface and underlined.

cells. The transfected cells were cultured in the absence of estrogen for 3 days, followed by treatment with either vehicle (ethanol) alone or 100 nM E $_2$  for 45 min. The binding of proteins on the estrogen response element in the pS2 promoter was determined by immunoprecipitation with specific antibodies. The status of protein complexes present on the promoter

was reflected by changes in the level of coprecipitated DNA analyzed by PCR using a primer pair flanking the estrogen response element. As shown in Fig. 9A, in pcDNA/T7-ERAP140-transfected cells, estradiol treatment induced a dramatic recruitment of ER $\alpha$ , AIB1, and T7-ERAP140 to the promoter. Western analysis (Fig. 9B) demonstrated that the

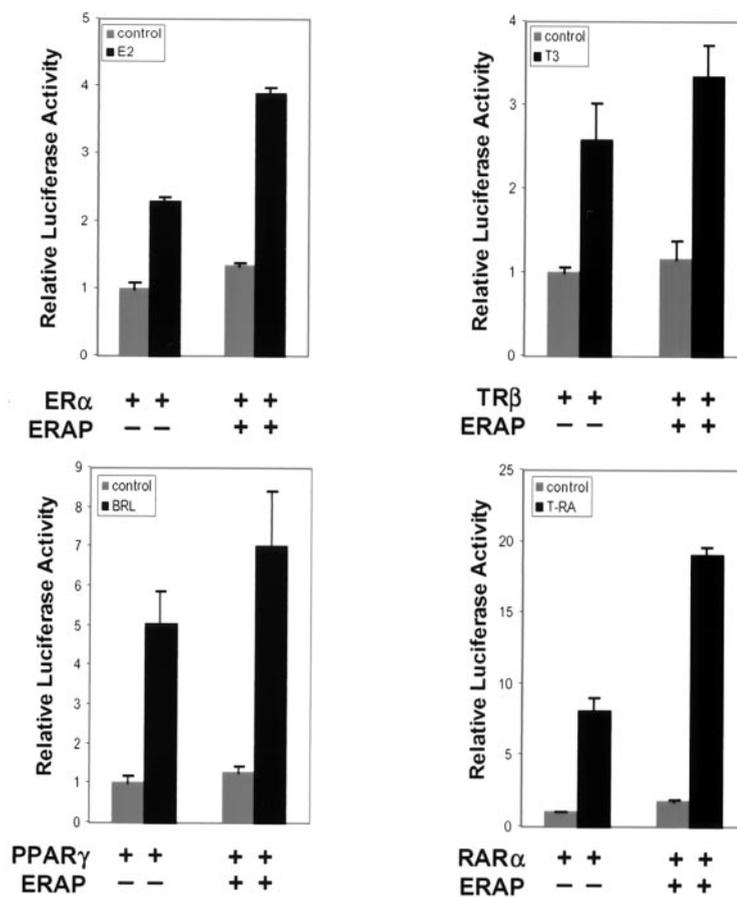
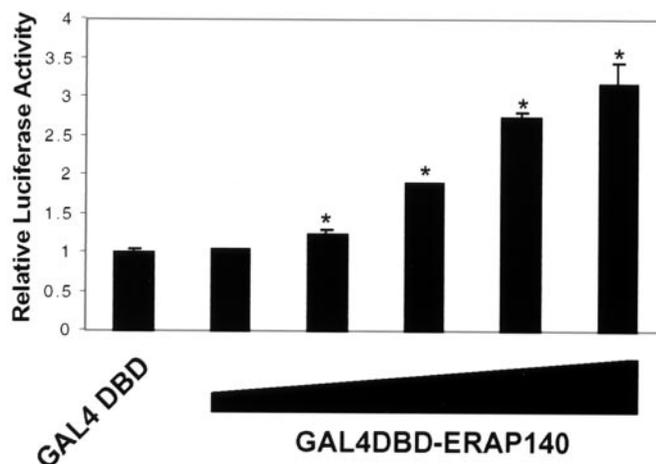
**A****B**

FIG. 8. ERAP140 enhances nuclear receptor transactivation and possesses intrinsic transcription activity when tethered to DNA. (A) HepG2 cells were plated under hormone-free conditions overnight before transfection. The cells were cotransfected with nuclear receptors and their respective reporter genes, and 100 ng of pcDNA3.1-ERAP140 was either included (+) in the transfection or not (-). At 20 h posttransfection, the cells were treated with vehicle (control) or 100 nM E<sub>2</sub>, 100 nM T<sub>3</sub>, 1  $\mu$ M BRL, or 100 nM T-RA as indicated and harvested after 24 h of incubation. Transcriptional activity was measured via luciferase assay. (B) Cos-1 cells were cotransfected with 100 ng of pCMX- $\beta$ Gal, 100 ng of (GAL4 X 5)SV40-Luc reporter, and 400 ng of GAL4 DBD or increasing concentrations (100, 200, 400, and 800 ng and 1  $\mu$ g) of the GAL4 DBD-ERAP140 fusion gene. The level of transactivation was represented by the relative luciferase activity. The level of coactivation by ERAP140 compared to that of GAL4 DBD alone is statistically significant ( $P < 0.05$ ) starting from a 200-ng concentration, as indicated by the asterisks. All experiments were performed multiple times with duplicate samples, and the results shown are representative of those studies. The error bars indicate standard deviations.

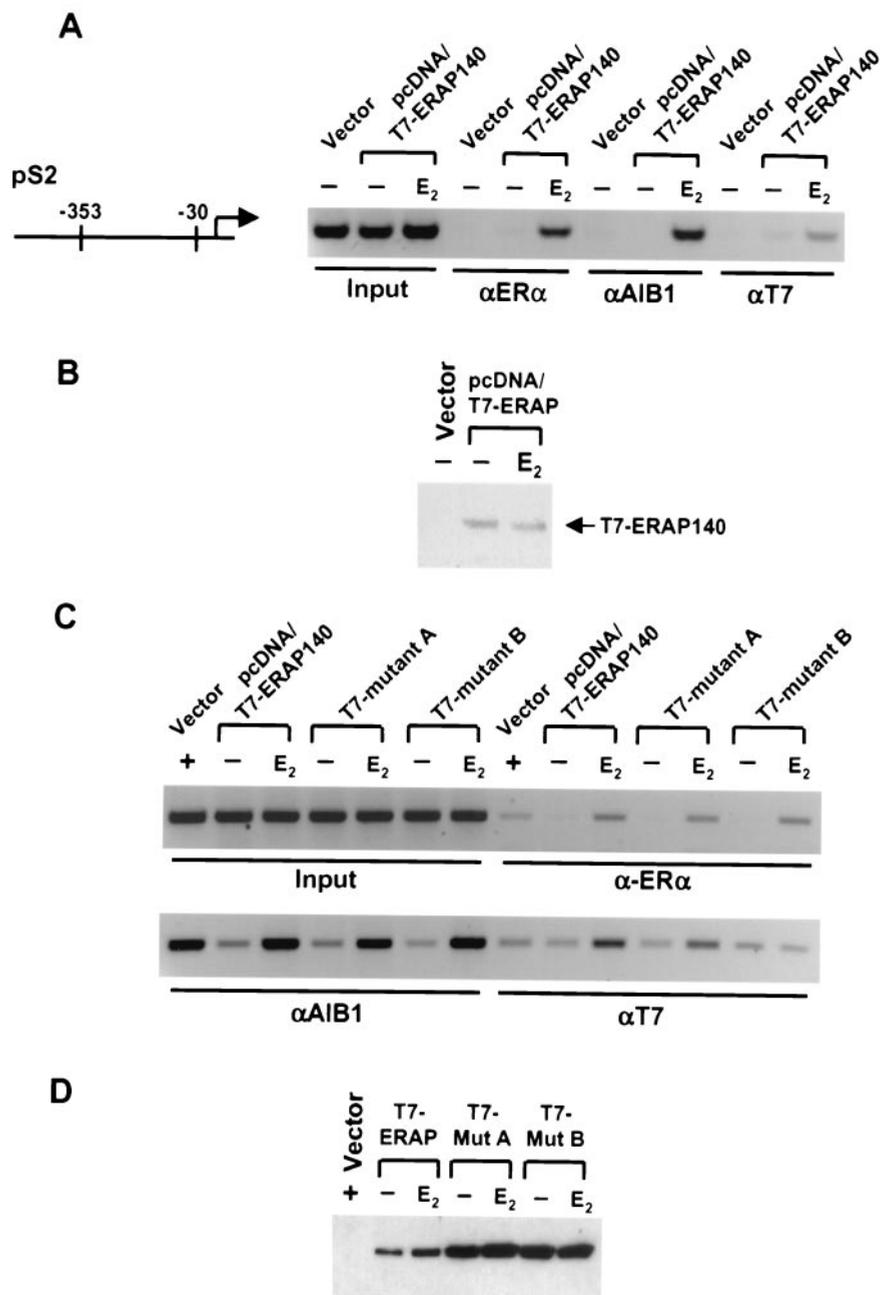


FIG. 9. In vivo, ERAP140 is recruited by ER $\alpha$  to the promoter of an endogenous ER target gene, pS2. (A and C) The in vivo binding of ER $\alpha$ , AIB1, and ERAP140 to the pS2 promoter was examined by the ChIP assay. Vector alone, pcDNA/T7-ERAP140 (A), or either of the two T7-tagged ERAP140 mutants (C), mutant A or mutant B, was transiently expressed in MCF-7 cells. Soluble chromatin was prepared from cells treated with 100 nM E<sub>2</sub> for 45 min (+) or untreated (-) and immunoprecipitated with antibodies against ER $\alpha$ , AIB1, and the T7 tag. Coprecipitated DNA was amplified using primers that flank the ERE in the pS2 promoter region; the 5' end of the primers relative to the transcriptional initiation site is indicated in the diagram in panel A. The presence of total pS2 promoter DNA in the soluble chromatin prior to immunoprecipitation was included as input. (B and D) Expression of T7-tagged wild-type or mutant ERAP140 protein in MCF-7 cells transfected with respective plasmids. Whole-cell extracts were prepared 2 days after transfection and subjected to Western blot analysis with an anti-T7 antibody.

increased binding of T7-ERAP140 to the pS2 promoter by E<sub>2</sub> was not due to changes in the protein expression level. Mutations at residues 523 and 524 in ERAP140 completely abolished ER $\alpha$  association in vitro (Fig. 6B). To verify that ERAP140 becomes recruited to the estrogen target gene pro-

motor via its ER $\alpha$  binding, we introduced L523A and I524A mutations into T7-ERAP140 (T7-mutant B), and compared its pS2 promoter binding with that of the wild-type T7-ERAP140 and the T7-mutant A containing mutations that do not impair ER $\alpha$  binding (Fig. 6B). As a result, both wild-type T7-

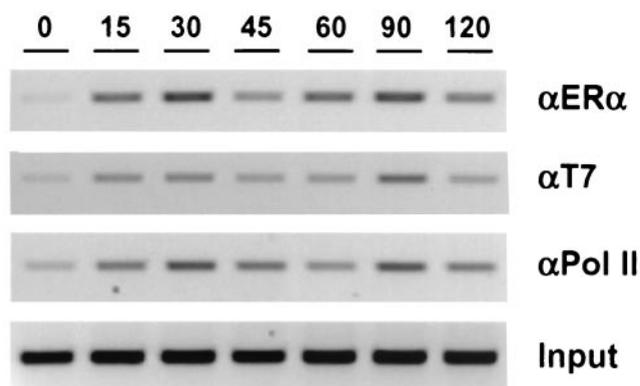


FIG. 10. ERAP140 is recruited to the pS2 promoter in a dynamic fashion. MCF-7 cells were transiently transfected with pcDNA/T7-ERAP140, and 2 days after transfection, the cells were treated with 100 nM  $E_2$  for the times indicated. Soluble chromatin was then prepared from the cells, followed by immunoprecipitation using either anti-ER $\alpha$ , anti-T7, or anti-RNA Pol II antibody. The coprecipitated DNA was amplified by PCR.

ERAP140 and T7-mutant A were recruited to the pS2 promoter after  $E_2$  stimulation, whereas T7-mutant B was not (Fig. 9C), suggesting that ERAP140 becomes associated with the promoter through its ER $\alpha$  interaction. Consistent with our data in Fig. 9A, ER $\alpha$  and AIB1 came to promoter association following  $E_2$  treatment. Overexpression of T7-mutant B did not affect binding of ER $\alpha$  or AIB1 to the promoter. The expression of either wild-type or mutant T7-ERAP40 proteins was analyzed by Western blotting and shown to remain the same with or without  $E_2$  treatment (Fig. 9D). Taken together, these findings provide *in vivo* evidence that, similar to other ER $\alpha$  coactivators, ERAP140 interacts with ER $\alpha$  in the presence of  $E_2$ , which in turn leads to ERAP140 association to the target gene promoter, resulting in transcriptional coactivation.

**ERAP140 is recruited to the estrogen target gene promoter in a cyclic fashion.** Having established that ERAP140 functions on the ER target gene promoter, we next sought to investigate the association of ERAP140 with the pS2 promoter at various time points in comparison to those of ER $\alpha$  and RNA polymerase II (Pol II). As shown in Fig. 10, ER $\alpha$  became rapidly bound to the pS2 promoter, within 15 min following  $E_2$  stimulation; the binding peaked by 30 min and had declined by 45 min, followed by a second cycle of promoter association in the presence of continuous estrogen stimulation. The assembly of T7-ERAP140 and RNA Pol II on the promoter showed an order and timing similar to those of ER $\alpha$ . These results suggest that ER $\alpha$ , ERAP140, and RNA Pol II act simultaneously on the same promoter.

## DISCUSSION

In the present study, we report the identification and characterization of a novel nuclear receptor coactivator, ERAP140, that is conserved among various species. ERAP140 interacts with both types of ER receptors, as well as a wide array of nuclear receptors, in a ligand-inducible fashion. The ER $\alpha$  association is mediated via a noncanonical LXXLL motif located in the central domain of ERAP140. When induced by  $E_2$ ,

ERAP140 complexes with ER $\alpha$  and is recruited to the promoter region of the ER target gene, which subsequently leads to transcriptional coactivation. In addition, ERAP140 exhibits intrinsic transcriptional activity when tethered to DNA.

Our findings strongly suggest ERAP140 functions as a receptor coactivator. First, ERAP140 was isolated on the basis of its interaction with ER $\alpha$  LBD in the presence of ER $\alpha$  agonists but not antagonists. In addition to both ER $\alpha$  and ER $\beta$ , ERAP140 exhibits specific ligand-inducible interactions with TR $\beta$ , PPAR $\gamma$ , and RAR $\alpha$  but not with RXR $\alpha$ . Thus, ERAP140 represents a novel coactivator with a distinct pattern of receptor selectivity. Second, the ER $\alpha$  binding mode of ERAP140 corresponds to its role as a coactivator. ERAP140, similar to other coactivators, requires the integrity of the ER $\alpha$  AF2 domain for receptor interaction. However, ERAP140 lacks the canonical LXXLL signature motif present in most of the coactivators. Instead, ERAP140 contains a central domain consisting of a short  $\alpha$ -helix that mediates ER $\alpha$  binding. This  $\alpha$ -helical structure likely resembles that of the LXXLL, which docks to the hydrophobic cleft on the surface of the receptor LBD. This notion is supported by our *in vitro* competition assays among ER $\alpha$ , ERAP140, and mGRIP1; the ERAP140 fragment encompassing the  $\alpha$ -helix can effectively block the binding of mGRIP1 to ER $\alpha$ , whereby a mutated form of the fragment is unable to compete ER $\alpha$ -mGRIP1 binding due to disruption of the  $\alpha$ -helix. The functional role of ERAP140 as a coactivator is further supported by *in vitro* transfection-based assays which demonstrate that ERAP140 enhances ligand-dependent transcription on synthetic receptor DNA response elements. More direct evidence that ERAP140 plays a role in receptor-mediated gene activation comes from our ChIP data. The ChIP technique allows a direct assessment of the ERAP140 action on the promoter of endogenous receptor target genes—in this case, the ER target gene, pS2—in relation to other coactivators. Following  $E_2$  stimulation, ERAP140 becomes recruited to the pS2 promoter through its ER $\alpha$  association, since an ERAP140 mutant that does not interact with ER $\alpha$  loses its promoter binding ability. The promoter occupancy by ERAP140 shows a cyclic pattern similar to that which has been proposed for other ER coactivator complexes (11, 41). The timing of ERAP140 bound to the promoter is synchronous with that of ER $\alpha$  and RNA Pol II. These findings are in line with the previous observations, which show that different cofactors are recruited to the promoter in a stepwise process and exhibit distinct and nonredundant roles in transcription initiation (13, 30, 41). Determination of the precise assembly of ERAP140, as well as other receptors and coactivators, on different target gene promoters may provide useful information regarding receptor-specific mechanisms by which ERAP140 regulates transcription.

ERAP140 shows no sequence similarity to other nuclear receptor coactivators, again supporting the hypothesis that ERAP140 belongs to a novel class of NR coactivators. Interestingly, homologues of ERAP140 have been identified in a variety of species, ranging from humans to *C. elegans*. The *Drosophila* homologue of ERAP140 is encoded by the L82 gene, which produces seven different isoforms, L82A through L82G. The longest isoform, L82A, consists of all three homology domains present in ERAP140, and all isoforms contain a carboxyl-terminal core that corresponds to the homology do-

main III. The L82 gene is responsible for the 82F late-late puff, which is a region of chromosome decondensation in response to ecdysone pulses during the larva-to-adult morphogenesis. Loss of L82 function leads to developmental delay and lethality at eclosion, resulting from failure to release the adult fly from the pupal case (44). Very recently, *Drosophila* homologues for two other coactivators have been identified (4, 5). The AIB1 homologue, *Taiman*, is required for follicle cell migration in the *Drosophila* ovary, whereas the TIF1 homologue, *Bonus*, plays a critical role in the development of several organs and tissues during embryogenesis and metamorphosis. In addition to *Drosophila*, ERAP140 homologues have been found in mouse and *C. elegans*. The mouse C7 gene was identified among genes that are up-regulated upon attachment to extracellular membrane; its encoded protein is predominantly localized to nucleoli (17). The function of the *C. elegans* homologue is presently unknown.

The protein sequence of ERAP140 reveals little in terms of functional motifs. A bipartite nuclear localization signal located at the N terminus suggests ERAP140 is a nuclear protein, and our Western analysis of different cellular fractions confirms ERAP140 is primarily localized in the nucleus (data not shown). The carboxyl-terminal domain III is conserved in all ERAP140 homologues; furthermore, this domain is also highly conserved in yeast and in a human gene, hOXR1, which was discovered in a search for genes that confer protection against oxidative damage (48).

Most NR cofactors described to date can be found in various cells and tissues; in contrast, ERAP140 expression is both cell and tissue type specific. It is especially abundant in the brain compared to all other tissues, and further, in situ hybridization data reveal that ERAP140 is exclusively expressed in neurons in the brain. The mammalian brain is clearly a target organ of steroid hormone action. Particularly, estrogen is a key regulator in the brain of functions associated with the neuroendocrine control of reproduction and associated behaviors. Moreover, estrogen has been shown to play a role in neuroprotection. Epidemiological studies suggest that estrogen exposure decreases the risk and delays the onset and progression of Alzheimer's and Parkinson's diseases and enhances recovery from neurological trauma, such as stroke (reviewed in reference 19). However, the mechanisms by which estrogen regulates brain function remain elusive. Studies have indicated that the mechanisms are likely via multiple pathways, among which the nuclear receptor pathway is considered of critical relevance. Reports of both ER $\alpha$  and ER $\beta$  knockout mice have revealed distinct phenotypes in the neuroendocrine system resulting in severe defects in sexual and field behavior in both female and male mice (reviewed in reference 14). Preliminary research on the influence of nuclear receptor coactivators in neuroendocrine function has demonstrated that these coactivators are critical for normal hormone-dependent development of the brain (reviewed in reference 45). Although SRC-1 knockout mice are fertile and have a growth rate similar to their wild-type littermates, they have decreased growth and development in response to steroid hormones (50). Further, reducing SRC-1 levels in neonatal rat brain interferes with hormone-mediated sexual differentiation of the brain (3). Disruption of another coactivator, CBP, also leads to brain dysfunction. In humans, mutation in the CBP gene causes Rubinstein-Taybi syndrome, which is characterized by severe mental retardation and craniofacial abnormalities (38).

Heterozygous CBP mutant mice have deficits in long-term memory. A novel neuron-specific cofactor, NIX1, was cloned recently by its interaction with the brain-specific orphan receptor ROR $\beta$ . NIX1 binds RAR, TR, and VDR in the presence of their ligands but does not bind RXR or steroid hormone receptors, and it inhibits receptor-mediated transcription (20). These reports suggest that nuclear receptors and their cofactors play essential roles in the nervous system. Our finding that the ERAP140 transcript is highly expressed in neurons raises the possibility that one of the ERAP140 functions is to mediate hormone-dependent gene activation in the brain. It will be interesting to determine whether the expression pattern of ERAP140 can be correlated with those of nuclear receptors and other cofactors and whether its expression can be manipulated to affect hormone signaling pathways in the brain.

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