Cloning and Characterization of RAP250, a Novel Nuclear Receptor Coactivator*  

(Received for publication, October 28, 1999, and in revised form, November 24, 1999)  

Françoise Cair,‡§, Per Antonson‡§, Markku Pelto-Huikko‡‡, Eckardt Treuter‡‡, and Jan-Åke Gustafsson  

From the Department of Biosciences at Novum, Karolinska Institutet, S-14157 Huddinge, Sweden, the Department of Developmental Biology, Tampere University Medical School, and the ‡‡Department of Pathology, Tampere University Hospital, P. O. Box 807, Fin-33101 Tampere, Finland  

Ligand-induced transcriptional activation of gene expression by nuclear receptors is dependent on recruitment of coactivators as intermediary factors. The present work describes the cloning and characterization of RAP250, a novel human nuclear receptor coactivator. The results of in vitro and in vivo experiments indicate that the interaction of RAP250 with nuclear receptors is ligand-dependent or ligand-enhanced depending on the nuclear receptor and involves only one short LXXLL motif called nuclear receptor box. Transient transfection assays further demonstrate that RAP250 has a large intrinsic glutamine-rich activation domain and can significantly enhance the transcriptional activity of several nuclear receptors, acting as a coactivator. Interestingly, Northern blot and in situ hybridization analyses reveal that RAP250 is widely expressed with the highest expression in reproductive organs (testis, prostate and ovary) and brain. Together, our data suggest that RAP250 may play an important role in mammalian gene expression mediated by nuclear receptor.

* This work was supported by grants from the Swedish Cancer Society and KavoBio AB, the Finnish Cancer Society and Medical Research Fund of Tampere University Hospital (Finland). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

†† These authors contributed equally to this work.

‡‡ To whom correspondence should be addressed. Tel.: 46-8-608-9160; Fax: 46-8-774-5538; E-mail: eckardt.treuter@csb.ki.se.

§§ To whom correspondence should be addressed. Tel.: 46-8-608-9160; Fax: 46-8-774-5538; E-mail: eckardt.treuter@csb.ki.se.

1 The abbreviations used are: NR, nuclear hormone receptor; aa, amino acids; AF, activation function; ER, estrogen receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GST, glutathione S-transferase; bp, base pair(s); kb, kilobase pair(s); DBD, DNA binding domain; LBD, ligand binding domain; mut, mutated; PCR, polymerase chain reaction; PPAR, peroxisome proliferator-activated receptor; RACE, rapid amplification of cDNA ends; RA, retinoic acid; RXR, retinoid X receptor; TR, thyroid hormone receptor; RLU, relative light units.

The nuclear receptor (NR)1 superfamily is a large group of structurally related transcription factors that regulate target gene transcription in response to ligands. The complex gene regulation programs regulated by NRs include biological processes such as growth, cell differentiation, and homeostasis (1). They can be divided into several subfamilies on the basis of characteristic DNA binding domains (DBDs) such as dimerization status, nature of the ligand, or structure of the DNA response element. NRs are characterized by a common domain structure, including a highly variable N-terminal region that contains a constitutive activation function (AF-1), a highly conserved DNA binding domain (DBD) responsible for recognition of specific DNA response elements, a conserved multifunctional C-terminal ligand binding domain (LBD), containing a dimerization and a ligand-dependent transactivation function (AF-2) (2). The liganded NRs bind to their cognate hormone response elements, located in the promoter or enhancer regions of target genes, and stimulate transcriptional activation by transmitting signals to the transcriptional machinery via direct protein-protein interactions (3–5). In addition, another class of proteins, called coactivators, are recruited and serve as bridging molecules between the transcription initiation complex and NRs (for reviews, see Refs. 6 and 7). Most of the coactivators interact with the AF-2 domain of NRs through one or several LXXLL motifs called NR boxes (8–12). Bona fide AF-2 coactivators include the three related members of the p160/SRC family, as well as the cointegrators CBP and p300 (for review, see Ref. 6). Because these coactivators possess intrinsic histone acetyltransferase activity and function in complex with other acetyltransferases, such as P/C/CAF, it has been proposed that functional connections exist between NR activation and the histone acetylation status. Evidence for the existence of NR-coactivator complexes came from biochemical studies identifying the TRAP/DRIP complex (13–16), which may function more directly through contacts to the basal machinery. In addition to coactivators, other AF-2-binding proteins, such as RIP140 (17, 18) or the nuclear orphan receptor SHP (19), may serve important regulatory functions by inhibiting NR activation.

To identify new potential coactivators, a mouse embryo cDNA library was screened using the yeast two-hybrid system with PPARα as bait. Here, we report the cloning and characterization of RAP250, a new NR coactivator.

EXPERIMENTAL PROCEDURES

Plasmids—All constructs were generated using standard cloning procedures and verified by restriction enzyme analysis and DNA sequencing. Details of each construction are available upon request. The partial mouse RAP250 cDNA fragment encoding amino acids 782–1138 was released from the EcoRI site of the pGAD10 clone isolated by the yeast two-hybrid system and subcloned into the EcoRI site of pGEX-4T1-1 vector (Amersham Pharmacia Biotech). GST-hERα (aa 249–595) and GST-hTRα (aa 122–410) have been described previously (19, 20). Mutated variant of RAP250 was constructed by two independent PCRs with the flanking primers and two mutagenesis primers: 5′-TTAACGAGCCCATTGGCGGTCAACGCACTACAGTGGG3′ and 5′-GTCGACTCTGTAGTGCGTTGACCGCCAATGGGCTC3′. The corresponding PCR products were isolated and combined together by an additional PCR with the flanking primers. The product of this PCR was digested by EcoRI and cloned into the corresponding site of pGEX-4T1. GST-RAP250 (aa 818–931) was generated by PCR and cloned into the EcoRI/SalI sites of pGEX-4T1.

This paper is available on line at http://www.jbc.org
NRs for in vitro translation have been expressed from the following previously described plasmids: pBKCMV-PPARa (T3), pSG5-cTRa (T7), pCMVhTRa (T3), pCMVhTRb (T7) (18), pBKCMV-RXRa (T3) (21), pThEBa (aa 1–595), and pThEBb (aa 1–485) (19). Plasmids with mutations of forms of TRa and RXRa have been described previously (21, 22). RAP250 cDNA was isolated by PCR using the NotI/Nhel sites of the plasmid pSG5Gal4(BGTBJ). These plasmids were used both for transient transfection experiments and/or to in vitro translate RAP250 partial fragments. The following eukaryotic expression vectors were used to express NRs: pSG5mPPARa, pSG5-cTRa (22), and pCMX-RXRa (21). pVP16-mRAP250 (aa 782–1138) was made by subcloning an EcoRI fragment from pGAD10-mRAP250 into pCMV-VP16. The luciferase reporter plasmids used were DR4-TRE (Luc-2), US-tk-Luc, PPRE-tk-Luc (18), and DR1-tk-Luc (23). pSG5-RAP250 plasmid used to transfect mammalian cells in the coactivation assay was obtained by subcloning the human full-length RAP250 cDNA (obtained by fusion of the 5’ end CDNA to the KIAA0181 plasmid) into pSG5 vector.

In Vitro Protein-Protein Interaction Assay (GST Pull-down Assay)—All the NRs that we tested in pull-down assays were in vitro transcribed and translated using rabbit reticulocyte lysate (TNT-coupled in vitro system Promega) according to manufacturer’s recommendations with [35S]methionine. Approximately 5 μg of GST fusion protein bound to glutathione-Sepharose 4B (Amersham Pharmacia Biotech) for 2 h at 4 °C and washed three times with phosphate-buffered saline (PBS). To produce pure GST fusion protein for electrophoretic mobility shift assay, the proteins were eluted with 4 volumes of 20 μm glutathione in 50 mm Tris-HCl (pH 8). Protein concentrations were determined by the Bradford dye binding procedure (Bio-Rad).

Electrophoretic Mobility Shift Assays—TRα and RXRa were synthesized in rabbit reticulocyte lysate by using the TNT-coupled in vitro transcription-translation system (Promega). Double-stranded synthetic oligonucleotides (5′-TCTGGTTGT-GGCTGGAGAGGAT-3′) for 30 cycles followed by a final elongation of 7 min. Each cycle consisted of 10 s at 94 °C, 10 s at 63 °C, and 1 min 30 s at 68 °C; 1 cycle consisted of 10 s at 94 °C, 10 s at 63 °C, and 1 min 30 s at 68 °C; 30 cycles followed by a final elongation of 7 min. Each cycle consisted of 10 s at 94 °C, 10 s at 63 °C, and 1 min 30 s at 68 °C; 100 mM HEPES (pH 7.9), 20% (v/v) glycerol, 100 mM KCl, 5 mM MgCl2, 0.2 mM EDTA, 0.01% Nonidet P-40, 1.5% bovine serum albumin, 1 mM dithiorthreitol, 0.2 mM phenylmethylsulfonyl fluoride supplemented with a protease inhibitor mixture (Complete, Roche Molecular Biochemicals). Beads were centrifuged (2000 × g) and washed three times for 15 min with incubation buffer without bovine serum albumin. Washed beads were resuspended in 60 μl of 1× SDS sample buffer, and an aliquot was subjected to SDS-polyacrylamide gel electrophoresis. Before autoradiography, gels were stained with Coomassie Blue to control for the stability of the GST fusion proteins and equal loading.
Fig. 1. RAP250 sequence. A, complete deduced amino acid sequence of human RAP250 cDNA. The longest open reading frame of hRAP250 starts with the first methionine. The longest open reading frame of the KIAA0181 cDNA clone, obtained from Kazusa DNA Research Institute (24), starts at methionine +78, whereas the cDNA sequence encoding the first 77 amino acids was obtained by RACE PCR. RAP250 contains two LXXLL motifs (boxed residues 887–891 and 1491–1495). The boxed part on protein sequence represents the location on human sequence of the partial clone originally isolated from the mouse embryo cDNA library (aa 782–1138). RAP250 contains two polyglutamine stretches (underlined) and, in between, a region rich in glutamine (20% Gln). The complete human protein sequence contains 2063 residues with a predicted molecular mass of 220 kDa. The nucleotide sequence contains an in-frame stop codon upstream of the first methionine (data not shown). B, in vitro translation of RAP250 cDNA. The human full-length RAP250 cDNA cloned in pSG5 was in vitro transcribed and translated using rabbit reticulocyte lysate with [35S]methionine. Radiolabeled protein was fractionated on a 7% SDS-polyacrylamide gel electrophoresis gel and visualized using autoradiography. The size of hRAP250 was estimated to be 250 kDa by comparison to the size of mPBP (TRAP220) (27) and the molecular markers. C, alignment of the mouse RAP250 partial clone and the homologue region on human protein. The residues shown in black boxes in mouse RAP250 differ from the corresponding human residues. The two RAP250 sequences share 90% identity. The 55 amino acids surrounding the LXXLL motif (LVNLL) are identical in both species, providing a large consensus sequence within the interacting region.
GenBank™ Accession Numbers—The human RAP250 nucleotide and protein sequences have been submitted to GenBank™ data base with accession number AF128458, and the partial mouse RAP250 nucleotide and protein sequences have been submitted with accession number AF135169.

RESULTS

Isolation and Cloning of RAP250 cDNA—We used the yeast two-hybrid system to screen a mouse embryo cDNA library with PPARα-LBD as a bait as described previously (18, 20). Of the isolated clones, more than 50% were isoforms of RXR, and a majority of the other clones were interacting parts of SMRT, N-CoR, TIF-2, and TRAP220 as described by Treuter et al. (18). However, one of the interacting clones revealed no homology with any described protein, and data base searches revealed a strong homology with a human EST sequence of 6504 bp named KIAA0181 (24). Compared with the human clone, this positive mouse clone only contained a partial cDNA sequence of 1.1 kb encoding 355 amino acids, corresponding to amino acids
RAP250 Is a Nuclear Receptor Coactivator

Human RAP250 mRNA Is Present in Many Tissues—Northern blot analysis of human RNAs revealed a widespread major RAP250 transcript of approximately 7.5 kb in length, which was present at different levels depending on the tissue (Fig. 2A). High levels were detected in reproductive organs, such as ovary, testis, and prostate, as well as in peripheral blood leukocytes, brain, and heart, and intermediate levels were observed in pancreas, kidney, liver, colon, spleen, and placenta. RAP250 mRNA levels were low but still detectable in small intestine, thymus, and skeletal muscle. Interestingly, in testis, a second transcript of approximately 4.5 kb in length was also detected. cDNA cloning and sequence analysis of this shorter mRNA indicated that it was an alternatively spliced form of RAP250 with an open reading frame encoding a 1070-amino acid-protein and encompassing amino acids 1–971 and 1494–2063 (data not shown). Thus, considering mRNA levels, testis appears to be the main RAP250 expressing organ. In peripheral blood leukocytes, a second 6-kb transcript was also detected that might represent either another alternatively spliced mRNA or a closely related but different gene product.

Ontogeny of RAP250 mRNA Expression—The levels of RAP250 mRNA in mouse and rat embryos were quite similar. RAP250 mRNA was widely detected during ontogeny. At embryonic day 9 (e9), clear signal was present in placenta, and lower expression could be seen in uterus (Fig. 2B). At this stage, neural tube expressed high levels of RAP250 mRNA and the expression in central nervous system was high throughout ontogeny (Fig. 2Bc). Strong expression was present in spinal cord and in cerebrum that was high and became more restricted during later stages of development (e17 onwards) and postnatal life. High expression was seen in cerebellum during development of this subregion of the brain (Fig. 2Bc). Also, sensory ganglia and retina showed high expression from e11 onwards (Fig. 2Bd). In the alimentary tract (oral cavity, stomach, and intestine), expression was seen from e13 and thereafter (Fig. 2B, Bb, and Bc). The developing teeth and salivary gland were also labeled (Fig. 2Bc). Olfactory epithelium was strongly labeled from e13 onwards (Fig. 2B, Bb, and Bc). Strong expression was present in liver (from e11) and kidney (from e13 onwards), and these...
levels decreased at later stages of development (Fig. 2, Bb and Bc). Lung had moderate signal from e13 and this level decreased during postnatal life (Fig. 2, Bc). Prominent signal was seen in thymus from e15 onwards, and in spleen from e17 and during early postnatal life, and subsequently, the expression decreased (Fig. 2, Bb and Bc). Low to moderate signal was seen in brown fat, as well as developing muscles, bones, and intervertebral discs. In adult mouse and rat, expression of RAP250 mRNA was more restricted than during embryonic development. High expression was observed in male and female rat genital organs. In testis, seminiferous tubules exhibited a strong signal and expression in separate tubules varied, indicating that RAP250 may be expressed in a stage-specific manner during spermatogenesis (Fig. 2, Bc). In dipped sections, RAP250 mRNA could be seen in primary spermatocytes. Prominent expression was also seen in the epithelium of prostate, whereas epididymis and seminal vesicles had low signals (Fig. 2, Bf). In ovary, the strongest signal was seen in interstitial cells and in the granulosa cells of different size follicles (Fig. 2, Bg). In the central nervous system, high expression was present in olfactory bulb, piriform cortex, hippocampus and cerebellar cortex, whereas other areas exhibited lower levels of RAP250 mRNA (Fig. 2, Bh).

**FIG. 5.** RAP250 forms an oligomeric complex with DNA bound NRs. Electrophoretic mobility shift assay analysis with in vitro translated NRs and bacterially expressed GST-RAP250 (aa 818–931) protein. A, RAP250/TRα/RXRα oligomeric complex formation on a TRE (DR4) in the presence of ligand using either wild type TRα/RXRα (lanes 3–6) or TRα/RXRα-mut (lanes 9–12). GST protein alone does not shift the receptors (lanes 1, 2, 7, and 8). B, RAP250/TRβ/RXRα oligomeric complex formation on a TRE (DR4) in the presence of ligand using TRα-mut/RXRα (lanes 3–6) but not using TRα-mut/RXRα-mut (lanes 9–12). GST protein alone does not shift the receptors (lanes 1, 2, 7, and 8). The mutations TRα-mut and RXRα-mut are helix 12 deletions known to not bind coactivators. Arrows indicate TRα/RXRα het erotramer (TR/RXR) and the ternary complex (TC) formed on DNA.

RAP250 strongly interacts with NRs in mammalian cells. A, COS-7 cells were transfected with NR expression vectors (TRα/RXRα, PPARγ/RXRα, or RXRα) and luciferase reporter genes (DR4, PRE, or DRI) together with either VP16 (gray bars) or VP16-mRAP250 (black bars). The activity of the luciferase reporter gene was measured 24 h after addition of appropriate ligands (T3/9-cis RA, BRL 96553/9-cis RA, or 9-cis RA, respectively). Results represent mean ± S.D. of at least two separate experiments carried out in duplicate and were normalized to the activity of each NR/reporter combination transfected with VP16, which was set as 1. RLU, relative light units.

**FIG. 6.** RAP250 interacts with PPARs, TRs, and ERs in vitro via the LXXLL Motif. The partial mouse RAP250 clone was originally isolated via its interaction with the PPARα-LBD bait in the yeast two-hybrid system. To determine whether the mouse RAP250 could interact with other NRs, we set up an in vitro protein-protein interaction assay with the mouse partial RAP250 clone (aa 782–1138) fused to GST, referred to here as GST-RAP250, and radioactively labeled in vitro translated NRs. As shown in Fig. 3, PPARs, TRs and ERs specifically interacted with GST-RAP250 but not with GST. The addition of appropriate ligands in the binding buffer increased the interaction between GST-RAP250 and most of NRs (Fig. 3B, lanes 3, and Fig. 4), indicating a ligand-dependent interaction in the cases of TRs, and a ligand-enhanced interaction for ERs and PPARs. To investigate whether the LXXLL motif (LVNLL, aa 891–895) of mouse RAP250 was responsible for the interaction
with NRs, the leucine core motif was mutated to AVNAL, as shown in Fig. 3A. Mutation of this motif abolished the interaction of GST-RAP250 with all tested NRs both in absence or presence of ligands (Fig. 3, lanes 5 and 6), indicating that the interaction between RAP250 and NRs was mediated by an LXXLL motif, the integrity of which is required to function as an NR box.

**RAP250 Contains Only One Functionally Active NR Box**—Whereas the NR-interacting RAP250 mouse fragment contains only one NR box domain, which is identical between mouse and human proteins (see Fig. 1C), analysis of the human RAP250 protein sequence revealed a second LXXLL motif (LSQLL, aa 1491–1495) that might possibly serve as an additional receptor recognition sequence. First, the full-length RAP250 clone was translated *in vitro* and used in a GST pull-down assay with ERα or TRα expressed as GST fusion proteins, in both the absence and presence of appropriate ligand. As expected, this transcript interacted in a ligand-dependent manner with the tested NRs (Fig. 4A). To further characterize the interaction between RAP250 and these NRs, eight overlapping cDNA fragments covering the human RAP250 sequence (Fig. 4B) translated *in vitro* and used in a GST pull-down assay. As seen in Fig. 4C, only fragment 4 (aa 819–1086), containing the first LXXLL motif, interacted strongly with NRs, whereas no interaction was observed with fragment 6 (aa 1491–1495), which contained the second LXXLL motif. The sequence of this region, SLSQLL, does not fit closely the consensus motif found in coactivators, which often contains a hydrophobic amino acid upstream of the first conserved leucine residue. This sequence difference might explain why the second motif did not function as an NR box. With the exception of fragment 5 (aa 1061–1338), which showed a very weak interaction with ERα, but not with TRα, none of the other RAP250 fragments interacted with the tested NRs. However, the strength of the interaction between fragment 4 and ER was manyfold higher, and thus, we consider this NR box-containing region to be the interacting region. As observed in Fig. 3, RAP250 interacts in a ligand-dependent manner with TRα and in a ligand-enhanced manner with ERα.
Taken together, these data indicate that the human RAP250, although containing two LXXLL motifs, may possess only one major NR-interacting-domain.

**RAP250 Interacts with NRs Bound to DNA**—To determine whether RAP250 interacted with NRs bound to their DNA response elements, we performed electrophoretic mobility shift assays. Termary complex formation with NR heterodimers was assessed using in vitro translated TRα/RXRα and the purified mouse RAP250 (aa 818–931) fused to GST. As seen in Fig. 5A, GST-protein alone did not interact with DNA bound TRα/RXRα in either the presence or absence of ligands (Fig. 5A, lanes 1 and 2). Addition of GST-RAP250 to the binding reaction resulted in a weak supershift in the absence of ligands (Fig. 5A, lane 3). However, the addition of ligands (T3 or 9-cis RA or both) resulted in a pronounced mobility shift of the TRα/RXRα dimer (Fig. 5A, lanes 4–6), which indicated the formation of an oligomeric complex containing GST-RAP250.

To further characterize the stoichiometry of this interaction, we used helix 12 mutated forms of TRα and RXRα. These mutants have previously been shown not to interact with co-activators (21, 22). When the mutated RXRα was used, the ligand-dependent interaction of GST-RAP250 in response to 9-cis RA was lost (Fig. 5A, lane 11). However, GST-RAP250 still interacted with the NR heterodimer in the presence of T3 (Fig. 5A, lanes 10 and 12). In a similar manner, when the mutated form of TRα was used, the ligand-dependent interaction of GST-RAP250 in response to T3 was lost (Fig. 5B, lane 4), but GST-RAP250 still interacted with the NRs in response to 9-cis RA (Fig. 5B, lanes 5 and 6). When the mutated receptors were used in combination, receptor heterodimers were detected on DNA, but no interaction of GST-RAP250 was detected (Fig. 5B, lanes 7–12). These results demonstrated that GST-RAP250 was able to interact with DNA-bound NRs in a ligand- and AF-2-dependent manner. Because the oligomeric complexes detected with GST-RAP250 and wild type receptors were located at the same position as those detected when one NRs was mutated, it seemed likely that one GST-RAP250 molecule bound per receptor heterodimer.

**RAP250 Differentially Interacts with Wild Type NRs in Transfected Mammalian Cells**—To determine whether RAP250 interacted with NRs in vivo, we used a mammalian transient transfection assay derived from the two-hybrid assay, which employed a herpesvirus VP16 activation domain fused to the mouse RAP250 clone (aa 782–1138). The VP16-RAP250 expression vector or a VP16 activation domain vector without RAP250 was transfected into COS-7 cells together with expression vectors for wild type NRs and a luciferase reporter gene containing appropriate response elements. As seen in Fig. 6, TRα/RXRα-mediated activity of the reporter gene was stimulated 3.8-fold by VP16-RAP250, as compared with VP16. A similar stimulation was observed for PPARγ/RXRα, (3.3-fold), whereas RXRα-mediated reporter gene expression was stimulated 10.5-fold. These in vivo data supported the observation made in vitro (Fig. 3) that RAP250 interacted with all tested NRs.

**RAP250 Activates Transcription via a Large Intrinsic Transcription Activation Domain**—In order to identify activation and/or repression domains within human RAP250, fragments of RAP250 were fused to the Gal4 DNA binding domain (DBD) and analyzed for transcription activation potential using transient transfection assays in COS-7 cells. In a first set of experiments, eight Gal4-RAP250 constructs containing about 300 amino acids each were assayed for their putative transcriptional activity in mammalian COS-7 cells (Fig. 7A). Two of the constructs were able to activate transcription, whereas none had significant repression activity. The construct containing amino acids 335–630 activated transcription approximately 5-fold as compared with Gal4-DBD alone, and the construct containing amino acids 577–855 activated transcription about 10-fold. Because these two fragments partially overlapped each other, a second set of constructs was made to determine whether there were two independent activation domains or whether the two fragments had one common activation domain in the overlapping region. Removal of 52 amino acids from the Gal4-RAP250-(335–630) construct generated a Gal4-RAP250-(335–577) construct that reduced the activation capacity from 5-fold to 3-fold, as shown in Fig. 7B. In a similar manner, 52 amino acids were removed from the Gal4-RAP250-(577–855) construct, generating Gal4-RAP250-(630–855), which reduced the activation capacity from 10-fold to 6-fold (Fig. 7B). Moreover, the fragment spanning amino acids 577–630, which overlaps the first set of fusion constructs (constructs 2 and 3), retained a weak activation potential of about 2-fold. An additional construct that contains all sequences showing activation potential, i.e. Gal4-RAP250-(335–855), activated transcription about 18-fold. Control studies demonstrated that all constructs were expressed and, with the exception of construct 7 (aa 1539–1771), at similar levels (Fig. 7C). From these data, we concluded that RAP250 contained one large activation domain localized between amino acids 335 and 855 and that removal of sequences within this activation domain gradually reduced its strength.

**RAP250 Functions as a Coactivator for NRs**—Based on the fact that fragments of RAP250 bind in vitro to several nuclear hormone receptors, it was important to investigate whether full-length RAP250 could also function as a coactivator for the transcriptional activity of these receptors. To investigate this hypothesis, expression vectors for TRα and RXRα were cotransfected with pSG5-hRAP250 or an empty vector together with a luciferase reporter gene, DR4-Tk-Luc for TRα, or DR1-Tk-Luc for RXRα. These assays were performed in the absence or presence of the appropriate ligand. As seen in Fig. 8, when TRα was used as a receptor, the activity of the luciferase reporter gene was measured 24 h after addition of ligands. Results represent mean ± S.D. of at least two separate experiments carried out in duplicate and were normalized to the activity of each NR/reporter combination transfected with pSG5 without ligand, which was set as 1.
Rap250 Is a Nuclear Receptor Coactivator

This study describes the structural and functional properties of Rap250, a novel NR coactivator isolated from a mouse embryonic library using the yeast two-hybrid system. Our results show that Rap250 interacts with multiple members of the NR family in a ligand-dependent manner, indicating that Rap250 is a coactivator of all these NRs. We also show that this interaction is dependent on an NR box and an intact AF-2 domain. Furthermore, Rap250 possesses an intrinsic transcriptional activation domain and functions as a NR coactivator in mammalian cells. A schematic representation of Rap250 is shown in Fig. 9. Together these results suggested that Rap250 may be a new AF-2 NR coactivator. Based on sequence analysis, Rap250 appears to be different from other NR coactivators characterized to date. For example, it shows no significant sequence homology with any known NR coactivator and has no bHLH/PAS domain as found in the p160 proteins or any motifs that would suggest histone acetylase or deacetylase activity.

Out of the two LXXLL motifs found in Rap250, only the first one functions as an NR box, in contrast to the p160 proteins that contain multiple NR boxes. In that respect, Rap250 resembles coactivators, such as TIF-1 (28, 29) or PGC-1 (30), that also have only one functional NR box. One model of coactivators binding to NR heterodimers proposes a bridging function of the p160 proteins by simultaneous binding of the two dimer-subunits with two adjacent NR boxes (31). However, this possibility clearly does not exist for Rap250, which has only one NR box. Another model, suggested by the presence of only one functional NR box, could involve two molecules of coactivator per NR-dimer, each of the subunits of the dimer binding one coactivator molecule via the NR box (20). In the case of Rap250, it would mean that each of the subunits of the NR-dimer binds one Rap250 molecule via the NR box. However, the electrophoretic mobility shift assay analysis (Fig. 5) does not suggest that this is the case for Rap250, because the complexes containing Rap250 and wild type NRs migrate at the same speed as when one of the NRs in the dimer has a mutation that prevents the coactivator from binding. An alternative could be that Rap250 is part of a larger complex of proteins, in which it would bind a NR via its NR box, in a manner similar to Trap220/Drp220 in the Trap/Drp complex (13, 14, 32, 33). Because Rap250 does not correspond to any of the subunits that have been identified (16, 34), it is possible that Rap250 either is part of a new coactivator complex or represents an unidentified member of the Trap/Drp complex.

The Rap250 activation domain is large and glutamine-rich with approximately 20% of Q residues (Figs. 1A and 9) but is not active when tested in yeast (data not shown). This is in agreement with previous findings demonstrating that glutamine-rich activation domains of transcription factors Oct-1, Oct-2, and Sp1, which activate transcription in mammalian cells, are inactive in yeast, probably reflecting some basic difference between the organization of yeast and mammalian promoters or transcription complexes (35). Interestingly, the enhancer-binding protein Sp1, which is a prototype for glutamine-rich transcription factors, was recently shown to interact with a transcription complex called CRSP, which contains Trap220 (25).

Rap250 mRNA is widely expressed, with the apparently highest expression in brain and reproductive organs, such as ovary, testis, and prostate (Fig. 2, A and B). Further work is required to determine whether Rap250 is of particular importance in these organs.

Acknowledgments—We thank Dr. T. Nagase for providing the KIAA0181 plasmid, and we also thank Drs. F. Saatciglu, P. Wiebel, J. K. Reddy, and R. M. Evans for providing plasmids and members of the Nuclear Receptor Unit at Novum for providing materials. We also thank Drs. Sadek, Jalaguier, and Tujague for helpful suggestions and critical reading of the manuscript. We acknowledge U. Jukarainen for expert technical assistance.

Note Added in Proof—After revision of our manuscript, we noticed that Rap250 is identical to ASC-2 (Lee, S.-K., Anzick, S. L., Choi, J.-E., Bubendorf, L., Guan, X.-Y., Jung, Y.-K., Kallioniemi, O. P., Kononen, J., Trent, J. M., Azorsa, D., Jhun, B.-H., Cheong, J. H., Lee, Y. C., Melter, P. S., and Lee, J. W. (1999) J. Biol. Chem. 274, 34283–34293)

REFERENCES

1. Mangelsdorf, D. J., Thummel, C., Beato, M., Herrlich, P., Schutz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P., and Evans, R. M. (1995) Cell 83, 835–839
2. Mangelsdorf, D. J., and Evans, R. M. (1995) Cell 83, 841–850
3. Beato, M., and Sanchez-Pacheco, A. (1996) Endocr. Rev. 17, 87–609
4. Hadaic, E., Desai-Yajnik, V., Helmer, E., Guo, S., Wu, S., Koudinova, N., Casanova, J., Raaka, B. M., and Samuels, H. H. (1995) Mol. Cell. Biol. 15, 4567–4571
5. Schulman, I. G., Chakravarti, D., Juguilon, H., Rono, A., and Evans, R. M. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 8288–8292
6. Xu, L., Glass, C. K., and Rosenfeld, M. G. (1999) Curr. Opin. Genet. Dev. 9, 140–147
7. McKenna, N. J., Lanz, R. B., and O'Malley, B. W. (1999) Endocr. Rev. 20, 321–344
8. Le Douarin, B., Nielsen, A. L., Garnier, J. M., Ichinohe, H., Jeannounguin, F., Losson, B., and Chambon, P. (1996) EMBO J. 15, 6701–6715
9. Torchio, J., Rose, D. W., Inostroza, J., Kamei, Y., Westin, S., Glass, C. K., and Rosenfeld, M. G. (1997) Nature 387, 677–684
10. Henry, D. M., Kalkhoven, E., Haare, S., and Parker, M. G. (1997) Nature 387, 733–736
11. McInerney, E. M., Rose, D. W., Flynn, S. E., Westin, S., Mullen, T. M., Krones, M., Inostroza, J., Torchio, J., Nolte, R. T., Assa-Munt, N., Milburn, M. V., Glass, C. K., and Rosenfeld, M. G. (1998) Genes Dev. 12, 3357–3368
12. Shiua, A. K., Barstad, D., Loria, P. M., Cheng, K., Kushner, P. J., Agard, D. A., and Greene, G. L. (1998) Cell 95, 927–937
13. Fondell, J. D., Ge, H., and Roeder, R. G. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 8329–8333
14. Fondell, J. D., Guermah, M., Malik, S., and Roeder, R. G. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 5959–5964
15. Rachez, C., Suldan, Z., Ward, J., Chang, C. P., Burakov, D., Erdjument-Bromage, H., Tempst, P., and Freedman, L. P. (1998) Genes Dev. 12, 1781–1800
16. Rachez, C., Lemon, B. D., Suldan, Z., Bromleigh, V., Gamble, M., Naar, A. M., Erdjument-Bromage, H., Tempst, P., and Freedman, L. P. (1999) Nature 398, 824–828
17. Cavailles, V., Dauvois, S., L’Horset, F., Lopez, G., Haare, S., Kushner, P. J., and Parker, M. G. (1995) EMBO J. 14, 3741–3751
18. Treuter, E., Albrektsson, T., Johansson, L., Leers, J., and Gustafsson, J.-A. (1998) Mol. Endocrinol. 12, 864–881
19. Johansson, L., Thomsen, J. S., Damdimopoulos, A. E., Spyrou, G., Gustafsson, J.-Å., and Treuter, E. (1999) *J. Biol. Chem.* **274**, 345–353
20. Leers, J., Treuter, E., and Gustafsson, J.-Å. (1998) *Mol. Cell. Biol.* **18**, 6001–6013
21. Wiebel, F. F., and Gustafsson, J.-Å. (1997) *Mol. Cell. Biol.* **17**, 3977–3986
22. Saatcioglu, F., Bartunek, P., Deng, T., Zenke, M., and Karin, M. (1993) *Mol. Cell. Biol.* **13**, 3678–3685
23. Feltkamp, D., Wiebel, F. F., Alberti, S., and Gustafsson, J.-Å. (1999) *J. Biol. Chem.* **274**, 10421–10429
24. Nagase, T., Seki, N., Ishikawa, K., Tanaka, A., and Nomura, N. (1996) *DNA Res.* **3**, 17–24
25. Kuiper, G., Enmark, E., Pelto-Huikko, M., Nilsson, S., and Gustafsson, J.-Å. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 5925–5930
26. Kosak, M. (1989) *J. Cell Biol.* **108**, 229–241
27. Zhu, Y. J., Qi, C., Jain, S., Rao, M. S., and Reddy, J. K. (1997) *J. Biol. Chem.* **272**, 25500–25506
28. Le Douarin, B., Zechel, C., Garnier, J. M., Lutz, Y., Tora, L., Pierrat, P., Heery, D., Gronemeyer, H., Chambon, P., and Lossow, R. (1995) *EMBO J.* **14**, 2020–2033
29. Thénot, S., Henriquet, C., Rochefort, H., and Cavailles, V. (1997) *J. Biol. Chem.* **272**, 12062–12068
30. Puigserver, P., Wu, Z., Park, C. W., Graves, R., Wright, M., and Spiegelman, B. M. (1998) *Cell* **92**, 829–839
31. Westin, S., Kurokawa, R., Nolte, R. T., Wisely, G. B., McInerney, E. M., Rose, D. W., Milburn, M. V., Rosenfeld, M. G., and Glass, C. K. (1998) *Nature* **395**, 199–202
32. Treuter, E., Johansson, L., Thomsen, J. S., Warnmark, A., Leers, J., Peit-Huikko, M., Sjöberg, M., Wright, A. P. H., Spyrou, G., and Gustafsson, J.-Å. (1999) *J. Biol. Chem.* **274**, 6667–6677
33. Yuan, C. X., Ito, M., Fondell, J. D., Fu, Z. Y., and Roeder, R. G. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 7939–7944
34. Ito, M., Yuan, C. X., Malik, S., Gu, W., Fondell, J. D., Yamamura, S., Fu, Z. Y., Zhang, X., Qin, J., and Roeder, R. G. (1999) *Mol. Cell.* **3**, 361–370
35. Kinsler, M., Braus, G. H., Georgiev, O., Seipel, K., and Schaffner, W. (1994) *EMBO J.* **13**, 641–645
36. Ryu, S., Zhou, S., Ladurner, A. G., and Tjian, R. (1999) *Nature* **397**, 446–450
