RAP80, a Novel Nuclear Protein That Interacts with the Retinoid-related Testis-associated Receptor*

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In this study, we describe the characterization of a novel nuclear protein, referred to as RAP80. The RAP80 cDNA was cloned from a human testis cDNA library and encodes a 719-amino acid protein containing two potential CXXC C1H, C2-type zinc finger motifs at its carboxy-terminal region. Analysis of its genomic structure revealed that the RAP80 gene covers more than 90 kb and consists of 15 exons and 14 introns. Fluorescence in situ hybridization mapped the RAP80 gene to human chromosome 5q35. RAP80 mRNA is expressed in many human tissues, but its expression is particularly high in testis. In situ hybridization showed that RAP80 is highly expressed in germ cells of mouse testis but is not differentially regulated during spermatogenesis. Confocal microscopy showed that RAP80 is localized to the nucleus, where it is distributed in a speckled pattern. Deletion analysis showed that a bipartite nuclear localization signal at the amino terminus is important in mediating nuclear transport of RAP80. Monohybrid analysis showed that RAP80 might function as an active repressor of transcription. Mammalian two-hybrid analysis demonstrated that RAP80 was able to interact with the retinoid-related testis-associated receptor (RTR), an orphan receptor that has been implicated in the control of embryonic development and spermatogenesis. Pull-down analysis showed that RAP80 and RTR physically interact in vitro. Deletion and point mutation analyses revealed that part of the hinge domain of RTR is required for this interaction. RAP80 is able to inhibit the interaction of RTR with the co-repressor N-CoR likely by competing with N-CoR for RTR binding. Our results suggest that RAP80 may be functioning as a modulator of RTR signaling.

The nuclear receptor superfamily is composed of a large number of ligand-dependent transcription factors that include nuclear orphan receptors for which a ligand has not yet been identified (1–4). Nuclear receptors share a common domain structure that includes an amino-terminal domain, a DNA-binding domain (DBD), hinge domain, and a ligand-binding domain (LBD). These domains are involved in the recognition of specific DNA response elements, receptor dimerization, nuclear localization, and ligand binding and contain repressor and transactivation functions. Repression and activation of transcription by nuclear receptors are mediated through interactions with co-repressor and co-activators, respectively. Nuclear receptors have been demonstrated to regulate many physiological processes, including embryonic development and cell growth and differentiation and have been implicated in a number of human diseases (5–8). Less, however, is known about the role of many nuclear orphan receptors, including the retinoid-related, testis-associated receptor (RTR), also referred to as germ cell nuclear factor (9, 10).

The orphan receptor RTR (named NR6A1 by the Nuclear Receptor Nomenclature Committee) has been cloned from several species, including mouse (9, 10), human (11–14), zebrafish (15), and Xenopus laevis (16). During embryonic development RTR is expressed in early embryonic stem cells, trophoblasts, and neuronal precursor cells while in the adult RTR expression is largely limited to testis and ovary (9, 11, 17–19). In the testis, RTR is most abundant in round spermatids suggesting that RTR controls gene transcription during a specific stage of spermatogenesis (9, 10, 17, 18). Several studies have suggested that RTR is also important during embryonic development. Targeted disruption of the RTR gene is embryonic lethal in mice (20). Embryos exhibit open neural tubes and an absence of posterior structures and do not survive beyond 10.5 days postcoitus probably due to cardiovascular failure. These studies suggest that RTR is essential for the postgastrulation and neurulation stages of mouse development. A recent study identified a role for RTR in the repression of the POU-domain transcription factor Oct4 (21). Overexpression of a dominant-negative RTR mutant in Xenopus showed that RTR has an essential function in anteroposterior differentiation during organogenesis (22).

RTR has been demonstrated to bind preferentially as a homodimer to DNA response elements consisting of the consensus sequence [AGGTCA]2 (23–26). However, little is still known about the transcriptional activity of RTR. In the absence of a putative ligand, RTR has been shown to function as a transcriptional repressor (27, 28). This repression is mediated at least in part through an interaction of RTR with the co-repressor N-CoR.

In this report, we describe the cloning and sequence of the full-length coding region of a novel gene referred to as receptor-associated protein 80 (RAP80). RAP80 encodes a 79.6-kDa nu-
clear protein. The RAP80 protein contains two Cys-X<sub>2</sub>-Cys-X<sub>2</sub>-His-X<sub>2</sub>-Cys zinc fingers near the carboxyl terminus. RAP80 mRNA is expressed in many tissues but is most abundant in testes. In situ hybridization localized RAP80 mRNA expression to the germ cells. The genomic structure of the RAP80 gene encompasses more than 90 kb and consists of 15 exons. The RAP80 gene was mapped to human chromosome 5q35. Confocal microscopy showed that RAP80 is localized largely to the nucleus. Using mammalian mono- and two-hybrid analysis, we characterized the transcriptional activity of RAP80 and its interaction with RTR. The results indicated that RAP80 is able to repress basal transcriptional activation indicating that it may function as a (co-)repressor for RTR. Deletion and point mutation analysis demonstrated that the hinge domain of RTR is important for this interaction. Previously, we reported that the hinge domain, in addition to helices 3–5 and 12, is also required for the interaction of RTR with the co-repressor N-CoR (28). We demonstrate that RAP80 is able to inhibit the interaction of the co-repressor N-CoR with RTR likely by competing with N-CoR for binding. These results suggest that RAP80 may play a role in modulating the transcriptional and biological activity of RTR.

**EXPERIMENTAL PROCEDURES**

**Cloning of RAP80**—RAP80 was initially identified by differential display using RNA from young proliferating and senescent normal human epididymal keratinocyte cells as previously described (29). A band representing RAP80 was excised, amplified, and ligated into a pCR II vector (Invitrogen, San Diego, CA). Sequence analysis revealed a 241-bp fragment containing a poly(A) tail. This fragment was used as a probe to screen a 470 pg phage cDNA library from human testis (CLONTECH, Palo Alto, CA), which yielded a cDNA encoding full-length RAP80. The full-length RAP80 cDNA was subcloned into pCDNA3.1 (Invitrogen). However, Northern blot analysis using this cDNA as a probe did not show any differential regulation in normal human epididymal keratinocyte cells (not shown).

**DNA Sequencing**—Plasmids were purified using Wizard miniprep or midprep kits from Promega. Automatic sequencing was carried out using a Dynamic ET Terminator Cycle Sequencing Ready reaction kit (PerkinElmer Life Sciences) and an ABI Prism 377 automatic sequencer. DNA sequencing was performed on sequence homology between the human and mouse RAP80 sequences to determine the genomic structure of the human RAP80 gene by in silico cloning.

**Plasmids**—The reporter plasmid pG5-CAT containing five copies of the GAL4 upstream activating sequence (UAS) upstream of the E1B minimal promoter and to repressor a (UAS)-CAT, and pM, encoding Gal4(DBD), was purchased from CLONTECH. The plasmid pM-N-CoR was described previously (28). The expression plasmids pBCleuSV-RTR encoding full-length ntrR, pBC5-VP16-RTR encoding the VP16(AD) fusions to full-length mTR, and pGEX-2TK-RTR encoding a GST-RTR fusion protein were described previously (23, 28, 31). The different pSG5-VP16-RTR deletion mutants were created by placing the VP16 activation domain at the amino terminus of various RTR fragments as described previously (28). Point mutations in the hinge domain of RTR were introduced using site-directed mutagenesis kits (Stratagene) following the manufacturer's protocol (28). The different pM-RAP80 deletion mutants were created by placing the Gal4(DBD) at the amino terminus of various RAP80 fragments. These fragments were generated by PCR. RAP80-specific 5'- and 3'-primers included either an EcoRI or BamHI restriction site, respectively, to allow the PCR fragments to be subcloned into the EcoRI and BamHI sites of pM. Details on the construction and sequence of each deletion are described in the text. The integrity of all constructs was confirmed by restriction digestion and automatic DNA sequencing.

**Mammalian Mono- and Two-hybrid Analysis**—CV-1 and CHO cells were plated in 6-well dishes at 2 × 10<sup>5</sup> cells/well and 20 h later transfected in Opti-MEM (Invitrogen) with various reporter and expression plasmid cDNAs as indicated in the legends using FuGENE 6 transfection reagent (Roche Molecular Biochemicals). The plasmid pB-actin-Luc was used as an internal control to monitor transfection efficiency. Cells were collected 48 h after transfection and then assayed for the level of CAT protein and luciferase activity. The level of CAT protein was determined by the CAT enzyme-linked immunobosorbent assay kit (Roche Molecular Biochemicals) according to the manufacturer's instructions. Luciferase activity was assayed with a Luciferase kit (Promega). Transfections were performed in triplicate, and each experiment was repeated at least two times. Expression levels of different Gal4(DBD) and VP16 fusion proteins were examined by Western blot analysis using antibodies against Gal4(DBD) and VP16 (CLONTECH), respectively. Because RTR functions as a repressor, VP16-RTR expression plasmids were used as described previously (28). Equal amounts of GST or GST-RTR protein were incubated with glutathione-Sepharose 4B beads and washed in phosphate-buffered saline (PBS).
FIG. 1. A, nucleotide and amino acid sequence of human RAP80. The deduced amino acid sequence is numbered on the left side of the sequence. The 719-amino acid open reading frame is shown. The three putative nuclear translocation signals are indicated by a dashed line. The two putative zinc finger motifs are shaded; the Cys and His residues involved in the tetrahedral configuration of zinc fingers are underlined. The consensus sequence of the zinc finger motifs is shown at the bottom. The putative ubiquitin interaction motif is indicated by a solid line. Arrowheads indicate the positions of introns. The start and stop codons are shown in boldface. The sequence was submitted to GenBank™ under accession number AF349313. B, schematic presentation of the genomic structure of the human RAP80 gene. Boxes indicate exons. The positions of the 15 exons are shown. Exons encoding the location of the two putative zinc finger motifs and the positions of the start and stop codon are indicated.
ered saline. [35S]Methionine-labeled RAP80 was obtained by in vitro translation using the TtV-coupled reticulocyte lysate system from Promega. The GST and GST-RTR beads were then incubated in 0.25 ml of binding buffer (20 mM Tris-HCl, pH 7.9, 100 mM KCl, 0.1% Nonidet P-40, 10% glycerol, 5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 0.5% nonfat dry milk) with the [35S]methionine-labeled RAP80. After 1-h incubation at room temperature, the beads were washed five times in binding buffer and then boiled in 30 μl of 2× SDS-PAGE loading buffer. Solubilized proteins were separated by 8% SDS-PAGE, and the radiolabeled proteins were visualized by autoradiography.

RESULTS

Cloning of Human RAP80 cDNA—As described under “Experimental Procedures,” a 241-bp PCR fragment encoding RAP80 was originally identified by differential display analysis. This fragment was isolated and subsequently cloned into the vector pCRII and sequenced. A cDNA clone containing the full-length coding region of RAP80 was obtained by screening a λgt10 human testis cDNA library. The full-length nucleotide and amino acid sequence of RAP80 is shown in Fig. 1A. The RAP80 nucleotide sequence revealed a long open reading frame, which started with an initiation codon at nucleotide 110 and ended with a stop codon at nucleotide 2267. The RAP80 mRNA encodes an acidic protein of 719 amino acids with a predicted molecular mass of 79.6 kDa and an isoelectric point of 5.42. Examination of the amino acid sequence indicated the presence of two zinc finger-like motifs near the carboxyl terminus of the murine homologue of RAP80. The amino acid sequence of RAP80 exhibited a 74% identity with this region. The putative zinc finger motifs are encoded by exons 10–12. A summary of the size of the introns and exons, and sequence at the splice sites is presented in Table I. The sizes at these junctions are consistent with the consensus AGA2G7/110G8/100A60G88 at the 5′ donor and Y3G4Ny97G100G100/G55 at the 3′ acceptor side of splice sites within eukaryotic genes (34). The first and tenth introns are particularly large, about 24 and 32 kb in size, respectively.

Chromosomal Localization of RAP80—The regional chromosomal localization was determined by FISH using a genomic DNA fragment containing the human RAP80 gene, as a probe. The initial experiment resulted in the specific labeling of the distal long arm believed to be chromosome 5. This localization was confirmed when a biotin-labeled probe specific for a region mapped to 5q21 was co-hybridized with a digoxigenin-labeled probe for RAP80. The results of the in situ hybridization are shown in Fig. 2A. The position of RAP80 was calculated to be at 96% of the distance from the centromere to the telomere of the chromosome arm 5q, an area that corresponds to band 5q35. An idiogram indicating the localization of human RAP80 is presented in Fig. 2B.

Genomic Structure of RAP80—Comparison of the human RAP80 cDNA sequence with high throughput genomic sequences (gtgs) in GenBankTM identified three clones, CTC-340P19, RP11-627M5, and CTC-499E20 (AC027317, AC026513, and AC034209, respectively; working drafts of unordered sequences), encoding parts of the human RAP80 gene. From the comparisons of the RAP80 cDNA sequence and the multiple genomic fragments contained in these gtgs clones, the genomic structure could be deduced. This analysis revealed that the RAP80 gene encompasses more than 90 kb and consists of 15 exons and 14 introns. The positions of the introns are indicated by an arrowhead in Fig. 1A. A schematic view of the genomic structure is presented in Fig. 1B. The putative zinc finger motifs are encoded by exons 10–12. A summary of the size of the introns and exons, and sequence at the splice sites is presented in Table I. The sequences at these junctions are consistent with the consensus AGA2G7/110G8/100A60G88 at the 5′ donor and Y3G4Ny97G100G100/G55 at the 3′ acceptor side of splice sites within eukaryotic genes (34). The first and tenth introns are particularly large, about 24 and 32 kb in size, respectively.
whether RAP80 expression was limited to a particular cell type and whether it was differentially regulated during spermatogenesis. The mature mouse testis consists of many different cell types, Sertoli cells, Leydig cells, and a variety of germ cells at different stages of differentiation.

In situ hybridization of sections from adult mouse testis using an antisense RAP80 probe showed that RAP80 mRNA expression is associated particularly with germ cells (Fig. 4B). Little expression was observed in surrounding Leydig cells. During spermatogenesis in the adult mouse, germ-cell differentiation advances in highly ordered waves along the axis of the seminiferous tubule, and each cross-section of a tubule can represent one of 12 stages of spermatogenesis (35). The intensity of the RAP80 hybridization signal appears to be very similar between different seminiferous tubules suggesting that RAP80 is not regulated during spermatogenesis. The latter was confirmed by Northern blot analysis using RNA from isolated spermatogonia, pachytenes, and spermatids subpopulations of germ cells. No significant differences in the level of RAP80 mRNA expression were found between these subpopulations of germ cells (not shown).

RAP80 is a Nuclear Protein—Examination of the amino acid sequence of RAP80 showed the presence of three putative nuclear localization signals (NLS) (36), NLS1 (R3RKKKVK) and a bipartite NLS2 (K19KDVETTSSVSIRKKR) at the amino terminus and NLS3 (R715RRK) at the carboxyl terminus (Fig. 1A).

These observations suggested that RAP80 might function as a nuclear protein. To investigate this further, we constructed plasmids encoding different EGFP-RAP80 fusion proteins. These plasmids were transfected into CV-1 cells, and the localization of the fusion proteins was examined by confocal microscopy. As shown in Fig. 5A, EGFP-RAP80 was largely restricted to the nucleus supporting the conclusion that RAP80 is a nuclear protein. RAP80 was distributed in a speckled pattern as observed for many nuclear proteins. To determine the role of the three nuclear localization signals, we examined the effect of several deletions on the nuclear localization of RAP80. RAP80 still localized to the nucleus after deleting NLS1 or NLS3 (Fig. 5B and C); however, RAP80 appeared in the cytoplasm after deleting both NLS1 and NLS2 or all three NLSs (Fig. 5D and E). These results suggest that NLS2 plays an important role in the nuclear transport of RAP80 and that NLS1 and -3 are not required.

RAP80 Contains a Repressor Function—Because RAP80 localized to the nucleus, we investigated whether it exhibited any
transcriptional activity. To examine whether RAP80 functions as an activator or repressor of transcription, we determined its effect on the transcriptional activation of a reporter gene using monohybrid analysis. For this purpose, CHO or CV-1 cells were co-transfected with (UAS)5-CAT and pM or pM-RAP80, and the effects of RAP80 on basal transcriptional activity were determined. As shown in Fig. 6, RAP80 decreased basal transcription in both cell lines, suggesting that RAP80 contains an active repressor function. Analysis of two deletion mutants showed that the repressor function was associated with the amino terminus of RAP80 (Fig. 6).

Interaction of RAP80 with RTR—Because the carboxyl-terminal region of mouse RAP80 (RIP110) has been reported to interact with the nuclear receptor RXR in yeast two-hybrid analysis (33), we studied the interaction of RAP80 with several nuclear receptors using mammalian two-hybrid analysis. These results demonstrated that RAP80 was able to interact with the orphan receptor RTR but not with the orphan receptor TAK1, or the retinoid receptors RAR/\alpha and RXR/\alpha, the estrogen receptor/\alpha, or the thyroid hormone receptor/\alpha either in the presence or absence of their respective ligand (not shown). We therefore focused our studies on the interaction between RTR and RAP80. CHO cells were co-transfected with (UAS)\alpha-CAT, pM-RAP80, and increasing amounts of VP16-RTR, and 48 h later reporter activity was measured. The results in Fig. 7A demonstrate that RAP80 and RTR were able to interact with each other in a dose-dependent manner. One might expect that increasing amounts of RTR would compete for RAP80 binding. To examine this, we co-transfected CHO cells with (UAS)\alpha-CAT, pM-RAP80, VP16-RTR, and increasing amounts of pZeoSV-RTR expression vector. These results demonstrated that increasing amounts of RTR reduced the levels of reporter activity likely by competing with VP16-RTR for binding to RAP80.

RAP80 Interacts with RTR in Vitro—Next we examined the interaction between RTR and RAP80 in vitro by GST-pull-down analysis. GST-RTR149–495 fusion protein and GST were immobilized on glutathione-Sepharose beads and then incubated with [35S]-methionine labeled RAP80. After extensive washing, labeled bound proteins were separated by SDS-electrophoresis and visualized by autoradiography. Fig. 8 shows that 35S-labeled RAP80 was able to bind to GST-RTR but not to GST alone, suggesting that this binding is specific for RTR. No interaction was found between RAP80 and GST-ROR/\alpha (not shown). This observation demonstrates that RAP80 is able to interact with RTR in vitro and supports the results obtained by two-hybrid analysis.

The Hinge Domain of RTR Is Essential in its Interaction with RAP80—To determine what region of RTR is required for the interaction of RTR with RAP80, we examined the effect of various carboxyl-terminal deletions within RTR on this interaction. Deletion of the LBD of RTR (RTR\alpha C5 in Fig. 9A) had little effect on the interaction of RTR with RAP80 suggesting that the LBD is not required for this interaction. Further carboxyl-terminal deletion up to Ile241 (mutant RTR\alpha C4) totally abolished the interaction of RTR with RAP80 indicating that the region between Ile241 and Leu268 is required for RTR-RAP80 interaction. Examination of deletions at the amino terminus of RTR indicated that deletion up to His187 had little effect, whereas further deletion up to Ser212 abrogated RTR-RAP80 interaction suggesting that the region between His187 and...
and Ser\(^{212}\) is important. The LBD of RTR (RTR\(_{\Delta N3}\)) did not interact with RAP80. The combined results obtained from the amino- and carboxyl-terminal deletions suggest that the region from His\(^{197}\) to Leu\(^{268}\) within the hinge domain is required for the interaction of RTR with RAP80.

We next examined the effect of several point mutations within the hinge region on the interaction between RTR and RAP80. The mutations L254P and Ser212 is important. The LBD of RTR (RTR\(_{\Delta N3}\)) did not interact with RAP80. The combined results obtained from the amino- and carboxyl-terminal deletions suggest that the region from His\(^{197}\) to Leu\(^{268}\) within the hinge domain is required for the interaction of RTR with RAP80.

We next examined the effect of several point mutations within the hinge region on the interaction between RTR and RAP80. The mutations L254P → AA and S265Y → AA had little effect on this interaction; however, the S265Y → GG mutation totally abolished the interaction of RTR with RAP80 (Fig. 9B). The latter confirms the importance of this hinge region in the interaction between RTR and RAP80.

To determine what regions in RAP80 are important in its interaction with RTR, the effect of several amino-terminal deletions were examined (Fig. 10). These results showed that deletion of the repressor domain at the amino terminus did not affect the interaction. However, deletion of the regions between 100–200 and 400–500 diminished the reporter activity in two-hybrid analysis suggesting that several regions of RAP80 are important for optimal interaction with RTR.

**RAP80 Inhibits the Interaction of RTR with the Co-repressor N-CoR—**Previous studies have demonstrated that RTR is able to interact with the co-repressor N-CoR (27, 28). In addition to helix 3 and helix 12 in the LBD, the hinge domain of RTR was found to be important for this interaction. Because the hinge domain is involved in RTR-RAP80 interactions, we were interested in examining whether RAP80 would interfere with the binding of N-CoR to RTR. The results in Fig. 11 demonstrate that increasing concentrations of RAP80 inhibited the interaction of N-CoR with RTR. Two-hybrid analysis using pM-N-CoR and VP16-RAP80 did not indicate any interaction between N-CoR and RAP80 (not shown). These observations suggest that RAP80 and N-CoR appear to compete with each other for RTR binding.

**DISCUSSION**

In this study, we describe the cloning of a cDNA encoding the full-length human RAP80 protein. Examination of the RAP80 protein sequence revealed several distinctive features, two putative zinc finger motifs, three nuclear localization signals, a Glu-rich region, a PEST sequence, and a potential ubiquitin-interaction motif between Thr\(^{487}\) and Glu\(^{490}\) (37). The two putative zinc finger motifs at the carboxyl terminus of RAP80 exhibit the consensus sequence Cys-X\(_2\)-Cys-X\(_{11}\)-His-X\(_2\)-Cys. The sequence separating the zinc finger motifs does not exhibit any homology with the consensus motif (T/S)GEKP (Y/F)X, typically found as interfinger spacer in members of the Krüppel-like zinc finger proteins (38) suggesting that RAP80 does not belong to the Krüppel-like zinc finger protein superfamily. The class CCHC-type zinc finger motifs found in RAP80 has been described in only a limited number of proteins. Members of the NZF and FOG transcription factor families contain zinc finger motifs with the consensus Cys-X\(_2\)-Cys-X\(_2\)-His-X\(_2\)-Cys (39–41). A different class of Cys-X\(_2\)-Cys-X\(_2\)-His-X\(_2\)-Cys motifs, referred to as “zinc knuckle” fingers, are contained in DNA-binding proteins from several retroviruses (42). RAP80 appears to be unique and unrelated to these nuclear proteins. Zinc finger motifs can exhibit multiple functions, including a role in DNA recognition, protein-protein interactions, and nuclear localization. We are in the process of examining whether RAP80 can recognize a specific DNA sequence and whether it functions as a DNA-binding protein.

Confocal microscopic analysis localized RAP80 to the nucleus, suggesting that RAP80 functions as a nuclear protein.
RAP80 is distributed in a speckled pattern, suggesting that it is part of a multiprotein complex. RAP80 contains three putative nuclear localization signals: at Arg3, Arg715, and a bipartite nuclear localization signal between Lys19 and Arg35 (36). Deletion analysis indicated that the bipartite nuclear localization signal is important in mediating nuclear transport of RAP80.

**FIG. 9.** The hinge domain of RTR is important in its interaction with RAP80. CHO cells were transfected with (UAS)5-CAT (0.5 μg), β-actin-LUC (0.1 μg), pSG5VP16 or pSG5VP16-RTR (0.2 μg), and various pM-RAP80 deletion mutants (0.5 μg), as indicated. The various RAP80 mutations are shown at the top. After 48 h, cells were collected and assayed for reporter activity. The relative level of CAT protein was calculated and plotted. Levels of the different Gal4(DBD)-RAP80 fusion proteins did not differ significantly.

**FIG. 10.** Effect of various deletions in RAP80 on the interaction of RAP80 with RTR. CHO cells were transfected with (UAS)5-CAT (0.5 μg), β-actin-LUC (0.1 μg), pSG5VP16 or pSG5VP16-RTR (0.2 μg), and different pM-RAP80 deletion mutants (0.5 μg), as indicated. After 48 h, cells were collected and assayed for reporter activity. The relative level of CAT protein was calculated and plotted. Levels of the different Gal4(DBD)-RAP80 fusion proteins did not differ significantly.

**FIG. 11.** Competition between the co-repressor N-COR and RAP80 for interaction with RTR. CHO cells were transfected with (UAS)5-CAT (0.5 μg), β-actin-LUC (0.1 μg), pM-N-COR (0.5 μg), VP16-RTR (0.1 μg), and pcDNA3.1-RAP80 (0.1, 0.3, or 0.5 μg) plasmid DNA as indicated. After 48 h, cells were collected and assayed for reporter activity. The relative level of CAT protein was calculated and plotted.
The nuclear localization and the presence of two zinc finger-like motifs suggested that RAP80 may function as a transcription factor. We therefore investigated whether it exhibited any transcriptional activity. Monohybrid analysis using different cell types showed that in several cell lines RAP80 was unable to enhance transcription of a reporter gene, suggesting that it did not function as a transcriptional activator under these conditions. Instead, Gal4(DBD)-RAP80 inhibited basal transcription, indicating that it may function as an active repressor. Deletion mutant analysis indicated that the repressor function is associated with the amino terminus of RAP80. This region has no similarity to any other known repressor domain.

RAP80 exhibits a 74% identity with a partial protein sequence referred to as RIP110 encoded by one of several sequences identified by yeast two-hybrid screening using the ligand binding domain of the RXRa receptor as bait (33). RIP110 appears to represent the carboxyl terminus of the mouse homologue of RAP80 and has not been studied further. Because of the observed interaction of RIP110 with RXR in yeast two-hybrid analysis, we investigated the interaction of RAP80 with a number of nuclear receptors in mammalian two-hybrid analysis. This analysis included the retinoid receptors RXRa and RARa, the estrogen receptor α, the thyroid hormone receptor α, and the orphan receptors RTR and TAK1 and was carried out in either the presence or absence of their respective ligand (not shown). These analyses revealed an interaction only between RAP80 and RTR (Fig. 7). Deletion and point mutation analysis identified a region within the hinge domain of RTR that is essential for its interaction with RAP80 while the ligand binding domain of RTR was not required. Several regions within RAP80 were important for optimal interaction with RTR. These observations suggest a function for RAP80 in modulating RTR activity. RAP80 may have multiple roles in RTR signaling. It may function as a co-repressor, as a mediator to recruit other proteins to RTR or target RTR to a particular complex or compartment such as nuclear matrix or proteasome. The fact that RAP80 inhibits basal transcriptional activity may suggest that it functions as a co-repressor.

Previous studies have demonstrated that RTR can function as an active repressor of transcription and is involved in the repression of transcription of the POU-domain transcriptional factor Oct4 (21, 28). Repression by nuclear receptors is mediated through interaction with multiprotein co-repressor complexes that contain histone deacetylase activity and affect chromatin structure. The protein N-CoR has been reported to physically interact with RTR and to function as a co-repressor for RTR. This interaction was shown to require the ligand binding domain as well as the hinge domain of RTR for optimal binding (28). Because both RAP80 and N-CoR interact with RTR and require the hinge region of RTR for binding, one might predict that the interaction of RAP80 and N-CoR to RTR interfere with each other. As shown in Fig. 11, RAP80 inhibits the interaction of N-CoR with RTR likely by competing with each other for RTR binding. The hinge region is required for the interaction of N-CoR with several nuclear receptors and appears to function as a structural determinant rather than serving as a direct interface in N-CoR binding (43, 44). These observations seem to indicate that RAP80 interferes with the binding of N-CoR to RTR through a mechanism of steric hindrance rather than competing for the same binding site.

RAP80 mRNA was found in many different human tissues but was most abundantly expressed in testis. In situ hybridization of sections from mouse testis indicated that RAP80 is expressed most highly in germ cells. Because no significant differences in the level or pattern of hybridization were observed between seminiferous tubules at different stages of spermatogenesis, expression of RAP80 mRNA appears not to be differentially regulated during spermatogenesis. This was supported by Northern blot analysis using RNA from different germ cell populations (not shown). Previous studies have demonstrated that RTR is differentially regulated during spermatogenesis and most highly expressed in spermatids (9, 10, 18). Because RTR and RAP80 are co-expressed in spermatids and in embryonal carcinoma cells and trophoblasts (not shown) (19), RAP80 may change RTR function and signaling in these cells. What the physiological significance is of the interaction between RAP80 and RTR awaits further analysis using functional assays. Because RAP80 mRNA is also found in tissues where RTR is not expressed suggests that RAP80 has additional functions and likely interacts with other (nuclear) proteins.

In summary, in this study we identify a novel nuclear protein, referred to as RAP80. This protein localizes to the nucleus and is able to repress basal transcription. We demonstrate that this protein interacts physically with the nuclear orphan receptor RTR. Deletion and point mutation analysis identified a region within the hinge domain of RTR that is essential for this interaction. This region is also required for the interaction of the co-repressor N-CoR. RAP80 and N-CoR appear to compete with each other for binding to RTR possibly through steric hindrance. Our results suggest that RAP80 may play a role in modulating the transcriptional and biological activity of RTR possibly by functioning as a co-repressor.

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