RAP250 Is a Coactivator in the Transforming Growth Factor β Signaling Pathway That Interacts with Smad2 and Smad3*

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RAP250 is a coactivator for nuclear receptors as well as other transcription factors. Recent studies have established RAP250 as an essential coactivator for many important biological processes, but its exact mechanism of action is not fully understood. To identify novel proteins that can associate with RAP250, we used a yeast two-hybrid system to screen cDNA libraries and identified the intracellular mediators of transforming growth factor-β (TGF-β) response Smad2 and Smad3 as direct interacting proteins. We show that the interaction between RAP250 and Smad2/3 is dependent upon the second LXXLL interaction motif in RAP250 and the MH2 domain in Smad2 and Smad3. Mouse embryonic fibroblasts lacking RAP250 have reduced expression of the TGF-β target gene PAI-1 after stimulation by TGF-β when compared with wild type cells. Furthermore, we demonstrate a cross-talk between TGF-β and liver X receptors (LXR) signaling pathways and show that stimulation of cells with TGF-β and LXR agonists have a synergistic effect on the expression of the LXR target gene ABCG1. Our data identify RAP250 as a new coactivator in the TGF-β signaling pathway that binds Smad2 and Smad3. Our data also suggest that the interaction between RAP250, Smad2, and Smad3 constitutes an important bridging mechanism linking LXR and TGF-β signaling pathways.

Transcription factors play an important role in regulation of gene expression where they bind to specific DNA sequences in promoter and enhancer regions and pass on signals that either repress or enhance transcription. The importance of transcriptional coregulators in this process is well established. Coregulators are auxiliary transcription modulators that can both induce (coactivators) and suppress (corepressors) the transcription potential of a given transcription factor. Today more than 200 different coactivators have been identified (1). Coactivators recruit multiprotein complexes that stimulate the process of transcription initiation. The accessibility for complete transcriptional networks to promoter regions is regulated by ATP-dependent chromatin remodeling by SWI/SNF family complexes that result in unwinding of DNA (2). Coactivators with intrinsic histone acetyltransferase activity covalently modify histones so that additional regulatory proteins are allowed to bind to DNA; examples are the general coactivators CBP2 and p300 (3). The mediator/TRAP/DRIP/ARC multiprotein complex has a central role in recruitment of RNA polymerase II by making contacts between transcriptional activators and the RNA polymerase II transcription machinery (4).

The three members of the SRC-1 family are among the most studied coactivators for nuclear receptors. These coactivators bind to nuclear receptors through LXXLL interaction motifs, also called NR box motifs (5). The LXXLL motif is a short hydrophobic domain that is sufficient for ligand-dependent interactions with nuclear receptors. Other LXXLL-containing coactivators that are important for nuclear receptor signaling are CBP/p300 (3), PGC-1 (6), MED1 (TRAP220/DRIP205/PBP) (7), and RAP250 (8).

RAP250 (9), also known as NcoA6/ASC-2/AIB3/TRBP/NRC, was isolated as a nuclear receptor coactivator, but it also acts on several other transcription factors and is considered as a general coactivator. It has an intrinsic glutamine-rich activation domain and two LXXLL interaction motifs. LXXLL-1 (amino acids 887–891) is located in the middle of RAP250 and binds to most nuclear receptors, but LXXLL-2 (amino acids 1491–1495) located in the C-terminal region is very selective in its binding and binds only liver X receptors (LXRα and LXRβ) (10). RAP250 has no intrinsic enzymatic activity but may recruit proteins with histone acetyltransferase (11, 12), methylase (13), and helicase activity (14). Knock-out studies in mice indicate an essential role for RAP250 since lack of RAP250 results in embryonic lethality (15–18). Transgene expression of a dominant negative form of RAP250 that includes the LXXLL-2 interaction domain results in a phenotype similar to that seen in LXRα knock-out mice with accumulation of cholesterol in the liver (19), suggesting that RAP250 could be an important factor in LXR signaling.

LXRα and LXRβ promote reverse cholesterol transport. Decreased atherosclerotic lesions were observed in the aorta of LDLR−/− and apoE−/− mice after activation of LXR where expression of both ABCA1 and ABCG1 was induced in the macrophages in the lesions. Furthermore, transplantation of macrophages from LXRα/β−/− mice into the same mouse models increased atherosclerosis in both recipient strains

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The abbreviations used are: CBP, CREB-binding protein; CREB, cAMP-response element-binding protein; TGF, transforming growth factor; LXR, liver X receptor; RXR, retinoid X receptor; MEF, mouse embryonic fibroblasts MH, mad homology; PAI-1, plasminogen activator inhibitor-1; CTGF, connective tissue growth factor gene; siRNA, small interfering RNA; siSmad4, siRNA against Smad4.
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(reviewed in Ref. 20). Recent studies have shown that transforming growth factor-β (TGF-β) limits atherosclerosis by modulating a number of processes, including the accumulation of lipids in the vessel wall. This effect is probably mediated by induced expression of ABCA1, ABCG1, and apoE in macrophages (21–23) that are also known LXR target genes.

In this study, we describe a novel function of RAP250 as a coactivator in TGF-β signaling. We show that RAP250 can bind directly to Smad2 and Smad3 through a short domain consisting of LXXLL interaction domain 2 and its C-terminal-flanking region. Our results suggest that RAP250 is a coactivator for Smad2 and Smad3 on TGF-β regulated genes and that RAP250, together with Smad2 and Smad3, can act as a coactivator for LXRs.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—pGBT9-RAP250 plasmids were generated using PCR and standard cloning methods. The bait plasmid used in the yeast two-hybrid screen was made with primers F6 and R7 (9), and shorter forms of this plasmid were made either with PCR or with restriction enzyme digestes. The LXXLL to AXAL mutation was generated with PCR using the following primers: 5′-TCGGCAAGTCAGCTTTGACAACCTC-TGGAGCTCCCA-3′ and 5′-GTCAGGCTTGCACCTGC-GCATTGTTGCTTTCCCTCA-3′. Smad plasmids for the mammalian two-hybrid system were generated with PCR with pCDNA3-FLAG-Smad2 or pCDNA3-FLAG-Smad4 (24) as template using the following primers: Smad2-MH2-F, 5′-GATCGAATTCCAATATCATCCATCTACTCTCT-3′; Smad2-MH2-R, 5′-GATCCTCTAGATTATGACATGCTTGAGC-3′; Smad4-MH2-F, 5′-GATCAGAATTCCTCCCGGACA-TTACTGGCCTGT-3′; Smad4-MH2-R, 5′-GATCTCTAG-ATCAGTCTAAAGGTTGTGGGT-3′. Smad4 plasmids for the yeast two-hybrid screen were generated using RNeasy mini kit (Qiagen) according to the manufacturer’s protocol. The concentration and quality of the purified total RNA were determined spectrophotometrically. Synthesis of single-stranded cDNA was carried out on 0.1 μg of RNA using the SuperScript II reverse transcriptase kit (Invitrogen) following standard protocol. Real-time reverse transcription–PCR assay on the basis of SYBR Green I technology was performed with ABI 7500 fast real-time PCR system (Applied Biosystems). All primer pairs span intron-exon boundaries, and for each pair of primers, a dissociation curve analysis was conducted to validate the specificity of the PCR amplification. 100 ng primer concentrations were used in all reverse transcription–PCR analyses. We calculated relative changes employing the comparative method using 18 S as the reference gene and controls as calibrators. Primer sequences used are as follows: RAP250-F, 5′-GCTCATGGGAAACAGAGCA-GTTA-T-3′; RAP250-R, 5′-GACACGGGAGTTAAGCTG-3′; mABCA1-F, 5′-GGTTCAGGAGGTAAAAGGCGC-3′; mABCA1-R, 5′-CATTAAGAAGGGCAAA-AATGTCA-3′; mSREBP1c-F, 5′-TGGTGCAGCCCTCAT-3′; mSREBP1c-R, 5′-CATTTCAAGGGAATGCTGTTAGCT-3′; mCTGF-F, 5′-CATATGTAAGGAGGCTGATGTTAAGGCGC-3′; mCTGF-R, 5′-CAAGGCTTGGGATTTAG-3′; hCTGF-F, 5′-CTGGTGCAGCCCTCAT-3′; hABCG1-F, 5′-CTTTCAAGGGAATGCTGTTAGCT-3′; hABCG1-R, 5′-CAAGGCTTGGGATTTAG-3′.

**RESULTS**

Identification of Smad2 and Smad3 as RAP250-interacting Proteins—To identify proteins that can interact with RAP250, we used the yeast two-hybrid system with RAP250 (amino acids 1300–1771) as bait. The bait does not contain the RAP250 transactivation domain, which is active in yeast, or the well characterized LXXLL-1 interaction domain. In a screen of a human ovary cDNA library under high stringency conditions, we isolated several clones that encoded Smad2 and its homolog...
Smad3. Smad2 and Smad3 are intracellular mediators of TGF-β response and have a similar structure consisting of an N-terminal mad homology (MH)1 domain and a C-terminal MH2 domain separated by a linker region, as reviewed in Refs. 28 and 29. All Smad2 clones encoded amino acids 272–467, including the entire MH2 domain, and all Smad3 encoded amino acids 22–425, including most of the MH1 and the entire linker and MH2 domains. These results suggest that it is the highly conserved MH2 domain that is responsible for the interaction with RAP250. The MH2 domain is a known protein-protein interaction domain and interacts with many different proteins including TGF-β type 1 receptors, transcription factors, nuclear receptors, and transcriptional coactivators, and this domain is also responsible for oligomerization of Smads.

To confirm the interaction between RAP250 and Smad2/3 and to map the interaction domain in RAP250, the liquid β-galactosidase assay was used. Fig. 1A shows that when Y187 yeast was transformed with bait and prey plasmids, the bait used in the screen did not interact with the empty prey vector but only with the Smad2 clone. The Smad3 clone was also positive in the liquid β-galactosidase assay (data not shown), and the strength of the interaction between Smad2 and 3 and RAP250 was high, about the same as between the known RAP250 interaction protein peroxisome proliferator-activated receptor-γ and LXXLL-1, as determined by yeast two-hybrid assays (Fig. 1, b and c). Deletion mutants of the RAP250 bait showed that the interaction domain in RAP250 could be localized to a region between amino acids 1467 and 1512 (RAP250 1467–1512 in Fig. 1A). Interestingly, this interaction domain contains RAP250 LXXLL-2, the unusual LXXL interaction motif that selectively binds LXR; alane substitutions of the LXXLL interaction motif core positions +1 and +4 (LXXLL to AXXAL) abolished the interaction with Smad2. Fig. 1B shows that the LXXLL-1 interaction motif in RAP250 can interact with a range of nuclear receptors but not with Smad2, which is in contrast to LXXLL-2 that only interacts with LXRs and Smad2 (Fig. 1C). This result suggests selectivity in the interaction between Smad2 and LXXLL interaction motifs.

To further investigate the interaction between Smad2 and RAP250 LXXLL-2, we made N- and C-terminal deletions of the RAP250 1467–1512 bait and found that the interaction domain could be narrowed down to 27 amino acids (RAP250 1485–1512) (Fig. 1D). The C-terminal region flanking the LXXLL interaction domain seems to be a part of the Smad interaction domain since a deletion in this region (RAP250 1467–1502) prevents the binding to Smad2. However, this bait can still interact with LXRs (Fig. 1D). Next, we used baits that were chimeras of RAP250 LXXLL-1 and LXXLL-2 interaction motifs in the yeast two-hybrid system (Fig. 1E). A construct that contained LXXLL-1 in its normal context (Del4) did not interact with the Smad2 clone, and replacing LXXLL-1 with LXXLL-2 (Del4-NR2) did not affect binding (Fig. 1F). The chimeric bait D4-NR2-D6, which contains the N-terminal-flanking region from LXXLL-1, LXXLL-2, and its C-terminal region, interacted as strongly with Smad2 as LXXLL-2 in its normal context (Del6). However, the construct with LXXLL-2 and the C-terminal region from LXXLL-1 (D6-NR2-D4) did not interact with Smad2. These results indicate that the Smad2/3 interaction domain in RAP250 consists of the LXXLL-2 motif and its C-terminal-flanking region.

Smad2 MH2 Interacts with RAP250 LXXLL-2 in Mammalian Cells—To examine whether Smad2 and RAP250 can interact in mammalian cells, we used the mammalian two-hybrid system. First, we confirmed that the RAP250 LXXLL-2 interaction motif could interact with LXRα and LXRβ as described previously (10) (supplemental Fig. S1, A and B). Next, RAP250-NR2 (amino acids 1467–1512 containing LXXLL-2), Smad2-MH2, and Smad4-MH2 (the dimerization partner of Smad2) were cloned into the mammalian expression vectors as fusions to GAL4-DBD (pM) and VP16-activation domain (pVP16) and assayed on a GAL4-responsive reporter gene in the presence of TGF-β. With RAP250-NR2 fused to GAL4-DBD (pM-NR2), enhanced reporter activity was detected together with VP16-Smad2-MH2, and to a certain extent, also with VP16-Smad4-MH2 but not with other tested constructs (Fig. 2A). These results confirm the yeast two-hybrid data that Smad2-MH2 can interact with the RAP250 LXXLL-2 interaction domain. Smad2-MH2 fused to Gal4-DBD (pM-Smad2) enhanced expression together with VP16-NR2 and VP16-Smad4-MH2 (Fig. 2B). The -fold change was lower in this experiment, perhaps due to the higher transcription activation activity of the Smad2-MH2 domain that harbors an intrinsic transcription activation domain. Gal4-Smad4-MH2 (pM-Smad4) interacted as expected with VP16-Smad2 but not with the other constructs (Fig. 2C). Similar interaction data were obtained when a transfected constitutively active TGF-β receptor was used in the assay (supplemental Fig. S2, A–C). Together, these results demonstrate that RAP250 LXXLL-2 can interact with the MH2 domain of Smad2 in mammalian cells. Fig. 2D shows that the Smad interaction domain in RAP250 when fused to Gal4-DBD (pM-NR2) can act as a TGF-β-induced activation domain since it specifically confers TGF-β-induced transcription of the reporter gene, suggesting that it can recruit endogenous Smad proteins. To further examine the mechanism of the TGF-β action on LXXLL-2, we used siRNA against Smad4 (siSmad4). Transfected siSmad4 reduced the mRNA levels of Smad4 by more than 90% (supplemental Fig. S3A) and reduced the induction of plasminogen activator inhibitor-1 (PAI-1) by TGF-β to about 50% (supplemental Fig. S3B). Transfected siSmad4 enhanced the TGF-β-stimulated transcriptional activity of pM-NR2 (Fig. 2E), further suggesting a regulatory role of endogenous Smad proteins on the activity of this motif.

RAP250 Is Required for Maximum Induction of PAI-1 after TGF-β Stimulation—The observed interaction between Smad2 and Smad3 with RAP250 suggested that RAP250 could be a coactivator for Smad2 and Smad3. To test this hypothesis, we used MEFs from wild type and RAP250 knock-out embryos and monitored the expression of the TGF-β target gene PAI-1, which is regulated by direct binding of Smad proteins to the promoter region (30, 31). As seen in Fig. 3A, PAI-1 is induced 12-fold in wild type cells but only 6-fold in RAP250 knock-out cells, suggesting that RAP250 is a coactivator in the TGF-β signaling pathway. The connective tissue growth factor gene (CTGF) was also less induced by TGF-β in RAP250 knock-out cells (5-fold) when compared with wild type cells (10-fold) (Fig. 3B). We also assayed the TGF-β target genes Smad7 and p21,
but no major regulation by TGF-β was monitored (data not shown). The fact that the activity in the RAP250 knock-out cells is not completely abolished probably reflects coactivator redundancy. It is well known that Smad proteins are able to interact with a number of coactivators including CBP/p300 and Mediator (32–34); hence, a completely blunted activity was not expected in the RAP250 knock-out cells.

**TGF-β Stimulates the Expression of LXR Reporter Genes and ABCG1**—Since RAP250 is known to be a coactivator for LXR and in view of our finding that Smad2 and Smad3 can interact...
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FIGURE 2. RAP250 interacts with Smad2 but not Smad4 in mammalian cells. A, mammalian two-hybrid assay in Huh7 cells using Gal4-DBD-tagged RAP250, pM-NR2, (amino acids 1467–1512), together with pVP16, pVP16-RAP250-NR2, pVP16-Smad2-MH2, and pVP16-Smad4-MH2. The assay was performed with TGF-β and the reporter plasmids UAS-tk-Luc and pCMVβ. Normalized luciferase activity is shown with the activity of pVP16 together with respective pM-fusion set to 1.0. RLU, relative light units. B, as in A but with Gal4-DBD-tagged Smad2-MH2. C, as in A but with Gal4-DBD-tagged Smad4-MH2. D, luciferase reporter assay in Huh7 cells transfected with either pM (Gal4-DBD) or pM-NR2 with UAS-tk-Luc and CMVβ-reporter plasmids and with or without TGF-β. E, luciferase reporter assay in Huh7 cells transfected with pM-NR2 and the reporter plasmids UAS-tk-Luc and CMVβ. The cells were also transfected with siRNA against Smad4 and stimulated with TGF-β as indicated. All experiments were performed at least three times. Values shown are the means of experiments performed in triplicate, and error bars indicate standard deviation.

In Fig. 4D, there is a synergistic effect of GW3965 and TGF-β on ABCG1 in wild type MEFs. However, in LXR double knockout cells, TGF-β had no effect on ABCG1 expression levels (Fig. 4E), although PAI-I mRNA was induced by TGF-β stimulation (supplemental Fig. S5, A and B), suggesting that the positive effect of TGF-β stimulation is mediated through LXR.

DISCUSSION

In this study, we have used the yeast two-hybrid system to identify proteins that directly interact with RAP250, and we identify the intracellular mediators of TGF-β signaling as RAP250-interacting proteins. Our results indicate that RAP250 is a new transcriptional coactivator for Smad2 and Smad3 and that the interaction between Smad2/3 and RAP250 influences the activity of Smad proteins both as transcription factors and as coactivators. The Smad interaction domain in RAP250 was mapped to a short unique domain containing LXXLL interaction domain 2, and we also show that it is the MH2 domain in Smad2 and Smad3 that is responsible for the interaction. MH2 domains are known to interact with a variety of different proteins (28, 29), but the LXXLL interaction motif is mainly associated with protein-protein interactions between nuclear receptors and their coactivators (35, 36). There are now a few reports of LXXLL interaction motifs involved in other protein-protein interactions (37), but so far, no interactions with Smad proteins have been described. In addition to LXXLL-2, its flanking C-terminal domain is required for the interaction between RAP250, Smad2, and Smad3. This is similar to the interactions between SRC-1 and nuclear receptors where the specificity of
protein-protein interactions is determined by amino acids C-terminally to the LXXLL interaction motif (38).

The Smad interaction domain in RAP250 shows no strong similarity to other Smad interaction domains such as the Smad-binding domains in Smad anchor for receptor activation (SARA) or the Smad-interacting motifs in FoxH1 (39, 40) or other proteins in the National Center for Biotechnology Information (NCBI) data base and appears to be selective in its binding since Smad2, Smad3, and LXRs are the only proteins that are known to bind to this domain. This domain may therefore represent a good target for inhibition of TGF-β activity in diseases where TGF-β signaling is unwanted such as fibrosis and cancer.

The role of RAP250 as a coactivator in the TGF-β signaling pathway was demonstrated using RAP250 knock-out MEFs. We show that the TGF-β target genes PAI-1 and CTGF are less induced in MEFs from RAP250 knock-out mice, indicating a role for RAP250 in its regulation. PAI-1 is a direct TGF-β target gene and binds Smad proteins in its promoter region after TGF-β stimulation. A possible role for RAP250 in the regulation of PAI-1 could be as a linker between CBP/p300 and mediator complexes since both RAP250 and Smad proteins are known to associate with CBP/p300, and the mediator components (11, 12, 34, 41), but other effects of RAP250 recruitment cannot be ruled out since RAP250 is known to associate with proteins that affect processes such as protein methylation and RNA processing (8). Our study also shows that costimulation by TGF-β and LXR agonists results in a dramatic induction of the ABCG1 expression. Since the role of ABCG1 in reverse cholesterol transport is well known and it is believed to have important antiatherogenic properties, simultaneous stimulation of these signaling pathways might be a way to reduce cholesterol accumulation. TGF-β has previously been reported to inhibit macrophage cholesterol ester accumulation by enhancing cholesterol efflux by up-regulation of ABCG1 expression (21), and the latter observation is in agreement with our findings here. However, the synergistic effect of LXR agonists and TGF-β on ABCG1 expression is a novel finding. In this case, we believe that RAP250 affects the role of Smad proteins as coactivators. Smad proteins have been shown to bind directly to nuclear receptors such as estrogen receptors (42) and vitamin D receptor (43), and it is interesting to speculate that RAP250 with its capacity to bind both LXR/RXR and Smad protein, such as CBP, may be a part of this complex.

![FIGURE 4. TGF-β and LXR have additive effects on ABCG1 expression.](image)

The experiment was performed three times, and the error bars indicate standard deviation.

![FIGURE 5. Model of RAP250- and Smad-binding structures.](image)

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