Role of p160 Coactivator Complex in the Activation of Liver X Receptor

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Objective—Liver X receptor (LXR) is a member of a nuclear receptor family regulating the expression of several key proteins involved in lipid metabolism and inflammation. In contrast to several other nuclear receptors, very little is known about the coactivators needed for the agonist-mediated transactivation by LXR. In this study, we have investigated the role of p160 coactivator complex in the regulation of ATP-binding transporter A1 (ABCA1), a clinically important gene transcriptionally upregulated by LXR/RXR (retinoid X receptor) heterodimer.

Methods and Results—Overexpression of LXRα, SRC-1, and p300, either alone or in combination, increased the luciferase activity driven by the wild-type ABCA1 promoter. The same coactivators bound to the ABCA1 promoter on oxysterol induction in chromatin immunoprecipitation assays. To the contrary, CARM-1 and P/CAF had no effect on ABCA1 transactivation, nor do they bind the promoter. When the DR-4 element was mutated from the ABCA1 promoter, only p300 was able to activate ABCA1 transcription in a ligand-independent manner.

Conclusions—The p160 coactivator complex members SRC-1 and p300, but not CARM-1 and P/CAF, coactivate LXR-mediated transcription of ABCA1 gene. In addition, p300 activates ABCA1 transcription independently of DR-4 element and LXR/RXR. (Arterioscler Thromb Vase Biol. 2004;24:703-708.)

Key Words: LXR • ABCA1 • HDL • coactivator • transcriptional regulation

Liver X receptor (LXR) is a nuclear protein regulating the transcription of key factors in lipid metabolism and inflammation.1,2 Its 2 isoforms, LXRα and LXRβ, both function usually as a heterodimer with retinoic X receptor (RXR).3 Transcriptional regulation by the LXR/RXR is achieved when the dimer bound to the DR-4 element of target promoters is activated by the ligand. This is accompanied by the dissociation of co-repressors and association of coactivators in the nuclear receptor complex. Although the activation mechanisms used by LXR are currently not well known, studies using other nuclear receptors have yielded a wealth of data leading to a general model for ligand-mediated nuclear receptor activation. In the noninduced state, nuclear receptors are believed to associate with co-repressors, such as silencing mediator for retinoic acid and thyroid hormone receptor (SMRT)4 and nuclear receptor co-repressor (N-CoR).5 At this point, the chromatin is in the compact state and the histones are non-acetylated because of the action of several histone deacetylases.6 On ligand binding, these co-repressor complexes dissociate and the agonist-bound receptors interact with distinct multi-protein coactivator complexes that contribute to the transmission of activating signals. There are several types of coactivator complexes that function together with the basic transcriptional machinery to remodel chromatin structure and initiate transcription.7-9 A chromatin remodeling complex (containing the catalytic subunits brahma [BRM] and brahma-related gene 1 [BRG-1] in humans) causes local changes in chromatin structure in an ATP-dependent manner.10,11 A family of p160 coactivators and associated molecules mediate binding to the nuclear receptors and also catalyze the acetylation and methylation of histones and other factors in the complex.12 Mediator complexes, such as thyroid hormone receptor-associated proteins (TRAP)13 and vitamin D receptor-interacting proteins (DRIP),14 recruit polymerase II to the site of transcription either by bridging nuclear receptors or by the associated p160 coactivator complex to the basal transcription machinery.

The aim of the current study was to investigate a possible role of the p160 coactivator complex in the transcriptional activation of ABCA1 by LXR and its oxysterol ligands. This complex consists of several proteins and functions by inducing local changes in the nucleosome structure via acetylation and methylation of histones and other components of the complex. p160 coactivators steroid receptor coactivator-1 (SRC-1), SRC-2 (also known as GRIP-1), and SRC-3 interact directly with nuclear receptors via LXXLL motifs (L indicates leucine; x, any amino acid), and with other members of this complex via AD1 and AD2 interaction domains.12 CBP (CREB-binding protein) and p300 are homologous coactivators capable of binding to the AD1 domain of p160 coactivators...
Chromatin Immunoprecipitation
Chromatin immunoprecipitation (ChIP) assays were performed with 293 cells stably overexpressing FLAG-tagged LXRα. This cell line was constructed by transfecting cells with FLAG-LXRα plasmid DNA vector (Invitrogen) and by selecting the transfected cells by growing them in DMEM medium containing 750 μg/mL neomycin. For the ChIP assays, 3×10^6 cells were seeded in 10-cm dishes in DMEM plus 10% FCS plus gentamycin and grown for 3 days. The medium was replaced with DMEM supplemented with 10% charcoal-stripped, delipidated serum (Sigma) and antibiotics. After 24 hours, the medium was substituted by DMEM plus 2 mg/mL T3-free bovine serum albumin (Calbiochem, San Diego, Calif) and antibiotics. The next day, half the cells were treated by adding 10 μmol/L 22(R)-OH for 1 hour, whereas the other half served as control. The cells were treated with a cross-linker (1% formaldehyde) for 10 minutes at RT and cross-linking quenched with 0.125 mol/L glycine for 5 minutes at RT. The cells were scraped into 50-mL tubes and washed twice with ice-cold PBS. The pellet was resuspended into 3 mL of buffer containing 3 mmol/L MgCl₂, 10 mmol/L NaCl, 10 mmol/L Tris-HCl pH 7.4, and 0.1% NP-40, incubated on roller for 10 minutes in the cold room and centrifuged at 2000g for 5 minutes at 4°C. The pellet was resuspended in 3 mL of PBS containing 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS and sonicated to yield chromatin fragments with an average length of 500 bp. The cell extract was incubated overnight with 5 μg of antibody against FLAG (F-7424; Sigma), RXR (sc-553; Santa Cruz Biotechnology, Santa Cruz, Calif), SRC-1 (sc-8995; Santa Cruz), p300 (sc-585; Santa Cruz), CARM-1 (#07-080; Upstate, Lake Placid, NY), or P/CAF (#07-141; Upstate). The antibody-protein-DNA complex was precipitated by incubating with Pansorbin staphylococcus cells (which contain a coat of protein A; Calbiochem) for 1 hour at 4°C. After co-precipitation, the cells were washed twice for 10 minutes at room temperature with 50 mmol/L Tris-HCl pH 8.0, 2 mmol/L EDTA, and 0.2% wt/vol sarkosyl, and 4 times with 100 mmol/L Tris-HCl pH 9.0, 500 mmol/L LiCl, 1% NP-40, and 1% deoxycholic acid. Cross-linked material was eluted with 1% SDS in 0.1 mol/L NaHCO₃. Cross-linking was reversed in 0.3 mol/L Na₂CO₃ for 4 hours at 67°C. After ethanol precipitation, proteins were digested with proteinase K and DNA precipitated after phenol-chloroform extraction. The DNA was used as template for PCR performed with Tth polymerase (Applied Biosystems, Foster City, Calif). ABCA1 oligonucleotides (5′-cccactcctcactgagctca-3′ reverse) spanning the promoter DR-4 element were used in the PCR.

Western Blotting
Nuclear extracts and cytosolic fractions were prepared from both 293T cells and cells transfected with various coactivators. Lysates from transfected cells were prepared by directly scraping the cells from 24 well-plate wells into 100 μL of hot Laemmli sample buffer containing 100 mmol/L β-mercaptoethanol; 50 μg of nuclear extracts or cytosolic fraction, or 20 μL of cell lysate, was separated on 10% SDS-PAGE gel, transferred onto nitrocellulose membrane, and blotted with the same antibodies as used for the ChIP experiments. Additionally, LXR antibody (LXRα, sc-1201) was used to detect endogenous LXR in Western blots.

Results
Expression Levels of Nuclear Receptors and Coactivators
The expression levels of LXR, RXR, and p160 coactivator family members were determined using Western blotting. Figure 1A shows the expression levels in whole cell extracts from both control cells and cells transfected with coactivators. The transfection of FLAG-tagged LXRα, SRC-1, p300, CARM-1, and P/CAF expression plasmids in 293T cells results in overexpression of these proteins compared with control cells. Transfection efficiency of the 293T cells was...
Figure 1. Expression levels of coactivators. A, Cell lysates from basal cells (− transfection) or cells transfected with various coactivators (+ transfection), either in the absence [− 22(R)-OH] or in the presence [+ 22(R)-OH] of LXR agonist were prepared, separated on SDS-PAGE gel, transferred onto nitrocellulose membrane, and blotted with specific antibodies against the proteins. B, Nuclear extracts (NE) and cytosolic fractions (rest) from 293T cells (− transfection) or cells transfected with individual plasmids (+ transfection) were prepared and blotted as mentioned. FLAG antibody (first line) was used to detect the overexpressed tagged LXR, whereas the LXR antibody (second line) detects endogenous and overexpressed LXR. Note that the panel for RXR refers to endogenous levels in either basal cells (− transfection) or the cells transfected with LXR (+ transfection).

Transcriptional Activation of ABCA1
Coactivators were transfected into 293T cells together with a firefly luciferase expression plasmid under the wild-type ABCA1 promoter (bases −844/+188, numbering based on Pullinger et al21) or with the same promoter in which the DR-4 element had been mutated. When the cells transfected with only luciferase construct were incubated with 10 μmol/L 22(R)-OH, the luciferase activity of the wild-type promoter was tripled (Figure 2A). Luciferase activity was significantly increased when the cells were transfected with expression plasmids encoding LXRα, SRC-1, p300, or combinations of these plasmids. In each case, the addition of hydroxysterol to the cells was associated with a further 2- to 3-fold increase in luciferase activity. On the contrary, transfecting the cells with CARM-1 or P/CAF plasmids either alone or in combination with SRC-1 and p300 led to no further increase above the maximal induction observed with the combination of LXRα, SRC-1, and p300 (Figure 2A). Statistical analysis of the results (Student t test, pair-wise comparison, 4 independent experiments performed in duplicate) indicated that in control and 22(R)-OH–induced cells, LXR overexpression versus control (P<0.01), SRC-1 or p300 overexpression versus LXR (P<0.05), and SRC-1 plus p300 overexpression versus expression of these components alone (P<0.05) was statistically significant. When SRC-1 expression plasmid was substituted with its close homolog GRIP-1, or when p300 was substituted with its homolog CBP, identical patterns of transcriptional activation to those observed with SRC-1 and p300 were obtained (data not shown). Taken together, these results show that maximal transcriptional activation of ABCA1 is achieved by co-expression of LXR, SRC-1, and p300 in the presence of oxysterols. In addition, the p160 coactivator family members SCR-1 and p300 can be substituted by their respective homologs, GRIP-1 and CBP.

When the DR-4 element in the ABCA1 promoter was inactivated by mutation, the basal level of promoter activity was reduced by 70% and the inducibility with hydroxysterol was almost lost in every combination of coactivators (Figure 2B). In contrast to the wild-type promoter, only p300 increased the luciferase activity of the mutant promoter. This means that in the ABCA1 promoter construct, the DR-4 element is the only element that uses SRC-1 as a coactivator. In contrast, p300 also acts as a coactivator independent of LXR/RXR heterodimer interaction, because transcription was still increased 5- to 7-fold in the mutated ABCA1 promoter construct on p300 overexpression. The combined effect of p300 and SRC-1 was observed only on wild-type and not on the mutated ABCA1 promoter (Figure 2). This indicates that p300 activates transcription by acting as a coactivator for LXR (via the intact DR-4 element), but it also activates ABCA1 transcription by coactivating one or several other transcription factors.

Another LXR agonist, T0901317, and RXR agonist, 9-CRA, produced an identical pattern of results compared with 22(R)-OH induction (Figure 2C). However, the response with T0901317 was unexpectedly weaker compared with the other 2 agonists.

Binding of Coactivators to the ABCA1 Promoter
We used chromatin immunoprecipitation (ChIP) to study the interaction of coactivators with DR-4 element of the ABCA1
promoter. These studies were performed in 293 cells stably overexpressing FLAG-tagged LXR. In noninduced cells, strong binding of LXR and RXR and very weak binding of SRC-1 and p300 to the DR-4 promoter element were observed (Figure 3). When the cells were induced with 22(R)-OH, the binding of SRC-1 and particularly p300, but not CARM-1 or P/CAF, was noticeably increased at this site. This difference cannot be explained by the absence of CARM-1 or P/CAF in these cells because these proteins were expressed in the nucleus of the cells (Figure 1B). The functionality of ChIP assays is based on antibodies that are capable of recognizing proteins after formaldehyde crosslinking. The antibodies against CARM-1 and P/CAF have previously been used to detect the binding of these coactivators in ChIP assays,26,27 further indicating that lack of binding to ABCA1 promoter was not the result of dysfunctional antibodies. The results from ChIP assays are consistent with the data on luciferase assays in showing that only SRC-1 and p300 activated the ABCA1 promoter (Figure 2). As a negative control in the ChIP assay, we used a genomic DNA 3 kb downstream of the ABCA1 DR-4 element. No binding of any of the proteins to this site was observed (Figure 3).

Discussion

In this study, we report for the first time to our knowledge the role of p160 coactivator family members in the transcriptional regulation by LXR/RXR heterodimer. We used 293T cells because these cells exhibit robust overexpression of proteins on transfection (Figure 1A). Even in the absence of LXR overexpression, ABCA1 promoter activity was tripled on addition of oxysterol ligand (Figure 2), indicating the presence of endogenous LXR. Further, in the nucleus of 293T

Figure 2. Regulation of ABCA1 promoter activity. The 293T cells were transfected with firefly luciferase expression plasmid under the control of ABCA1 wild-type promoter (A, C) or the same promoter in which the DR-4 element had been mutated (B). The cells were cotransfected with expression plasmids for LXR, SRC-1, p300, CARM-1, and P/CAF. The luciferase activity 24 hours after transfection from noninduced (control, white bars), oxysterol-treated [22(R)-OH, black bars], T0901317-induced (light gray in panel C), or 9-CRA incubated (dark gray in panel C) cells was determined. The results are expressed relative to the wild-type promoter (no overexpression of LXR or coactivators) under non-inducing conditions. The vertical lines in panels A and B indicate which cotransfections resulted in statistically significant differences (see text for details). A representative experiment performed in duplicate is shown. Please note the different scales of relative luciferase activities in A and B.
cells, all the proteins studied are also present without overexpression (Figure 1B). These facts support the use of the 293T cell model for the regulation of ABCA1 transcription by LXR/RXR.

The transcriptional activity of the ABCA1 promoter was enhanced by the overexpression of LXRα, SRC-1, and p300, but not by CARM-1 and P/CAF (Figure 2). These results are in agreement with previous studies reporting the role of SRC-1 and p300 for the regulation of transcription by estrogen and progesterone receptors. An unexpected result was that in contrast to androgen, estrogen, and thyroid hormone receptors, LXR activity was not enhanced by the overexpression of CARM-1 or P/CAF (Figure 2). Although distribution of these overexpressed proteins was normal, their activities were not otherwise demonstrated in this study. The co-stimulatory effect of CARM-1 was observed in previous studies only when the levels of transfected nuclear receptors were low and the agonist was present. Even in cells that had not been transfected with LXR, we found no stimulatory effect of CARM-1 on LXR activity (data not shown). Negative results on the role of P/CAF for LXR-mediated activation were also observed (Figure 2A). This coactivator exists in a large multi-protein complex and activates retinoic acid, estrogen, androgen, and thyroid hormone receptors. The current findings suggest that although CARM-1 and P/CAF are able to coactivate some nuclear receptors, they play little role in LXR activation.

The data from luciferase assays are supported by the chromatin immunoprecipitation results, in which the binding of SRC-1 and p300, but not those of CARM-1 and P/CAF, to the ABCA1 promoter was observed on addition of LXR ligand to the cells (Figure 3). When Wagner et al. studied the changes in the acetylation and methylation status of ABCA1 promoter, they made 2 observations that further support our results: histone acetylation was increased on ligand addition whereas the methylation status of the histones was unchanged. This can be explained by the fact that the histone acetyltransferase p300 is recruited to the promoter, acetylating histones, and activating transcription, whereas methyltransferase CARM-1 does not bind the promoter nor has an effect on luciferase activity. Together, all these results suggest that there are differences between the nuclear receptors with respect to the use of p160 coactivator family members in transcriptional activation. It is also noteworthy that the coactivators SRC-1 and p300 can be replaced by their close homologs GRIP-1 (also called SRC-2) and CBP, respectively, to achieve maximal induction of ABCA1 gene activity (data not shown). This redundancy between the family members is possibly a way to ensure that in all tissues and cell types the necessary coactivators (or one of their close homologs) are always present to maximize gene activation.

Mutating the DR-4 element in the ABCA1 promoter led to 70% reduction in basal promoter activity (Figure 2). This is in agreement with previous findings indicating the role of intact DR-4 for LXR-mediated and oxysterol-mediated upregulation of ABCA1 gene. A novel finding in this study was the differential role of SRC-1 and p300 in the regulation of mutated ABCA1 promoter. Only p300 was able to upregulate transcription from the mutated promoter, and the combined effect of p300 and SRC-1 observed in the intact ABCA1 promoter was lost when the DR-4 element was mutated (Figure 2). These findings can be explained by the fact that SRC-1 is mostly associated with nuclear receptor-mediated transcriptional activation, whereas p300 can also coactivate several other transcription factors, the binding sites of which are present in the ABCA1 promoter.

Based on our current results (and those published by other groups), a model for the activation of LXR is proposed (Figure 4). In the noninduced state, co-repressors N-CoR and SMRT are bound to LXR. On agonist binding, these co-repressors dissociate and are replaced by p160 coactivator family members (either SRC-1 or GRIP-1) and p300/CBP. This either alone or in combinatorial effect with ATP-dependent chromatin remodeling and/or mediator complex leads to transcriptional activation. Based on this study, binding of CARM-1 to the AD2 domain of p160 coactivators and the interaction of P/CAF with p160 coactivator complex are not needed for LXR-mediated transcription. The nuclear receptors and coactivators investigated in this study are gray.
mechanisms. Activating signal co-integrator 2 (ASC-2) has been shown to interact specifically with LXR via its second LXXLL motif and to activate transcription.36 Precise mechanism is not currently known, although ASC-2 exists in a complex containing methyltransferases. Further, LXR can interact with multi-protein bridging family 1 (MBF-1).37 MBF-1 directly interacts with transcription factor IID complex, making it a possible bridging molecule between LXR and basal transcription machinery. Future research is needed to resolve the relationship between these different activating mechanisms, as well as the role of ATP-dependent chromatin remodeling and mediator complexes for the LXR-induced transcriptional activation.

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