Additional Sex Comb-like 1 (ASXL1), in Cooperation with SRC-1, Acts as a Ligand-dependent Coactivator for Retinoic Acid Receptor*

Received for publication, November 28, 2005, and in revised form, March 16, 2006 Published, JBC Papers in Press, April 10, 2006, DOI 10.1074/jbc.M512616200

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Additional sex comb-like 1 (ASXL1, 170 kDa), a mammalian homolog of Drosophila ASX, was identified as a protein that interacts with retinoic acid receptor (RAR) in the presence of retinoic acid (RA). Systematic binding assays showed that the C-terminal nuclear receptor box (LVMQLL) of ASXL1 and the activation function-2 activation domain (AF-2 AD) core of the RAR are critical for ligand-dependent interaction. The interaction was confirmed using in vitro glutathione S-transferase pulldown and in vivo immunoprecipitation (IP) assays. Confocal microscopy revealed that ASXL1 localizes in the nucleus. In addition to the intrinsic transactivation function of ASXL1, its cotransfection together with a RA-responsive luciferase reporter increased the RA activity. This ASXL1 activity appears to be mediated through the functional cooperation with SRC-1, as shown by GST pulldown, IP, chromatin IP, and transcription assays. In the presence of ASXL1, more acetylated histone H3 was accumulated on the RA-responsive promoter in response to RA. Finally, stable expression of ASXL1 increased the expression of endogenous RA-regulated genes and enhanced the antiproliferative potential of RA. Overall, these results suggest that ASXL1 is a novel coactivator of RAR that cooperates with SRC-1 and implicates it as a potential antitumor target of RA in RA-resistant cancer cells.

The all-trans and 9-cis forms of retinoic acid (RA)3 strongly influence various biological processes, such as cell differentiation, proliferation, and development (1, 2). These pleiotropic effects of RA are transduced by two families of nuclear receptors (NRs), the retinoic acid receptors (RARs, -ß, and -y) and the retinoid X receptors (RXRs, -ß, and -y). RA-bound receptors associate with cis-acting RA-responsive elements (RARE), located in the regulatory regions of target genes, and activate gene transcription. Both RARs and RXRs contain two activation functions (AFs) (3) as follows: the ligand-independent N-terminal AF-1 and the RA-induced AF-2 associated with the ligand-binding domain (LBD). Transcriptional regulation by NRs (including RARs and RXRs) involves the binding and recruitment of coactivators to target gene promoters (4).

In the past decade, a large number of coactivators that associate with retinoid receptors and enhance their ability to activate the transcription of target genes have been cloned and characterized (5, 6). Functional studies have suggested that the coactivators lack apparent NR specificity and that different coactivators have two roles in NR activation (7–14). First, they generate a transcriptionally permissive state within the promoter region by modifying the chromatin structure via the histone acetyltransferase activity of p160 proteins in concert with other factors, including GCN5/PCAF and p300/CBP, the histone methyltransferase activity of CARM1/PRMT1, and the ATP-dependent chromatin remodeling activity of SWI/SNF complexes. Second, they recruit the RNA polymerase II complex to the promoter region by bridging the transcriptional machinery to NRs via interactions with either p300/CBP or the TRAP/SMCC/DRIP/ARC complex. These coactivators share a typical receptor interaction region composed of one or more copies of a consensus leucine-rich motif, LXXLL, also called the NR box (15). Biochemical and structural studies have shown that the NR box is critical for interactions with the ligand-bound AF-2 core domain of NRs, thereby providing a molecular basis for NR-coactivator recruitment and NR activation (16).

Although the mechanisms of action of RAR (and RXR) coactivators have been well documented at the molecular level, the physiological roles of such coactivators in RA-mediated biological processes are poorly understood. One set of RA-regulated genes is the Hox gene family, which plays a crucial role in development and cell differentiation (17–19). Most interestingly, Hox genes show a colinear response to RA in cell culture, and each Hox gene has a limited window of sensitivity to RA in animals (20, 21). RAR binds to RAREs in Hox regulatory regions in the presence of RA and activates Hox gene expression (22–24). Despite the critical role of RAR in Hox gene expression, little is known about how RAR regulates Hox gene activation and what other factors are involved. In Drosophila, the Polycomb (PcG) and trithorax (TrxG) group proteins maintain spatial patterns of Hox gene expression in appropriate segments (25, 26). In general, PcG proteins are repressors that maintain the off-state, whereas TrxG proteins are activators that maintain transcription. Like their Drosophila counterparts, the mammalian PcG/TrxG proteins maintain the correct expression patterns of the Hox genes. In addition, evidence has accumulated that PcG/TrxG proteins play a critical role in certain human cancers. Recent knock-out studies with M33, a mouse PcG, have suggested that mammalian PcG/TrxG participate in the regulation of Hox gene expression in response...
to RA (27). However, the molecular basis underlying the mechanism is not clear.

Here we address the hypothesis that unidentified PcG/TrxG proteins are present in mammals and mediate the RA response through a functional association with RAR. To identify such factor(s), we performed a yeast two-hybrid screen and found Additional sex comb-like 1 (ASXL1) (28), a mammalian homolog of Drosophila ASXL, which interacts with RAR in the presence of RA. In this study, we demonstrate that mammalian ASXL1 interacts with the AF-2 AD core of RAR (and RXR) through a novel, promiscuous NR box (LVMQLL) and enhances transcriptional activity of the receptors in certain cells. We also show that ASXL1 associates specifically with SRC-1 and cooperates synergistically in the transcriptional activation. In addition, overexpression of ASXL1 in HeLa cells results in increased cytotoxicity of RA. Therefore, ASXL1 may represent a new RAR coactivator that cooperates with SRC-1 functionally and mediates the RA response in vivo.

EXPERIMENTAL PROCEDURES

Cell Lines and Cell Culture—HeLa, MCF-7, and NIH3T3 cells were maintained in DMEM supplemented with 5% heat-inactivated fetal bovine serum (FBS) and antibiotic/antimycotic (all from Invitrogen) in a 5% CO2 atmosphere at 37 °C. For transcription assay, FBS was pretreated with charcoal.

Plasmids and Cloning—All cDNAs were constructed according to standard methods and verified by sequencing. The multiplicity yeast expression plasmids used in the two-hybrid assays were described elsewhere (29). Deletion mutants of the desired genes were created by PCR amplification and subcloned into the pBMT116 or pASV3. FLAG (2×)-tagged mASXL1 genes were placed on pCDNA3 vector (Invitrogen). Gal4-tagged mASXL1 deletions were placed on pG4MpolyII vector. GFP- and HcRed-tagged constructs were created into pEGFP-C3 and pHcRed (BD Biosciences), respectively. For GST-fused proteins, either mGEX2T or mGEX4T-1 (Amersham Biosciences) was used.

Yeast Two-hybrid Screening and Assays—A HeLa cDNA library in the prey plasmid pACT2 (BD Biosciences) was screened for proteins that interact with hRAR using yeast reporter strain L40. Experimental procedures were the same as reported previously (29) except LexA-fused hRARs DEF was used as bait in this study. To map the RAR/RXR interaction domain in ASXL1, deletion derivatives of ASXL1 were fused with a VP16 AD by subcloning into pASV3, and to also localize the ASXL1-binding motif on the RAR/RXR, AF-2 AD core mutants of RAR DEF or RXR DE were fused with a LexA DBD by subcloning into pBMT116. The level of interaction was determined by quantitative β-galactosidase assays.

Glutathione S-transferase Pulldown Assays—Various GST fusions of ASXL1 were purified on glutathione-Sepharose beads (Amersham Biosciences) by standard methods. The indicated NR proteins were translated in vitro in rabbit reticulocyte lysate (Promega, Madison, WI) supplemented with [35S]methionine (Amersham Biosciences). Detailed experimental procedures were described previously (29) except that assays were performed in the presence of a ligand in this study.

Immunoprecipitation Assays—After transfection with the indicated plasmid DNA, either NIH3T3, MCF-7, HeLa, or HEK293 cells were washed in phosphate-buffered saline (PBS), and cell lysates were prepared by adding 1 ml of TEN modified buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.1% Nonidet P-40, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride) supplemented with protease inhibitors (Roche Applied Science). Lysates were percolated by preincubation with protein A/G-agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) for 15 min, incubated at 1:200 dilution of anti-NRs or SRC-1 antibody (all from Santa Cruz Biotechnology) for 2 h at 4 °C, and further incubated for 1 h at 4 °C with beads. Beads were washed three times with PBS, and the immune complexes were released from the beads by boiling. Following electrophoresis on 10% SDS-PAGE, immunoprecipitated products were analyzed by Western blotting using anti-GFP rabbit polyclonal antibody (Sc-8334; Santa Cruz Biotechnology) or anti-FLAG M2 monoclonal antibody (F-3165; Sigma) (1:1000).

Confocal Microscopy—NIH3T3 cells seeded on coverslips in 6-well plates, transfected with GFP-mRAR and/or HcRed-mASXL1. One day after transfection, 1 μM ATRA was treated. Other procedures were the same as reported previously (29).

Transient Transfection and Luciferase Assay—HeLa or NIH3T3 cells were seeded in a 12-well culture plate at a density of 1.5 × 104 cells/well. Transfection was performed using Lipofectamine Plus reagent (Invitrogen) with Gal4-tk-luciferase or RARE-tk-luciferase. Depending on the experimental conditions, Gal4-ASXL1, Gal4-RAR (or RAR), or Gal4-RXR (or RXR) expression vector was cotransfected. After 4 h of transfection, cells were washed, fed with 5% charcoal-stripped medium, and incubated for an additional 20 h in the presence of ligand. Cells were then washed with ice-cold PBS, collected, resuspended in 100 μl of luciferase lysis buffer (Promega), and subjected to three freeze-thaw cycles. Luciferase activity was measured by adding 20 μl of luciferin into 30 μl of lysates using an analytical luminescence luminometer according to the manufacturer’s instructions (Promega). β-Galactosidase activity was determined in 96-well plates that were read at 405 nm using an enzyme-linked immunosorbent assay reader. The luciferase activities were normalized to the β-galactosidase activity.

Stable Transfection and RT-PCR—HeLa cells were transfected with either mASXL1 (or mASXL1ΔPHD) subcloned into the G418-resistant vector pcDNA3 (2× FLAG-tagged) or empty vector in the presence of Lipofectamine Plus reagent (Invitrogen) for 48 h and treated with 1 mg/ml. After 5–7 days, cells were incubated with fresh culture medium containing G418, and G418-resistant colonies were selected for 2 weeks. Cells stably expressing FLAG-mASXL1 (or mASXL1ΔPHD) was verified by Western blotting using anti-FLAG antibody (Sigma).

For RT-PCR, HeLa cells stably expressing FLAG-mASXL1 (or mASXL1ΔPHD), grown in DMEM with 5% charcoal-stripped FBS, were treated with 2 μM ATRA as indicated. Total RNA was extracted using TRIzol Reagent (Invitrogen), and 5 μg of RNA was reverse-transcribed using Superscript II reverse transcriptase (Invitrogen) and random oligo(dT) primers (New England Biolabs, Beverly, MA). RT products were amplified by PCR using pairs of primers as follows: for the RARβ2 coding sequence (421 bp), forward, 5′-TGGATGTTCAGTCAAGTCT-C3′, and reverse, 5′-CCCCATTCAAGACCTTCT-3′; for the PDCD4 coding sequence (504 bp), forward, 5′-ATGGATGTAGAAAATGAGCAG-3′, and reverse, 5′-GTAGCTCTGGTTTAA-3′; for control G6PDH (421 bp), forward, 5′-ATGGATGTTCAGTCAAGTCT-3′, and reverse, 5′-GCCATCAGGACCTACA-3′.
A schematic representation of human and mouse ASXL1. B, schematic representation of the human ASXL family. The chromosomal location of each gene is shown.

**FIGURE 2.** Identification of ASXL1 by yeast two-hybrid screen. A, locations of identified clones in human ASXL1 (B3, C8, A346, and A104). These clones were identified by yeast two-hybrid screen using a Gal4 AD-fused HeLa cDNA library and LexA DBD-fused RAR (DEF; ligand-binding domain or LBD) as bait. B, interaction of ASXL1 with hRAR-DEF (DEF) in the presence of AtRA, SRC-1 (amino acids 1205–1441) and NcoR-1 (amino acids 1953–2440) were used for comparison. Interactions were monitored by introducing LexA DBD-fused RAR (DEF) and Gal4 AD-fused test partner into yeast strain L40, and by β-galactosidase (β-gal) assays. Fold β-galactosidase activity indicates relative value compared with Gal4 AD empty control. In all experiments 1 μM of AtRA was treated. Me2SO (DMSO) was used as a control for AtRA. C, interaction of ASXL1 with other nuclear receptor family. Yeast two-hybrid assays were performed with other NRs indicated in the presence of their cognate ligands as follows: for RAR, 1 μM AtRA; RXR, 1 μM 9-cis-RA; ER, 1 μM estradiol (E2); GR, 5 μM deoxycorticosterone (DOC); TR, 1 μM triiodo-L-thyronine (T3). Mouse full-length ASXL1 was shown by mASXL1.
antibody (05-614; Upstate), or anti-acetylated histone H3 antibody (06-599; Upstate) on a rotator at 4 °C overnight. Immunoprecipitation was performed using protein A/G Plus-agarose for 1 h at 4 °C with rotation. Cross-linked, precipitated chromatin complexes were then recovered and reversed according to the manufacturer’s protocol (Upstate, Chicago). Final DNA pellets were recovered and analyzed by PCR using a pair of primers that encompass the \( \text{RAR}\textsubscript{H9252}2 \) promoter region (288 bp).

The primers used were forward 5’-AAGCTCTGTGAGAATCCTG-3’ and reverse 5’-GGATCCTACCCCGACGGTG-3’.

RESULTS

Isolation of \text{ASXL1} —From a yeast two-hybrid screen of a HeLa cDNA library, using the ligand-binding domain (or DEF) of hRAR\textsubscript{H9251} as bait, we obtained partial C-terminal fragments of a protein with an amino acid sequence identical to that of human \text{ASXL1}. The longest fragment corresponded to amino acids 659–1541 of human \text{ASXL1} For further studies, we used the mouse full-length cDNA that was kindly provided as a KIAA clone (mKIAA0978 or GenBank\textsuperscript{TM} accession number AK122413) from the Kazusa DNA Research Institute (Japan). A conserved domain search of data bases revealed a C4HC3 cluster, also known as a PHD, at the extreme C terminus of \text{ASXL1} (Fig. 1A). A BLAST search using the conserved PHD region identified two paralogs of \text{ASXL1} at different chromosome loci, which were designated \text{ASXL2} (30) and \text{ASXL3} (31). The alignment of the human \text{ASXL} family indicated that there were three homologous regions as follows: the \text{ASXN}, \text{ASXM}, and PHD domains (Fig. 1B).

Mapping the Interaction Domains between RAR and \text{ASXL1} —The AF-2 AD core of NR is critical for the NR-mediated transcriptional activation. It locates in helix 12 of NR LBD and serves to recruit coactivators, such as SRC-1/p160/TIF2 and CBP/p300, in the presence of a ligand. To test whether the AF-2 core AD of RAR/RXR is responsible for the interaction with \text{ASXL1}, LexA DBD fusions of RAR or RXR AF-2 mutants and \text{ASXL1} clone A104 harboring a Gal4 AD fusion were introduced into yeast, and the \( \beta \)-galactosidase activity was measured. As indicated in Fig. 3, wild-type RAR DE and RXR DE showed ligand-dependent interactions with \text{ASXL1}, whereas the interaction was abolished in the deletion mutants of the AF-2 AD core, as shown by the dominant negative and most of the AD core point mutants, except the Glu\textsubscript{3}Gln mutant. Consistently, all mutants except the Glu\textsubscript{3}Gln mutant showed no transcriptional activity when fused to Gal4 DBD and
transfected with the Gal4-responsive luciferase (Gal4-LUC) reporter in NIH3T3 cells (Fig. 3, B and D). A similar pattern of interaction was observed with SRC-1. These results suggest that the AF-2 AD core of RAR/RXR is involved in the interaction with ASXL1. Because the AF-2 AD core of NR is required for coactivator binding and consequently enhancing the transcriptional activity of NR, ASXL1 may be categorized in the coactivator family.

To map the RAR binding domain within ASXL1, yeast two-hybrid assays were performed using VP16 AD fusions of ASXL1 and its deletion derivatives, and LexA DBD fusion of RAR DEF in the absence and presence of AtRA. The original clone (A104) isolated from the screen contained amino acid residues 1088–1541. A conserved motif search in this region revealed a single putative NR box (LVMQLL; amino acids 1107–1112). Deletion analysis indicated that ASXL1 fragments with this region retained the interaction, whereas fragments lacking the region abrogated the interaction (Fig. 4A). The expression level of each mutant was nearly equal, as determined by Western analysis using anti-VP16 antibody (data not shown). The amino acid alignment indicated that the C-terminal NR box is well conserved within the ASXL family and in mammals (Fig. 4B). To determine whether amino acid residues in the region are critical for the interaction with ASXL1, four point mutants (combinations of Leu → Ala and Val → Leu) and one deletion were generated by PCR and subcloned into pASV3 vector. As shown in Fig. 4C, all three leucines and the NR box are required for ligand-dependent binding to RAR. Notably, replacement of Val-1083 with Leu resulted in increased interaction with RAR, suggesting that this NR box is evolutionarily promiscuous. Combined, our interaction data suggest that the C-terminal NR box in ASXL1 and the AF-2 AD core in RAR are important for the ligand-dependent interaction between two proteins, at least in yeast.

Interaction In Vitro and in Mammalian Cells—To confirm the physical interaction of NRs with ASXL1 in vitro and in mammalian cells, we performed GST pulldown and immunoprecipitation (IP) assays. For GST pulldown assays, GST-fused ASXL1 was expressed in Escherichia coli, purified, and mixed separately with in vitro translated, 35S-labeled NRs. Consistent with the yeast two-hybrid data, the 35S-labeled mRAR, mRXR, cTR, or hER was retained by ASXL1 only in the presence of the cognate ligand (Fig. 5A).

For IP assays, various cells were transfected with FLAG-tagged mouse full-length ASXL1 expression vector in the absence and presence of NR
both the wild-type (WT) and the V1083L mutant retained the ATRA-dependent interaction with RAR, whereas the NR box mutant (ΔNR, deletion of amino acids 1107–1112) was defective in RAR binding (Fig. 5C). Again, the interaction of the V1083L mutant was slightly weaker than that of the wild type. These in vitro and in vivo findings confirmed the interaction observed in yeast, further emphasizing that the newly identified NR box is required for the ATRA-dependent interaction of ASXL1 with RAR.

To determine the subcellular localization and interaction of ASXL1 and RAR in vivo, GFP-tagged RAR and HcRed-tagged ASXL1 were cotransfected into NIH3T3 cells. Confocal microscopy showed that both RAR and ASXL1 were colocalized in the nucleus (Fig. 5D). The presence of ATRA had no effect on the nuclear colocalization of two proteins (data not shown).

**Effect of ASXL1 on the Transcriptional Activity of RAR**—Before investigating the significance of the physical interaction between ASXL1 and RAR, we determined whether ASXL1 alone shows autonomous transcriptional activity when recruited to the promoter. To this end, Gal4 DBD-fused ASXL1 was constructed and cotransfected with the Gal4-LUC reporter into HeLa cells. As shown in Fig. 6A, overexpression of Gal4-ASXL1 resulted in a great increase in luciferase activity. Similar effects were observed in NIH3T3 and MCF-7 cells (data not shown).

Next, the effect of ASXL1 on RAR (or RXR) activity was investigated using three transient transfection experiments. First, cells were transfected with Gal4-RAR DEF (or Gal4-RXR DE), Gal4-LUC reporter, and increasing amounts of ASXL1 expression vector in the presence of ATRA (or 9-cis-RA). In a second set of transfections, RAR (or RXR) and RARE (or RXRE)-tk-luciferase reporters were used instead. In both experiments, ASXL1 strongly enhanced the RA-induced transcriptional activity of RAR (and RXR) in a dose-dependent manner (Fig. 6B–D). In the final set of transfections, the expression vector for wild-type ASXL1, ΔNR, or the V1083L mutant was treated under conditions similar to those used for the second set of transfections. No stimulatory effect on RAR activity was observed with the ASXL1 lacking the NR box (ΔNR), which was defective in RAR binding (Fig. 6E). In contrast, an increase in V1083L mutant expression led to even greater enhancement of ATRA-induced transcriptional activity of RAR than in the wild type. From these transfection assays, we concluded that ASXL1 mediates ATRA-dependent transcriptional activation by RAR, for which the defined NR box is critical. Combined with the interaction data, our results suggest that ASXL1 is a novel member of the RAR coactivator family in certain mammalian cells.

**Cooperation of ASXL1 with SRC-1**—To understand the molecular basis underlying the ASXL1 regulation of RAR activity, we first sought to elucidate the effect of other known coactivators on the autonomous transcription activity of ASXL1. Transfection with increasing amounts of CBP, pCAF, or SRC-1 expression vector revealed that only SRC-1 could potentiate ASXL1 activity (Fig. 7A). The effect of SRC-1 was further demonstrated by other transfection assays. Increasing amounts of either the SRC-1 or the ASXL1 expression plasmid slightly enhanced RAR activity under low level expression conditions, but the expression of both SRC-1 and ASXL1 under the same conditions resulted in a cooperative, synergistic increase in the RA-dependent activity of RAR (Fig. 7B).

How does ASXL1 cooperate with SRC-1 to enhance RAR activity? To answer this question, we determined whether ASXL1 can associate with SRC-1. IP with an anti-SRC-1 antibody and subsequent WB with anti-FLAG antibody demonstrated the interaction between two proteins (Fig. 7C). To map the ASXL1 region responsible for SRC-1 binding, HeLa cells were transfected with FLAG-tagged full-length ASXL1 or
two half-fragments (ASX(1–655) and ASX(655–1514)). As shown in Fig. 7D, full-length and ASX(1–655), but not ASX(655–1514), was present in the immunoprecipitate of SRC-1. Further mapping within the N-terminal region indicated that ASX(300–655) is responsible for the interaction with SRC-1 (Fig. 7E). This interaction was confirmed by GST pulldown using GST-ASX(300–655) and in vitro translated SRC-1, and by WB using anti-SRC-1 antibody (Fig. 7F). To determine whether the interaction of ASXL1 with SRC-1 via ASX(300–655) contributes to the autonomous transcriptional activity of ASXL1, we performed similar transfection assays as described in Fig. 7A. When recruited alone to the promoter through the Gal4 DBD fusion, ASX(300–655), but not ASX(1–299), showed modest autonomous activation activity similar to the longer fragment of ASX (1–655; see Fig. 7G). The modest activation function of ASX(300–655) and ASX(1–655) was strongly stimulated by SRC-1. Conversely, ASX(1–299), which lacks the SRC-1 binding domain, did not respond to SRC-1. Therefore, both the ability to bind SRC-1 and the autonomous activation of ASXL1 are required for its coactivator function. The SRC-1 binding domain is depicted in Fig. 7H. Although the data are not shown, IP assays using SRC-1 deletions indicated that ASX(300–655) could associate with the C-terminal domain of SRC-1, which also contains the transcriptional activation domain AD2 (32) (Fig. 7I).

Function of ASXL1 Overexpression in Vivo—To correlate the role of ASXL1 in transcriptional activation with its function in vivo, we generated HeLa cells stably expressing FLAG-tagged ASXL1 or ASXL1 lacking the PHD domain (H9004 PHD), and tested first whether the cells show enhanced RAR activity by luciferase reporter assays. As indicated in Fig. 8A, the RA-dependent RAR activity was greatly increased in the cells stably expressing ASXL1. In contrast, H9004 PHD showed modest enhancement, suggesting that the C-terminal PHD is one of the important domains mediating ASXL1 activity. We next examined the effect of ASXL1 on the expression of endogenous RA-regulated genes, RAR and PDCD4 (34), using RT-PCR. As shown in Fig. 8B, the mRNA expression of both genes was much higher in ASXL1-expressing cells in the presence of AtRA when compared with the control level of GAPDH. A weak increase in expression was also observed with H9004 PHD. Finally, to determine the biological effect of ASXL1 overexpression on cell growth, we measured cell proliferation rates in response to AtRA using MTT assays. In both time course and dose-dependent experiments, the cells expressing ASXL1 were more sensitive to AtRA (Fig. 8C), suggesting
that ASXL1 is involved in the cytotoxic signaling of AtRA through increasing expression of RARB2 and PDCD4 genes, which are known to repress cell growth in response to AtRA (34, 35).

Recruitment of ASXL1 to the RARB2 Gene Promoter—Finally, to test whether ASXL1 is involved in the transcriptional activation of the chromatin-integrated, endogenous AtRA-regulated gene by RAR, we used...
ChIP assays and examined the AtRA-dependent recruitment of ASXL1 to the RARβ2 promoter. To this end, HeLa cells were transfected with FLAG-empty or ASXL1 expression vector in the absence or presence of AtRA. Chromatin DNA fragments were precipitated with the indicated antibodies and amplified with primers selective for the AtRA-responsive RARβ2 promoter (Fig. 9A). The AtRA-dependent recruitment of RAR to the promoter was further increased when ASXL1 was overexpressed. ASXL1 was also recruited to the promoter in an AtRA-dependent manner. Similar results were obtained using acetylated histone H3. Interestingly, the chromatin association of SRC-1 was absolutely dependent on the presence of both AtRA and ASXL1 under our experimental conditions. In contrast to acetylated H3 or SRC-1, HDAC1 was disassociated from the RARβ2 promoter in the presence of AtRA and ASXL1. From these results, we speculate that ASXL1 forms an AtRA-dependent complex with RAR and SRC-1 on the endogenous chromatin-integrated promoter, and the SRC-1 of the complex attracts other coactivators with histone acetyltransferase activity, such as p300/CBP, and releases HDAC, thereby resulting in the accumulation of more acetylated H3 on the chromatin template (Fig. 9B). Finally, the ASXL1-induced accumulation of acetylated H3 may enhance the RAR-mediated transcriptional activity.

DISCUSSION

Despite a detailed molecular understanding of how various coactivators function in RAR-mediated transcriptional activation, there is no clear evidence to support the in vivo biological functions of RA, such as in differentiation and development. To explain this missing link, one can use the homeotic (Hox) gene, for which expression is tightly regulated by RA according to the developmental stage. During mammalian development, spatially and quantitatively appropriate Hox gene expression is essential for the anterior-posterior specification of axial structures (17–19). The PcG and TrxG proteins play a crucial role in maintaining Hox gene expression, probably by modifying the chromatin configuration. Both proteins form large, chromatin-associated multi-protein complexes in mammals (36, 37). Therefore, one can speculate that RA, likely together with RAR, modulates Hox gene expression by cooperating with PcG or TrxG complexes. To date, however, no com-
ponent of the complex is known to cooperate in RA signaling. In this study, we identified human Additional sex comb-like 1 (ASXL1) (28), a mammalian homolog of Drosophila ASX, which interacts with RAR in the presence of RA. We demonstrated that ASXL1 interacts with the AF-2 AD core of RAR via a novel, promiscuous NR box (LVMQQL) and enhances RA-induced transactivation of RAR in certain cells (NIH3T3, HeLa, and MCF-7). We also showed that ASXL1 associates specifically with SRC-1 and cooperates synergistically in transcriptional activation. Therefore, our data suggest that ASXL1 is a new RAR coactivator that functionally cooperates with SRC-1. Although it remains to be investigated whether ASXL1 mediates RA-dependent RAR activation, like PcG/TrxG, the following observations support this possibility. Genetic studies in Drosophila have indicated that ASX is an Enhancer of trithorax and Polycomb (ETP) gene, functioning in both anterior and posterior transformations (38), which are related to the expression pattern of Hox genes. It has been speculated that ASX is a chromatin protein based on its role in position-effect variegation and the presence of the PHD domain at the C terminus, common to PcG and TrxG proteins (39).

Recent gene trap studies in mammals have identified a mouse homolog (mASXL1) of Drosophila ASX as a developmentally regulated, RA-inducible gene (40); mASXL1 was localized in spatially defined regions along the anterior-posterior axis associated with the spinal cord, which is characteristic of Hox expression. In our additional study (data not shown), ASXL1 functioned as a corepressor of RAR activity in other mammalian cells, supporting its dual functions in Drosophila. The detailed mechanism underlying this transcriptional repression is under investigation.

Structurally, ASXL1 contains notable domains, including putative N- and C-terminal NR binding motifs, and a PHD finger at the extreme C terminus. The alignment of human ASXL family proteins, including ASXL2 and ASXL3, indicated that there are three homologous regions as follows: the ASXN, ASXM, and PHD domains. The N-terminal NR boxes (located in ASXM) are not present in ASXL3, whereas those in ASXL1 and ASXL2 are highly conserved in various species. In our study, we observed that these structurally predicted N-terminal NR boxes are defective in RAR binding. Instead, we found a novel NR box (LVMQQL) at the C-terminal region (located between ASXM and PHD) that is conserved in both the ASXL family and vertebrate species. Mutation studies indicated that this NR box is required for ligand-dependent RAR binding and RAR-mediated transcriptional activation. Interestingly, a change from Val to Leu resulted in enhanced activity, suggesting that this newly discovered NR box is evolutionarily promiscuous. Moreover, this motif is not conserved in Drosophila ASX, whereas Drosophila ASX contains N-terminal NR boxes that are thought to be nonfunctional based on our data. Therefore, this property of Drosophila ASX may account for the lack of RA-mediated RAR signaling in Drosophila, which may still be evolving to acquire RA signaling seen in vertebrates.

Another structural feature of ASXL1 is its possession of a PHD finger at the extreme C terminus. Because PHD fingers are frequently present in chromatin-associated proteins, including p300 (41), ASXL1 may be involved in chromatin-mediated transcriptional regulation, likely via nucleosome binding (42). Our study suggests that the PHD of ASXL1 contributes to RAR-mediated transcriptional activation in vivo. However, whether ASXL1 PHD is functionally associated with chromatin remains to be determined. Finally, we defined one transcriptional activation domain located near the conserved ASXM domain spanning amino acids 300–655. This region serves as a binding site for SRC-1, as described below in detail. The amino acid alignment of the ASXL family indicates that the N-terminal end of ASXM (amino acids 300–361) is highly conserved within the family, even in the Drosophila homolog. Thus, it will be interesting to examine whether this region is the minimal transactivation domain of ASXL1.

Because interactions among coactivators, such as CBP-SRC-1 and CBP-p300, routinely occur in transcriptional activation in eukaryotes, we tested which factors are involved in ASXL1-stimulated transcriptional activation. Of the three factors examined, SRC-1 was found to mediate both the autonomous transcriptional activity of ASXL1 and the ASXL1 stimulation of RAR activity. In addition, binding assays demonstrated that the effect of SRC-1 resulted from the direct interaction between SRC-1 and ASXL1.

Further data indicated that the transactivation domain (Ad; amino acids 300–655) of ASXL1, newly defined in this study, interacts with the C-terminal AD2 (amino acids 1217–1441) of SRC-1 (Fig. 7H), suggesting that one AD cooperates with the other AD in transcriptional activation by RAR. SRC-1 AD2 is also responsible for the interaction with the histone methyltransferases CARMA1 and PRMT1 (43, 44). In this respect, it will be of interest to determine whether ASXL1 can cooperate with CARMA1 or PRMT1 in the presence of SRC-1. The other domain, AD1, is responsible for the interaction with the histone acetyltransferases p300 and CBP and the additional recruiting of p/CAF (45, 46). The p160/SRC-mediated recruitment of these chromatin-modifying enzymes to an enhancer/promoter may result in the local chromatin remodeling required for ligand-dependent transcriptional activation by nuclear receptors, including RAR. Recently, weak AD3 was identified during a search for factors that interact with the N-terminal basic helix-loop-helix/Per-Arnt-Sim domain (47, 48). Therefore, the SRC family has diverse functions via the associations between various coactivator proteins, including ASXL1 and three ADs located in different regions.

In addition to its role in development, as described above, we propose that ASXL1 plays another role in regulating cell growth. It is well documented that RA regulates cell growth by inducing cell cycle arrest or programmed cell death (3). Therefore, RA and its derivatives have been used as therapeutic agents in various cancers, including acute promyelocytic leukemia (49). Recent evidence suggests that PcG/TrxG proteins are also involved in regulating cell proliferation and tumorigenesis (50). Therefore, ASXL1, a vertebrate PcG/TrxG protein, may mediate RA-regulated cell growth by modulating RAR activity. In this regard, we investigated the role of ASXL1 in RA-mediated growth regulation of HeLa cancer cells. The RA cytotoxicity was more profound in cells overexpressing ASXL1 than in cells not expressing ASXL1. Furthermore, we found that ASXL1 was a coactivator for RAR in RA-sensitive cancer cell lines, including HeLa, whereas it was a corepressor in three RA-resistant cancer cell lines (data not shown), suggesting that the role of ASXL1 in transcriptional regulation is closely related to the RA-induced growth control in vivo.

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