Knockdown of XAB2 Enhances All-Trans Retinoic Acid–Induced Cellular Differentiation in All-Trans Retinoic Acid–Sensitive and –Resistant Cancer Cells

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Abstract
Xeroderma pigmentosum group A (XPA)–binding protein 2 (XAB2) is composed of 855 amino acids, contains 15 tetrapeptide repeat motifs, and associates with Cockayne syndrome group A and B proteins and RNA polymerase II, as well as XPA. In vitro and in vivo studies showed that XAB2 is involved in pre-mRNA splicing, transcription, and transcription-coupled DNA repair, leading to preimplantation lethality, and is essential for mouse embryogenesis. Retinoids are effective for the treatment of preneoplastic diseases including xeroderma pigmentosus and other dermatologic diseases such as photocaging. We therefore focused on defining the effect of XAB2 on cellular differentiation in the presence of ATRA treatment. In the present study, we showed that overexpression of XAB2 inhibited ATRA-induced cellular differentiation in human rhabdomyosarcoma cell line, and that knockdown of XAB2 by small interfering RNA (siRNA) increased ATRA-sensitive cellular differentiation in the human promyelocytic leukemia cell line HL60 at both physiologic (10\(^{-8}\)–10\(^{-6}\) mol/L) and therapeutic (10\(^{-5}\) mol/L) concentrations of ATRA. Moreover, we found that XAB2 was associated with retinoic acid receptor \(\alpha\) (RAR\(\alpha\)) and histone deacetylase 3 in the nuclei. Finally, using siRNA against XAB2, we showed that the ATRA-resistant neuroblastoma cell line IMR-32 underwent cellular differentiation induced by ATRA at a therapeutic concentration (10\(^{-6}\) mol/L). These results strongly suggest that XAB2 is a component of the RAR corepressor complex with an inhibitory effect on ATRA-induced cellular differentiation and that XAB2 plays a role in ATRA-mediated cellular differentiation as an important aspect of cancer therapy. [Cancer Res 2007;67(3):1019–29]

Introduction
The retinoids are natural and synthetic derivatives of vitamin A that regulate a variety of important cellular functions such as differentiation and growth-suppressive effects in normal, prema-

lignant, and malignant cells (1, 2). These findings provided a basis for the use of retinoic acid in clinical cancer therapy and chemoprevention as differentiation therapy (3–6). For instance, all-trans retinoic acid (ATRA) has dramatically changed the clinical outcome of patients with acute promyelocytic leukemia from one that was highly lethal to one that now seems to be highly curable (7, 8). Acute promyelocytic leukemia is characterized by a chromosomal translocation, t(15;17), resulting in the fusion product PML-RAR\(\alpha\) (retromyelocytic leukemia gene on chromosome 15 and retinoic acid receptor \(\alpha\) gene on chromosome 17; refs. 9–11). The PML-RAR\(\alpha\) protein is stably associated with histone deacetylase (HDAC)–containing corepressor complexes, which leads to transcriptional repression of the program required for granulocytic differentiation of acute promyelocytic leukemia blasts at physiologic levels of retinoic acid (10\(^{-8}\) mol/L; refs. 12–14). At therapeutic concentrations (10\(^{-6}\) mol/L), retinoic acid dissociates the corepressor complex and recruits coactivator complexes, restoring the regulation of target genes (8, 11, 15).

Through metabolites such as ATRA, retinoids exert their effect on gene transcription mediated by the retinoic acid receptor (RAR) or retinoid X receptor (RXR) via the binding of RAR-RXR heterodimers or RXR-RXR homodimers to retinoic acid response elements (RARE; refs. 16–18). The ligand-dependent activation of genes via RAR or RXR depends on cofactor complexes interacting with RAR or RXR. In the absence of ATRA, RAR-RXR heterodimers interact with nuclear corepressors, such as silencing mediator of retinoid and thyroid hormone receptors (SMRT) and nuclear receptor corepressor (N-CoR), which recruit HDACs to induce chromatin modifications and transcriptional repression (18–20). Binding of retinoic acid permits dissociation of the corepressor complex and recruitment of coactivator complex, which in turn recruits histone acetyltransferase, thus leading to a permissive chromatin modification for opening promoter accessibility and finally to transcriptional activity (21). N-CoR/SMRT recruits HDAC3 to serve as effective corepressors of DNA-bound unliganded RAR-RXR (22, 23). Moreover, N-CoR and SMRT function not merely as platforms for recruitment of HDAC3 but also directly stimulate HDAC3 enzymatic activity (22, 24, 25). In acute promyelocytic leukemia, it has been reported that the PML-derived oligomerization domain of PML-RAR\(\alpha\) is essential for such biological properties as enhanced binding of the corepressor N-CoR/SMRT with HDAC and blocking myeloid cell differentiation (26, 27). These molecular findings suggest that abnormal recruitment of HDACs by RAR complexes is a key pathogenic mechanism.
in acute promyelocytic leukemia, and that the repressor complexes, including HDACs itself, as well as RAR, are the molecular target of retinoic acid in differentiation therapy (28–32).

Xeroderma pigmentosum group A (XPA)–binding protein 2 (XAB2), which we independently isolated under the name of HDART by virtue of its sequence similarity to Drosophila CRN, is composed of 855 amino acids. XAB2 contains 15 tetratricopeptide repeat motifs and functions in a nucleotide excision repair complex (33). Cloned by virtue of its ability to interact with XPA protein in the yeast two-hybrid system, XAB2 thus derives its name as XPA-binding protein 2, and is found to form a complex with the nucleotide excision repair machinery (33). XAB2 knockout mice have been reported to show preimplantation lethality (34, 35).

Xeroderma pigmentosum is a rare human hereditary disease characterized by hypersensitivity to sunlight, a high incidence of skin cancer in sun-exposed areas, and accelerated neurodegeneration (36, 37). Cells from xeroderma pigmentosum patients are hypersensitive to killing by UV irradiation, with early development of skin cancer being caused by defective DNA repair (38–41). Preneoplastic diseases including xeroderma pigmentosum were previously shown to be successfully treated with retinoids (1, 4). Expanding on these observations, we focus in this article on the role of XAB2 as a novel target for retinoic acid–based differentiation therapy for cancers, defining in particular the biological and physiologic function of XAB2 in the RAR/HDAC3 complex. We now show that XAB2 functions as a corepressor of the RAR/HDAC3 complex, and that knockdown of XAB2 by small interfering RNA (siRNA) enhances sensitivity to ATRA in the acute promyelocytic leukemia cell line HL60 and the rhabdomyosarcoma cell line MM-1-19P, as well as the ATRA-resistant neuroblastoma cell line IMR-32.

Materials and Methods

Cells, antibodies, and reagents. HEK293T, HepG2, HL60, and MM-1-19-P were grown in DMEM (Life Technologies, Inc., Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Life Technologies). 100 units/mL penicillin, and 100 μg/mL streptomycin (Life Technologies). MM-1-19-P human rhabdomyosarcoma cell line, HL60 human promyelocytic leukemia cell line, and retinoic acid–resistant human neuroblastoma cell line IMR-32 were grown in RPMI 1640 (Life Technologies) supplemented with 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin, using Laminin six-well multiwell plates (BD Biosciences, San Jose, CA).

The following antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA): anti-Flag M2 monoclonal antibody (mAb), anti–green fluorescent protein (GFP) polyclonal antibody (pAb), antimitulin mAb, anti-HDAC1 pAb, anti-HDAC3 pAb, anti-HDAC4 pAb, anti-HDAC6 pAb, anti-RARα pAb, Texas red–conjugated antiohistone H4 polyclonal antibody, Texas red–conjugated antiohistone H4 polyclonal antibody, and FITC–conjugated antiohistone H4 polyclonal antibody, and FITC–conjugated antiohistone H4 polyclonal antibody. Texas red–conjugated antiohistone H4 polyclonal antibody was obtained from Mo Bi Tec (Gottingen, Germany); phycocerythrin–conjugated CD11b and 7-amino-actinomycin D were obtained from BD Pharmingen (San Jose, CA) and anti-acetyl-histone H4 polyclonal antibody was obtained from Upstate (Chapel Hill, NC).

siRNA against human XAB2. We selected the target sequences from +90 to +108 and +1673 to +1691 downstream of the start codon of human XAB2 mRNA (National Center for Biotechnology Information accession no. NM_020196; sense siRNA s1, 5′-GAACACCACCCUCUUGGAACAdTdT-3′; sense siRNA s2, 5′-GAAUCUAAUGUAUAAATdTdT-3′). Moreover, control siRNA was prepared to examine nonspecific effects of siRNA duplexes (5′-GAACACUCUUCCUGUCAAdTdT-3′). These selected sequences also were submitted to a BLAST search against the human genome sequence to ensure that only one gene of the human genome was targeted. siRNAs were purchased from Japan Bioservice (Saitama, Japan). siRNA duplex formation (annealing) was done as previously described (42). A total of 60 pmol of siRNA duplexes were transfected into 0.5 × 10⁶ cells using Nucleofector (Amaxa, Inc., Gaithersburg, MD). After 48 h of transfection, cells were prepared for examination.

Cellular differentiation. MM-1-19-P cells (1 × 10⁶/mL) were transfected with 2 μg of pcDNA3-XAB2 or pcDNA3 plasmids asmock control, and pEGFP-C1 plasmid (0.4 μg) was cotransfected for the identification of transfected cells. After 24 h of transfection,cells were treated with 2 μmol/L ATRA for an additional 24 h and were submitted to fluorescence microscopy analysis. The effect of exogenous XAB2 on cells was observed by morphologic analysis of cells through the use of phase-contrast microscopy merged with green fluorescence of GFP. Myocytic differentiation of rhabdomyosarcoma cells was observed with phase-contrast microscopy, with two pathologists identifying the morphologic changes to myocytic differentiation. For quantification, the percentage of GFP-positive, myocyte–differentiated cells among a total of 1,000 GFP-positive cells was shown in the graph. All experiments were done in triplicate and the results presented as means with SE.

After transfection of siRNA, IMR-32 cells (1 × 10⁶/mL) were incubated in the presence of 2 μmol/L ATRA for an additional 48 h. Neuroepithelial differentiation of neuroblastoma cells was observed as previously described (43); cells with extending processes longer than twice the diameter of the cell body were counted as being differentiated, with two pathologists identifying the morphologic changes to formation of neurite-like outgrowth. For quantification, the percentage of differentiated cells among 1,000 cells was shown in the graph. All experiments were done in triplicate and the results presented as means with SE.

Relative quantitative reverse transcription-PCR assay. In experiments assessing expression of RARβ and CYP26 mRNA, after HEK293T or HL60 cells were treated as described above, total RNA was then extracted and cDNA was produced as described previously (44). PCR primers for the coding sequences of RARβ and CYP26 were as follows: RARβ, forward 5′-ATGTTCGACTGATGTTGTT-3′, reverse 5′-CCACTCTAAGACACTTGCTG-3′; CYP26, forward 5′-GATGAAAGCCCCAGAAATAC-3′, reverse 5′-ATGGCG-GATGCGAGATGGAG-3′. These primers were designed for the coding sequences that are located on different exons to eliminate amplifying genomic DNA contamination in the PCRs. The quantities of mRNA were adjusted equally by using PCR of β-actin (forward primer 5′-CAAGATGCATGGGCTGCTGTT-3′ and reverse primer 5′-TCTTCTGCATCC-TGGCGGC-3′ as the internal control).

Chromatin immunoprecipitation assay. Chromatin immunoprecipitation assay was done using 1 × 10⁶ of HL60 cells in the presence or absence of 1 μmol/L ATRA for 72 h. Protein-DNA complexes were prepared using EZ ChIP Chromatin Immunoprecipitation Kit (Upstate) according to the manufacturer’s instructions. After washing and reverse cross-linking, immunoprecipitated DNA was purified and PCR was done. The following PCR primers were used to detect the immunoprecipitated promoter DNA: RARβ promoter, forward 5′-CTCTGGCTGTCGCTTTGTCG-3′, reverse 5′-AAAAGAGCTTCTCCAGTGTG-3′; CYP26 promoter, forward 5′-GATTGATTGCCGGCAGC-3′, reverse 5′-CATCTGCAGGTTTCTC-3′. The PCR reactions were done as described above.

Coimmunoprecipitation assay. For coimmunoprecipitation assays involving endogenous XAB2 and HDACs, nuclear extracts were prepared from 1 × 10⁶ HL60 cells as previously described (45). Nuclear extracts were then isolated by centrifugation at 15,000 × g for 30 min. Immunoprecipitation assays were done subsequently by incubating nuclear extracts with 2 μg of control immunoglobulin and protein G-Sepharose beads (Amersham Pharmacia, Piscataway, NJ) at 4°C for 1 h. After centrifugation, supernatants were incubated with 2 μg of anti-XAB2 mAb at 4°C for 1 h, followed by the addition of protein G-Sepharose beads for 1 h (45). Cell lysates and nuclear extracts were prepared and coimmunoprecipitation assay, SDS-PAGE, and

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7 Unpublished observation.

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Western blot analysis were conducted according to the methods described elsewhere (44, 45).

Confocal laser microscopy. For fluorescence microscopy experiments, HL60 cells were incubated for 24 h in the presence or absence of ATRA and then attached to microslide glass (Matsunami Glass, Inc., Tokyo, Japan) coated with poly-L-lysine and fixed with ice-cold acetone/methanol (1:1). After washing with ice-cold PBS, cells were blocked with mouse and goat immunoglobulin isotypes, followed by incubation with anti-XAB2 mAb, RAB6 pAb, or anti-HDAC3 pAb (each at a concentration of 10 μg/mL), then washed and incubated with FITC-conjugated antimmunoglobulin and/or Texas red–conjugated ant-immunoglobulin antibodies (each at a concentration of 5 μg/mL). Confocal microscopy was done as described elsewhere (44, 45).

Chloramphenicol acetyltransferase assay. To examine the potential effect of XAB2 on ATRA-mediated transcriptional activity, HepG2 cells were transiently transfected with pRARE-chloramphenicol acetyltransferase (CAT), which is a RARE-containing RAR reporter plasmid, cloned in frame with CAT under the control of a thymidine kinase minimal promoter. Cells were seeded onto 35-mm-diameter dishes at a density of 1.75 × 10^5 per well and incubated overnight in FCS-supplemented medium. The following day, cells were transfected with pRARE-CAT (1 μg), pSV-β-gal (0.5 μg), and pcDNA3-XAB2 (0.5 or 1.0 μg), or pcDNA3 as mock control. The amount of expression vector was adjusted to 1 μg with pcDNA3 empty vector in each transfection. Twenty-four hours later, culture medium was removed and cells were washed thrice with serum-free MEM (Life Technologies), and then treated with fresh medium containing 100 nmol/L ATRA. Cells were harvested after 24 h and assayed for β-galactosidase and CAT activity with the use of the β-Galactosidase Enzyme Assay System (Promega, Madison, WI) and CAT Enzyme Assay System (Promega) according to the manufacturer’s instruction. For transfection efficiency, CAT activity was normalized to β-galactosidase activity. The CAT activity in control cells treated with DMSO vehicle and a mock vector was set as equal to one and all other values are expressed as fold induction. Data represent mean from triplicate experiments ± SE.

Statistics. Student’s t test was used to determine whether the difference between control and experimental samples was significant (P < 0.05).

Results

Overexpression of XAB2 inhibits cellular differentiation and transcriptional activation induced by ATRA. To determine an effect of XAB2 on cellular differentiation, we first examined whether cellular differentiation is induced by overexpression of exogenous XAB2. For this purpose, we used the human rhabdomyosarcoma cell line MM-1-19-P which is mainly composed of small polygonal cells and terminally differentiates to myotube-like giant cells following treatment with ATRA (46). As shown in Fig. 1A, MM-1-19-P cells transfected with mock vector differentiated to myotube-like giant cells following treatment with ATRA (arrowheads in a). On the other hand, ATRA did not induce any morphologic changes in MM-1-19-P cells transfected with XAB2 expression plasmid (Fig. 1A, b). For further confirmation and quantification of ATRA-induced cellular differentiation of MM-1-19-P cells, we next conducted cotransfection experiments with XAB2-expressing plasmids (pcDNA3-XAB2) or mock plasmid (pcDNA3) and GFP-expressing plasmids (pEGFP-C1) for visualization. As shown in Fig. 1B, no change was observed both in MM-1-19-P cells transfected with mock and GFP-expressing plasmids and in MM-1-19-P cells transfected with XAB2- and GFP-expressing plasmids (a and c). On the other hand, myocytic cellular differentiation following treatment with ATRA was significantly observed in mock-transfected MM-1-19-P cells, which were cotransfected with GFP-expressing plasmid (Fig. 1B, b). However, cellular differentiation to myotube-like giant cell induced by ATRA was not detected in MM-1-19-P cells transfected with XAB2 expression plasmid (Fig. 1B, d). To quantify the number of ATRA-induced differentiated cells in the mock- and XAB2-transfected MM-1-19-P cell population, we counted the myotube-like giant cells expressing GFP under examination by fluorescence and bright-field microscopy. As shown in Fig. 1C, MM-1-19-P cells transfected with mock and GFP vectors exhibited an increase in the number of differentiated cells (Fig. 1C, #). However, ATRA-induced differentiation of XAB2-transfected MM-1-19-P cells was significantly inhibited (Fig. 1C, ##). These results suggest that XAB2 has an inhibitory effect on ATRA-mediated cellular differentiation.

To confirm the inhibitory effect of XAB2 on ATRA-induced cell differentiation, CAT assays were done with the RAR reporter plasmid containing a RARE under the control of a thymidine kinase minimal promoter (pRARE-CAT). HepG2 cells were transiently transfected with pcDNA3-XAB2, which allows for the constitutive expression of XAB2, and were grown in the presence or absence of ATRA. In mock (pcDNA3)-transfected cells, CAT activity was induced 5-fold by ATRA. However, the ATRA-induced CAT activation was suppressed in a dose-dependent manner by XAB2 (Fig. 1D). These results suggest that XAB2 suppresses ATRA-activated transcription.

To further determine whether the overexpressed XAB2 protein plays an inhibitory role in ATRA-induced activation of transcription, we analyzed ATRA-targeted gene expression pattern in HEK293T cells by reverse transcription-PCR (RT-PCR). For this purpose, two RARα target genes, RARβ and CYP26, were selected (47). HEK293T cells were transiently transfected with pcDNA3-XAB2 to allow for the constitutive expression of XAB2 and were grown in the presence or absence of ATRA. Total RNA was purified and RT-PCR using RARα-, CYP26-, and β-actin-specific primers was done. As shown in Fig. 1E, in mock (pcDNA3)-transfected cells, endogenous RARα and CYP26 mRNA expression was induced by ATRA (lanes 1 and 2). However, the ATRA-induced RARα and CYP26 expression was suppressed in a dose-dependent manner by XAB2 (Fig. 1E, lanes 3–6). Exogenous XAB2 transfection did not affect the expression level of endogenous RARα and CYP26 mRNA (Fig. 1E, lanes 1, 3, and 5). These results confirm our earlier data indicating that XAB2 suppresses ATRA-activated gene transcription.

Knockdown of XAB2 by siRNA enhances cellular differentiation of HL60 cells induced by ATRA. To further define an inhibitory effect of XAB2 on ATRA-induced cellular differentiation, we next used the human promyelocytic leukemia cell line HL60, which is induced to differentiate to neutrophilic cells by various drugs including ATRA (48). For this purpose, we prepared two separate siRNA against XAB2, as described in Materials and Methods, to ensure the specificity of the knockdown effect. As shown in Fig. 2A, these siRNAs against XAB2 (ss1 and ss2) effectively knocked down XAB2 expression in HL60 cells (top, lanes 2 and 4). Because XAB2 in HL60 cells was not significantly knocked down by control siRNA (Fig. 2A, top, lanes 1 and 3) and expression level of tubulin as a housekeeping protein was not affected by both of siRNAs (Fig. 2A, bottom), this inhibitory effect of XAB2 by siRNAs in HL60 cells was specific. In addition, the expression levels of HDAC3 and RARα were not changed in the presence or absence of siRNA treatment (data not shown). We next examined ATRA-induced cellular differentiation of HL60 cells in the presence or absence of siRNAs against XAB2 through flow cytometric analysis of cell-surface expression level of CD11b (49). As shown in Fig. 2B, ATRA-induced HL60 cell differentiation was enhanced in a dose-dependent manner with treatment with siRNAs against XAB2 as compared with the level of differentiation.
observed in HL60 cells treated with control siRNA. These data suggest that specific knockdown of XAB2 enhances ATRA-induced cellular differentiation at physiologic levels (10^{-9}–10^{-8} mol/L) as well as at therapeutic levels (10^{-7} mol/L).

To further confirm whether knockdown of XAB2 enhances ATRA-induced gene expression, we analyzed ATRA-targeted gene expression pattern in HL60 cells by RT-PCR, in the presence or absence of siRNA against XAB2. As shown in Fig. 2C, ATRA-induced mRNA expression of RARβ and CYP26 was increased in a dose-dependent manner (lanes 1, 4, 7, and 10). The increased expression of RARβ and CYP26 mRNA was further enhanced in HL60 cells treated with siRNA against XAB2 (Fig. 2C, lane 4 versus lanes 5 and 6, lane 7 versus lanes 8 and 9, and lane 10 versus lanes 11 and 12). This enhanced expression of RARβ and CYP26 mRNA was again observed to be dependent on ATRA doses (Fig. 2C, lanes 5 and 6, 8 and 9, and 11 and 12). These data suggest that specific knockdown of XAB2 enhances not only cellular differentiation but also induction of ATRA-targeted genes at physiologic (10^{-9}–10^{-8} mol/L) and therapeutic (10^{-7} mol/L) levels.

XAB2 is associated with HDAC3. Retinoid-dependent cellular differentiation of embryonal carcinoma cell lines was previously shown to be mediated by HDAC activity (12, 15, 50). We thus examined whether XAB2 involvement in retinoid-mediated transcriptional regulation is through HDAC activity. For this purpose, the potential association of XAB2 with HDAC activity on RAR-regulated transcription was examined via RARE-chloramphenicol acetyltransferase (CAT) assays, which were done on the RAR reporter plasmid containing a RARE under the control of
thymidine kinase minimal promoter (pRARE-CAT). HepG2 cells were transiently transfected with pcDNA3-XAB2 to allow for the constitutive expression of XAB2 and were then grown in the presence or absence of ATRA. As shown in Fig. 3A, transcrip-
tional activity was significantly enhanced by ATRA addition (Fig. 3A, #), and this ATRA-induced transcrip-
tional activity was decreased in the presence of overexpression of XAB2 (Fig. 3A, ##). Moreover, the addi-
tion of trichostatin A or sodium butyrate, specific inhibitors of HDAC, completely antagonized the repression by XAB2 of RAR reporter activity (Fig. 3A, ###). To further confirm the antagonistic effect of HDAC inhibitors on XAB2-mediated repression, we analyzed the expression level of endogenous retinoic acid–targeted genes RARβ and CYP26. As shown in Fig. 3B, the mRNA level of RARβ and CYP26 was enhanced by ATRA addition (lane 2), and this ATRA-induced enhancement of RARβ and CYP26 mRNA expression was decreased by XAB2 overexpression (lane 3). In contrast, the addition of trichostatin A or sodium butyrate completely antagonized the repression by XAB2 of ATRA-induced RARβ and CYP26 mRNA expression (Fig. 3B, lanes 4 and 5).

These data suggest that the XAB2-mediated inhibitory effect of ATRA-induced transcriptional activity is associated with HDAC activity.

To further explore XAB2 association with HDACs, we next evaluated the potential interaction of XAB2 with HDACs in a transcrip-
tional component through coimmunoprecipitation assays. HEK293T cells were transfected with an expression plasmid of GFP-XAB2 alone or together with expression plasmids of Flag-HDAC1, HDAC3, HDAC4, and HDAC6 (Fig. 3C, top). The Flag- HDACs were immunoprecipitated with antibody against the Flag tag, and coimmunoprecipitated GFP-XAB2 protein was detected by Western blotting with anti-GFP antibody. As shown in Fig. 3C, the GFP-XAB2 protein was communoprecipitated with Flag-HDAC1, HDAC3, HDAC4, and HDAC6 from HEK293T cell lysates (middle, lanes 1 and 3–5). No cellular protein was detected by the anti-GFP antibody in immunoprecipitants of lysates of HEK293T cells transfected with Flag-vector control plasmid (middle, lane 2). The expression level of GFP-XAB2 was not affected by the presence or absence of Flag-HDACs (Fig. 3C, bottom).
Various HDACs have previously been reported to be involved in RARα-mediated transcriptional regulation via interaction with N-CoR/SMRT (20, 51, 52). However, the identity of the precise HDACs involved remains controversial, partly because of the different cell types or assay systems being used. We therefore attempted to determine the specific HDACs among HDAC1, HDAC3, HDAC4, or HDAC6 that are associated with XAB2 in our system. For this purpose, immunoprecipitation assays detecting endogenous HDACs and XAB2 proteins were done on nuclear extract of HL60 cells. As shown in Fig. 3D, endogenous HDAC1, HDAC3, HDAC4, HDAC6, and XAB2 were expressed in HL60 cells (lane 1), with each control immunoglobulin G (IgG) not able to precipitate endogenous HDAC1, HDAC3, HDAC4, HDAC6, and XAB2 (lane 2). In this experimental system, HDAC3 was clearly detected in the complex precipitated by XAB2 whereas very slight amount of HDAC1 was coprecipitated with XAB2. On the other hand, no portion of HDAC4 or HDAC6 was detected in the complex (lane 2). These data indicate that XAB2 is associated mainly with

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**Figure 3.** XAB2 is associated with HDAC3. A, HepG2 cells were transiently transfected with pRARE-CAT (2 μg), pSV-β-gal (0.4 μg), and pcDNA3-XAB2 (1.0 μg) or pcDNA3 empty vector (1.0 μg). Twenty-four hours after transfection, cells were treated with 100 nmol/L ATRA or DMSO vehicle as a control in the presence or absence of trichostatin A (TSA; 10 nmol/L) or sodium butyrate (Buty; 1 mmol/L). Cells were then harvested after 24 h and assayed for β-galactosidase and CAT activity. For transfection efficiency, CAT activity was normalized to β-galactosidase activity. CAT activity in control cells treated with DMSO vehicle and a mock vector was set as equal to one and all other values are expressed as fold induction. Columns, mean from triplicate experiments; bars, SE. #, ##, and ###, significant changes compared with control (P < 0.05). B, HEK293T cells were transiently transfected with pcDNA3 empty or pcDNA3-XAB2 vector (1.0 μg). Twenty-four hours after transfection, cells were treated with 1 μmol/L ATRA or DMSO vehicle as a control in the presence or absence of trichostatin A (10 nmol/L) or sodium butyrate (1 mmol/L). Cells were then harvested after 24 h and total RNA was extracted. RT-PCR assays using primers for RARα, CYP26, and β-actin (as internal control) were then done. All experiments were done at least thrice with similar results. C, HEK293T cells were transfected with pEGFP-C1-XAB2 and pFLAG-CMV-HDACs (lane 1, HDAC1; lane 3, HDAC3; lane 4, HDAC4; lane 5, HDAC6). pFLAG-CMV empty vector was transfected along with pEGFP-C1-XAB2 (lane 2). After 24-h transfection, cells were harvested and nuclear extracts were immunoprecipitated with anti-Flag M2 mAb and then Western blotted with anti-Flag M2 mAb (top) or anti-GFP polyclonal antibody (middle). Ten micrograms of nuclear extracts were Western blotted with anti-GFP polyclonal antibody to show equal expression of transfected GFP-XAB2 protein (bottom). Asterisk, nonspecific bands. D, in the absence of ATRA, nuclear extracts were prepared from HL60 cells and immunoprecipitation (IP) assays were conducted with anti-XAB2 antibody or control immunoglobulin (IgG). Immunoprecipitation complexes, along with 10% input of nuclear extract, were then separated by 4% to 20% SDS-PAGE, followed by Western blotting with indicated antibodies. Representative results obtained from three independent experiments.
HDAC3 in our system and that XAB2 associates with HDAC3-dependent transcriptional components that interact with RARE. 

**ATRA leads to dissociation of XAB2 from RARα and HDAC3.**

As shown above, XAB2 associates with HDAC3-dependent transcriptional components that interact with RARE. ATRA leads to dissociation of XAB2 from RARα and HDAC3. As shown above, XAB2 is associated mostly with HDAC3 and is involved in the suppression of ATRA-mediated cellular differentiation. We next examined potential alterations in the complex containing endogenous XAB2, RARα, and HDAC3 after ATRA treatment. For this purpose, we conducted immunoprecipitation experiments using nuclear extracts of HL60 cells incubated in the presence or absence of ATRA. As shown in Fig. 4A, XAB2 was found with RARα and HDAC3 in immunoprecipitation complexes of nuclear extracts of untreated cells (Fig. 4A, lanes 1 and 4). Following treatment of HL60 cells with ATRA for 24 to 72 h, XAB2 and HDAC3 were dissociated from RARα (Fig. 4A, lanes 2 and 3) and, similarly, RARα and HDAC3 were dissociated from XAB2 (Fig. 4A, lanes 5 and 6). At these time points, Western blot analysis of nuclear extracts revealed that the expression level of HDAC3 was decreased by ATRA treatment (Fig. 4B, middle), whereas the expression levels of XAB2 and tubulin were not changed. These data indicate that XAB2 forms a complex with RARα and HDAC3 and that, following ATRA treatment, XAB2 is disengaged from the complex without degradation while HDAC3 is degraded, as previously reported (22).

To further characterize the association among XAB2, HDAC3, and RARα, we did fluorescence microscopic analysis to determine the subcellular localization of these structures. As shown in Fig. 4C, XAB2 clearly colocalized with RARα, as well as HDAC3, in the nuclei of untreated HL60 cells (a–c and g–i). Following treatment of HL60 cells with ATRA, XAB2 was not found to colocalize with RARα (d–f). Moreover, whereas XAB2 was localized in the nuclei after ATRA treatment (e and j), HDAC3 was not detected in the nuclei after ATRA treatment (k). Taken together, these data indicate that XAB2 associates with HDAC3 and RARα in the nuclei of untreated HL60 cells, with ATRA treatment resulting in the subsequent disengagement of XAB2 and HDAC3 from the complex. Furthermore, ATRA treatment induces change of localization of XAB2 in the nuclei while HDAC3 is degraded as reported previously (22).

To further confirm that XAB2, RARα, and HDAC3 bind to the same ATRA-targeted gene promoters and that XAB2 and HDAC3

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**Figure 4.** XAB2 forms a complex with RARα and HDAC3 and is dissociated from the complex following ATRA treatment. A, nuclear extracts of HL60 cells were immunoprecipitated with anti-RARα polyclonal antibody or anti-XAB2 mAb in the presence of absence of ATRA treatment (24 or 48 h) and Western blotted with anti-XAB2 mAb, anti-HDAC3 polyclonal antibody, or anti-RARα polyclonal antibody. B, 10 μg of nuclear extracts of HL60 cells were Western blotted with anti-XAB2 mAb, anti-HDAC3 polyclonal antibody, or anti-RARα polyclonal antibody, or anti-tubulin mAb in the presence of absence of ATRA treatment (24 or 48 h). C, HL60 cells were treated with ATRA (1 μmol/L) or DMSO vehicle for 24 h and submitted to analysis using confocal laser microscopy as described in Materials and Methods. D, chromatin immunoprecipitation assay was done using HL60 cells treated with ATRA (1 μmol/L) or DMSO vehicle for 24 h. Extracted chromatin samples were used directly in the PCR for the input control (lanes 1 and 2). Goat polyclonal IgG was used as negative control (cIgG; lanes 3 and 4). Antibodies for XAB2 (lanes 5 and 6), HDAC3 (lanes 7 and 8), RARα (lanes 9 and 10), and acetylated histone H4 (Ac-H4; lanes 11 and 12) were used for immunoprecipitation of the endogenous proteins. PCR was done using primers for RARα and CYP26 promoters containing RARE as described in Materials and Methods. All experiments were done at least thrice with similar results.
disengage from these genes following ATRA treatment, we did chromatin immunoprecipitation assay with HL60 cells. For this purpose, HL60 cells were treated in the presence or absence of ATRA for 24 h, followed by cross-linking by formaldehyde, and chromatin-protein complexes were immunoprecipitated using control IgG, anti-XAB2 pAb, anti-HDAC3 pAb, anti-RARα pAb, or anti–acetyl-histone H4 pAb. Chromatin immunoprecipitation assays against endogenous RARβ and CYP26 promoters that contain RARE sequences were done (47). As shown in Fig. 4D, endogenous XAB2 and HDAC3 were recruited to RARβ and CYP26 promoters in the absence of ATRA (lanes 5 and 7). However, in the presence of ATRA, endogenous XAB2 and HDAC3 were released from RARβ and CYP26 promoters constitutively in the presence or absence of ATRA (Fig. 4D, lanes 9 and 10). Moreover, to determine the acetylation level of histones around the RARE region of RARβ and CYP26 genes, chromatin immunoprecipitation assays with anti–acetyl-histone H4 pAb were done. In the absence of ATRA, histone acetylation levels of RARβ and CYP26 promoters were decreased (Fig. 4D, lane 11). On the other hand, in the presence of ATRA, histone acetylation level of these promoters was significantly increased (Fig. 4D, lane 12). These data suggest that XAB2 forms a complex with RARα and HDAC3 on the same ATRA-targeted genes and that, following ATRA treatment, XAB2 and HDAC3 are disengaged from the complex, which is associated with increased level of promoter histone acetylation.

Knockdown of XAB2 by siRNA induces differentiation of ATRA-resistant neuroblastoma cell line. In view of XAB2 inhibitory effect on ATRA-mediated transcriptional activity and cellular differentiation, we examined the effect of XAB2 on ATRA-mediated cellular differentiation of ATRA-resistant cells. For this purpose, we conducted siRNA experiments using the ATRA-resistant human neuroblastoma cell line IMR-32 (53). We prepared two specific siRNAs against XAB2 as described in Materials and Methods, which effectively knocked down XAB2 expression in IMR-32 cells (Fig. 5A, top, lanes 2 and 4). Because XAB2 in IMR-32 cells was not significantly knocked down by control siRNA (Fig. 5A, top, lanes 1 and 3) and expression level of tubulin as a housekeeping protein was not affected by both siRNAs (Fig. 5A, bottom), the
observed inhibition of XAB2 expression by its siRNA was specific. In addition, the expression levels of RARα and HDAC3 were not altered by treatment of XAB2-specific siRNA (data not shown). We next examined the effect of ATRA on cell differentiation of IMR-32 cells with XAB2 expression being knocked down by siRNA. As shown in Fig. 5B, changes consistent with cell differentiation following treatment with ATRA were not observed in ATRA-resistant IMR-32 cells treated with control siRNA (a). On the other hand, ATRA-resistant IMR-32 cells treated with two separate siRNAs against XAB2 exhibited ATRA-induced cell differentiation (b and c). Quantification of cell differentiation by ATRA in IMR-32 cells is shown in Fig. 5C. As shown in microscopic observation study (Fig. 5f, a–c), neurite-like differentiation of ATRA-resistant IMR-32 induced by ATRA treatment was significantly increased in cells treated with siRNAs against XAB2 (Fig. 5C, # and ##) compared with cell treated with control siRNA (Fig. 5C, ###). Although the precise mechanisms of ATRA resistance in IMR-32 have not yet been revealed (53), these data suggest that XAB plays an inhibitory role in ATRA-induced cell differentiation and that knockdown of XAB2 in selected ATRA-resistant tumors induces ATRA-mediated cellular differentiation.

Discussion

In this study, we showed that overexpression of XAB2 inhibited ATRA-induced cellular differentiation of the human rhabdomyosarcoma cell line MM-1-19-P and that knockdown of XAB2 by siRNA enhanced ATRA-mediated cellular differentiation of the human promyelocytic leukemia cell line HL60. Moreover, we found that XAB2 was associated with RARα and HDAC3 in the nuclei. Finally, we showed that, following treatment with siRNA specific against XAB2, the ATRA-resistant neuroblastoma cell line IMR-32 underwent cellular differentiation when incubated with ATRA at a therapeutic concentration.

XAB2 has been isolated by virtue of its ability to interact with XPA in the yeast two-hybrid system (33). Xeroderma pigmentosum patients show striking hypersensitivity to sunlight and an extremely high incidence of skin cancer in sun-exposed areas, as well as frequently progressive neurologic degeneration (40). Antibodies against the XAB2 protein has been shown to inhibit transcription in non–UV-irradiated normal cells, strongly suggesting that XAB2 can be a novel factor involved in the transcription process itself. In addition, it has previously been shown that retinoids are effective in treating preneoplastic diseases including xeroderma pigmentosum and other dermatologic diseases such as photoaging. We thus aimed to analyze the effect of XAB2 on retinoid-mediated transcription in the present study, especially on ATRA-mediated cellular differentiation, as cancer therapy. Our in vitro analyses shown in Figs. 1 and 2 showed that XAB2 has an inhibitory effect on ATRA-induced cellular differentiation of ATRA-sensitive neoplastic cell lines and that ATRA-mediated transcriptional activity is inhibited by XAB2 in a dose-dependent manner. Moreover, expression levels of endogenous RARα and HDAC3 are not affected by overexpression of exogenous XAB2 or by knockdown of XAB2 by siRNA (Figs. 2, 3, and 5). These results suggest that XAB2 plays an inhibitory role in ATRA-induced cellular differentiation by being functionally associated with the transcriptional repressor complex existing in RARE.

Our next focus in this study was to analyze the molecular mechanisms of XAB2-mediated inhibitory effect on ATRA-induced cellular differentiation. As shown in Figs. 3 and 4, XAB2 forms a complex with RARα and HDAC3 and is dissociated from RARα and HDAC3 in the presence of ATRA treatment. In the absence of ligands for RAR-RXR dimers such as ATRA, genes targeted by the receptors are repressed (reviewed in ref. 3). This is due to the recruitment of HDAC-containing complexes that are tethered through corepressors, such as N-CoR/SMRT, to the nonliganded RAR-RXR dimers. Moreover, the repressive activity of the N-CoR/SMRT complex results from direct or indirect association with HDAC3, leading to the formation of a stoichiometric core complex with N-CoR/SMRT (24, 54). Our data shown in Figs. 3 and 4 suggest that XAB2 is associated with corepressor complexes that bind to RARα and/or HDAC3 to repress transcriptional activity in the absence of ATRA. On the other hand, on binding of ligands to RAR, the corepressor interface is destabilized to generate a novel interaction surface for coactivators such as histone acetyltransferases and cAMP-responsive element binding protein-binding proteins or p300, leading to transcription initiation (3). To switch from corepressors to coactivators following ligand binding to RARα-RXR, specific adaptors are required for dismissal and subsequent degradation by ubiquitination of corepressors N-CoR/SMRT/HDAC3 (22, 24). TBL1/TBLR1 has been reported to serve as specific adaptors for the recruitment of the ubiquitin-conjugating proteosome complex to degrade N-CoR/SMRT/HDAC3 (55). In this
regard, although it is presently unclear whether XAB2 is degraded or recycled via an unknown pathway, XAB2 remains localized in the nuclei after ATRA treatment (Fig. 4) while still disengaging a corepressor complex containing N-CoR/SMRT, HDAC3, and XAB2 from RARα-RXRα on RARE (Fig. 4), potentially mediating corepressor-coactivator exchange.

Our findings suggest XAB2 interaction with corepressor complex containing HDAC3 and RARα through biochemical assays revealing protein-protein association and cellular assays showing biological effects (Figs. 3 and 4). However, we also raise doubts about the functional implication of XAB2 binding to HDAC3 and RARα. As shown in Fig. 3, endogenous XAB2 was mainly associated with HDAC3, whereas a small amount of HDAC1 was coprecipitated with XAB2. In fact, the precise mechanism of HDACs involvement in retinoid signaling is controversial (56). We therefore could not exclude the possibility that HDAC1 is involved in XAB2-associated retinoid signaling, a topic which will be elucidated in future studies. Another issue raised by our findings is whether XAB2 is involved in other nuclear receptor systems than RARα, such as vitamin D receptor or thyroid hormone receptor (24). Indeed, corepressor complex such as N-CoR/SMRT/HDACs also regulates other nuclear receptor systems than RARα (19), suggesting that XAB2 may be possibly involved in other transcriptional systems. Whereas data shown in our present study were obtained in transient transfection systems, we plan to examine the inhibitory effect of XAB2 on retinoid signaling or other nuclear receptor systems with stably transfected systems of overexpression and siRNAs in future studies.

On the basis of our results, we propose a model to describe the role of XAB2 in cell responsiveness to retinoids (Fig. 6). In this model, unliganded RARα-RXRα heterodimers bind to RARE and recruit HDAC3 complexes containing XAB2, followed by suppression of target genes for differentiation blockade (Fig. 6, a). On binding of ligands such as ATRA to RARα, N-CoR/SMRT/HDAC3 corepressors are dismissed and subsequently degraded by ubiquitination, which is mediated by specific adaptors such as TBL1/TBL1R (Fig. 6, b and c). Subsequently, coactivator complexes such as cAMP-response element binding protein–binding protein or p300 are recruited, leading to target gene activation and differentiation programs (Fig. 6, d). The potential clinical relevance of this model is revealed by the fact that pharmacologic concentrations of ATRA are required in the presence of XAB2 to disrupt this complex. However, combining ATRA treatment with knockdown of XAB2 by siRNA allows for the dissociation of the repressor complex containing HDAC3 and XAB2, leading subsequently to cellular differentiation.

Work over the past decades has shown that retinoids and their cognate receptors are required for the proper functioning of a number of organs, including the skin, and of the neuronal system and for prevention and treatment of selected cancers (17, 57, 58). One of the important objectives of cancer differentiation therapy is to selectively induce growth inhibition through RAR-dependent pathways. Our findings therefore should have broad implications for potential therapy not only for ATRA-sensitive cancer such as acute promyelocytic leukemia and rhabdomyosarcoma but also for ATRA-resistant solid tumors including neuroblastoma.

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