Inhibition of the Transforming Growth Factor β1 Signaling Pathway by the AML1/ETO Leukemia-associated Fusion Protein*

The t(8;21) translocation, found in adult acute myelogenous leukemia, results in the formation of an AML1/ETO chimeric transcription factor. AML1/ETO expression leads to alterations in hematopoietic progenitor cell differentiation, although its role in leukemic transformation is not clear. The N-terminal portion of AML1, which is retained in AML1/ETO, contains a region of homology to the FAST proteins, which cooperate with Smads to regulate transforming growth factor β1 (TGF-β1) target genes. We have demonstrated the physical association of Smad proteins with AML1 and AML1/ETO by immunoprecipitation and have mapped the region of interaction to the runt homology domain in these AML1 proteins. Using confocal microscopy, we demonstrated that AML1 and ETO and/or AML1/ETO, colocalize with Smads in the nucleus of t(8;21)-positive Kasumi-1 cells, in the presence but not the absence of TGF-β1. Using transient transfection assays and a reporter gene construct that contains both Smad and AML1 consensus binding sequences, we demonstrated that overexpression of AML1B cooperates with TGF-β1 in stimulating reporter gene activity, whereas AML1/ETO represses basal promoter activity and blocks the response to TGF-β1. Considering the critical role of TGF-β1 in the growth and differentiation of hematopoietic cells, interference with TGF-β1 signaling by AML1/ETO may contribute to leukemogenesis.

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The t(8;21) is the most frequent chromosomal translocation in de novo acute myelogenous leukemia, and it joins the AML1 gene on chromosome 21 to the ETO gene on chromosome 8, resulting in the formation of an AML1/ETO chimeric transcription factor. AML1 is a member of the runt family of transcription factors, and gene disruption experiments in mice have shown that AML1 is critical for the development of definitive (fetal liver) hematopoiesis (1–3). A family of ETO proteins has recently been described that share homology with the Drosophila gene nervy, and although the function of ETO is not well defined, ETO was recently shown to recruit corepressor molecules (4, 5) and associate with histone deacetylase activity. The most prominent functional consequence of the AML1/ETO fusion is the conversion of AML1 from a context-dependent transcriptional activator into a transcriptional repressor (by its fusion with ETO). The mechanism of repression by AML1/ETO is believed to be the recruitment of corepressor complexes via interactions of mSin3 and N-CoR with the ETO portion of the fusion protein. Nonetheless, transcriptional repression by AML1 and activation by AML1/ETO have been reported (6–8).

Although it is unclear how the altered transcriptional activity of AML1/ETO contributes to leukemogenesis, AML1/ETO was shown to cause abnormal differentiation and increased self-renewal capacity of hematopoietic cells (9, 10). In addition, it was reported that AML1/ETO can block differentiation of hematopoietic cells in response to several different stimuli (including granulocyte-colony-stimulating factor and the combination of vitamin D3 and TGF-β (5, 11)). Based on this observation, and considering the critical role of TGF-β in the regulation of cell proliferation and differentiation of both hematopoietic and nonhematopoietic cells, we examined whether proteins involved in the TGF-β signaling pathway can interact with AML1 and/or AML1/ETO.

Smad proteins have been identified as central mediators of TGF-β1 signaling (for a review, see Refs. 12 and 13). Activation of TGF-β1 receptors results in phosphorylation of cytoplasmic Smad2 and Smad3, which form heteromeric complexes with Smad4 and translocate to the nucleus, where they participate in the regulation of TGF-β1-inducible target genes recognizing the sequence CAGAC (which is the Smad binding motif). Activation of gene expression by Smads may require interaction with sequence-specific DNA-binding nuclear proteins, including FAST1 (14–16) and FAST2 (17, 18), AP1 (19, 20), Sp1 (21, 22), OAZ (23), Milk and Mixer (24), and TFE3 (25, 26). These interactions play a critical role in TGF-β1 signaling, as binding of Smad-interacting proteins to their consensus DNA binding sites enhances Smad-specific transcriptional regulation (27).

The possibility that AML1-related proteins could cooperate with TGF-β1 signaling was previously suggested (28, 29). The t(3;21) fusion product, AML1/EVI-1, was shown to interact with Smad3 and was proposed to interfere with TGF-β1-mediated growth inhibition of myeloid cells via the EVI-1 portion of the protein (30). However, recent reports indicate that AML1 proteins can form complexes with a variety of Smad proteins (including those involved in bone morphogenetic protein or activin signaling) (31–33).

We identified a region of homology between the Smad interacting domain in FAST1 and FAST2 and the N-terminal por-
After overnight culture, protein expression was induced with 0.1 mM 35S-Labeled proteins were synthesized in vitro using TNT coupled reticulocyte lysate system (Promega, Madison, WI). The beads were washed four times, and the immune complexes were assessed by immunoblotting using an anti-AML1 (RHD) antibody.

**EXPERIMENTAL PROCEDURES**

**Coimmunoprecipitation Experiments**—COS-1 cells were transfected with cytomegalovirus promoter-based AML1A, AML1B, AML1/ETO, and Flag-tagged Smad3 expression plasmids and were cultured for 18 h. Cells were lysed and sonicated in a buffer containing 50 mM HEPES, 50 mM NaCl, 0.1% Tween 20, 20 mM β-glycerol-PO₄, 20 mM sodium pyrophosphate, 0.3 mM sodium azide, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and phosphatase inhibitors. Cell extracts were incubated with anti-Flag monoclonal antibodies (Sigma) and protein A/G-agarose. The immune complexes were washed four times, and the immune complexes were assessed by immunoblotting using an anti-AML1 (RHD) antibody.

**In Vitro GST Pulldown Assays**—AML1-GST and Smad-GST fusion protein plasmids were transformed into *Escherichia coli* BL21, and then cultured in the presence or absence of 200 μM TGF-β1 for 18 h. Cells were lysed and sonicated in a buffer containing 50 mM HEPES, 50 mM NaCl, 0.1% Tween 20, 10% glycerol, 50 mM NaF, 20 mM β-glycerol-PO₄, 20 mM sodium pyrophosphate, 0.3 mM sodium azide, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and phosphatase inhibitors. Cell extracts were incubated with anti-Flag monoclonal antibodies (Sigma) and protein A/G-agarose. The immunoprecipitates were washed four times, and the immune complexes were assessed by immunoblotting using an anti-AML1 (RHD) antibody (Oncogene, Cambridge, MA). In *Vitro* GST Pulldown Assays—AML1-GST and Smad-GST fusion protein plasmids were transformed into *Escherichia coli* BL21, and protein expression was obtained, as described previously (34). Briefly, after overnight culture, protein expression was induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside for 3–4 h. The bacterial pellets were lysed in PBS-T (phosphate-buffered saline, 0.1% Tween 20) buffer with the aid of sonication. Equivalent amounts of GST fusion proteins were incubated with 20 μl of 50% glutathione-Sepharose 4B beads for 45 min at 4 °C. The beads were washed and then incubated with 2 μl of 35S-labeled Smad3 and AML1 proteins in PBS-T buffer for 1–3 h. 35S-Labeled proteins were synthesized *in vitro* using TnT coupled reticulocyte lysate system (Promega, Madison, WI). The beads were washed six times with PBS-T, the bound proteins were released from the beads by boiling at 95 °C in sodium dodecyl sulfate gel loading buffer, and the proteins were analyzed by SDS-10% polyacrylamide gel electrophoresis and autoradiography.

**Confocal Microscopy Colocalization Studies**—Kasumi-1 cells were cultured for 2 h in the presence or absence of 200 μM TGF-β1. The cells were attached to slides and fixed in methanol at −20 °C for 10 min. After blocking with 10% normal donkey serum, the cells were incubated overnight with rabbit anti-AML1 antibody (1:100) (Oncogene, Cambridge, MA) or anti-ETO antibody (1:500, the generous gift of Dr. P. Erickson) and goat anti-Smad2 or Smad3 antibodies (1:50) (Santa Cruz Biotechnology) in phosphate-buffered saline containing 5% normal donkey serum. Cells were washed three times and incubated with Cy3-conjugated (red) anti-goat IgG (1:200) and fluorescein isothiocyanate-conjugated (green) anti-rabbit IgG (1:500) (Jackson ImmunoResearch Laboratories) at room temperature for 1 h. Cells were washed in PBS, mounted, and examined using a Zeiss Axiosvert 100M confocal immunofluorescence microscope. For the negative control, parallel cultures were incubated in the presence of 5× excess of the immunogen (where available) or with preimmune serum. Colocalization is demonstrated by a yellow signal, which is generated by the overlay of the red and green signals.

**Transient Transfection Reporter Gene Assay**—Double-stranded, complementary oligonucleotides (containing sequences from the mouse germine Igo promoter) were used to construct an Igo-luc reporter gene that contains both AML1 and Smad consensus binding sites. The sequence of the oligonucleotide was 5′-CGCGTGGCCCACATGTTGCAGACACACCTGTCTCCACCCACGCACGACACCAGGCCCAGACTAGCCTGGAGGC-3′; and the annealed oligonucleotides were subcloned into the *Mu*1 and *Xho*I sites of the pGL2basic vector, which contains a minimal promoter. NIH3T3 cells were cultured in Dulbecco’s modified Eagle’s medium (DME) with 10% calf serum and were cotransfected with the Igo reporter gene plasmid and full-length cDNAs encoding AML1A, AML1B, or AML1/ETO subcloned into the pSVRe-MSV-tk-neo expression vector, using the calcium phosphate method (Promega, Madison, WI), as described previously (35). To control for the transfection efficiency, 0.5 μg of DNA of a pSVβ-galactosidase reporter plasmid was also cotransfected in each sample. The amount of DNA was kept constant, at 7.5 μg in most experiments, using the empty pSVβ vector.
After 24 h, the medium was changed to DME, 0.2% calf serum, with or without 200 pM TGF-β1, for 18 h. The cells were lysed in reporter lysis buffer, and luciferase activity was measured as per the manufacturer’s recommendation (Promega). Data are expressed as fold activation of luciferase activity, after normalization for βSVβ-galactosidase activity, assigning the activity of the promoter in the presence of the empty vector the value of 1.

RESULTS AND DISCUSSION

We first determined whether AML1A, a short isoform of AML1 (amino acids 1–250) that contains the region of homology with FAST1, can interact with Smads, by transiently overexpressing Flag-tagged Smads alone or with AML1A in COS-1 cells and performing immunoprecipitations using anti-Flag antibodies. As shown in Fig. 1, AML1A interacted with Smad3, and the interaction was observed in both the presence and absence of TGF-β1. Although in some experiments, including the one shown here, the interaction was increased by TGF-β1 stimulation, this was not uniformly observed. Overexpression of AML1/ETO or AML1B (the larger 479-amino acid isoform of AML1) with Flag-tagged Smad3, followed by immunoprecipitation with the Flag antibody, demonstrated the interaction between both of these proteins and Smad3. These interactions were seen in both the presence and absence of TGF-β1 stimulation and usually (as shown for AML1A in Fig. 1C and AML1/ETO in Fig. 1D) but not always (AML1B in Fig. 1D), this interaction was increased by TGF-β1. Similar variability in TGF-β1 inducibility of AML-Smad interactions was noted by Pardali et al. (32). Using this assay, we demonstrated that other Smad family members (including Smad2 and Smad4) also interact with AML1 proteins (data not shown), similar to that recently reported for AML2 (by Hanai et al. (31)).

These results suggest that the Smad interacting domain in AML1 proteins is localized to its N terminus, as neither AML1A nor AML1/ETO contains the C-terminal portion of AML1B. To more precisely map the region of interaction, we used in vitro transcribed and translated forms of AML1/ETO in Fig. 1D).
ETO and AML1B (Fig. 2C) and full-length GST-Smad3. As shown in Fig. 2D, full-length AML1/ETO interacted with Smad3, although this interaction is weaker than the interaction of AML1B with Smad3. Deletion of the middle third of the RHD from AML1/ETO (or from AML1B) eliminated their interaction with Smad3; this region partially overlaps with the region in AML1 that shares homology with FAST1. In contrast, deletion of the Ets interacting domain (EID), which is located at the C terminus of the RHD (34), had much less effect on the ability of AML1/ETO or AML1B to associate with Smad3. These findings suggest that the more N-terminal portion of the AML1 RHD plays a critical role in the interaction of AML1 proteins with Smad3. The stronger interaction of Smad3 with AML1B than with AML1/ETO could reflect the loss of a second Smad interacting domain, which has been found at the C terminus of AML1B (32) and in AML2 (31). Examination of various GST-Smad3 full-length or mutant proteins showed that Smad3 proteins containing the C-terminal MH2 domain most efficiently bound several different AML1 constructs (data not shown), similar to that reported by Pardali et al. (32).

Given the observed physical interaction between overexpressed AML1 (and AML1/ETO) and Smads in COS cells, we examined the intracellular localization of the endogenous proteins in Kasumi-1 cells, a t(8;21)-positive AML1 cell line, using immunostaining for AML1 and Smads in the presence and absence of TGF-β1. In the absence of TGF-β1, endogenous Smad2 (Fig. 3) and Smad3 (data not shown) are found predominantly in the cytoplasm, whereas AML1 is found in the nucleus. TGF-β1 stimulation resulted in the translocation of Smads to the nucleus and their colocalization with AML1. The same pattern of immunofluorescence staining was obtained using the anti-ETO antibody indicating colocalization of AML1/ETO and/or ETO with Smad2 or Smad3. This result suggests that the physiologic association of AML1 proteins and Smads is TGF-β1-inducible; it contrasts with the cytoplasmic AML1B-Smad3 complexes detected by Pardali et al. (32), following overexpression of both proteins in DG75 B cells (32). Hanai et al. (31) have recently shown the inducible association of AML1 with Smads, but only for Smad4. It is possible that receptor activation, which leads to the association of Smad4 with regulatory Smads (R-Smads), strengthens the protein-protein interactions involving R-Smads by incorporating Smad4 into a multimeric complex.

To determine the functional significance of the observed association between AML1 proteins and Smads we prepared an Igα promoter reporter gene construct that contains both AML1 and Smad consensus binding sequences in close proximity (28, 29). We performed transient transfection experiments and observed clearly divergent effects of AML1B and AML1/ETO on the promoter activity, as shown in Fig. 4A. AML1B alone and TGF-β1 both exerted a stimulatory effect on promoter activity, whereas AML1/ETO blocked the effects of TGF-β1 and repressed basal promoter function. Whereas AML1A was shown to activate the IκB promoter in DG75 B cells (29), AML1A (alone or with TGF-β1) weakly suppressed promoter activity in COS cells, perhaps due to its ability to bind to the AML1 consensus binding site but lack of the C-terminal AML1B transactivation domain. Similarly, when Smad3 was overexpressed together with one of the AML1 proteins, AML1B cooperated with Smad3 and AML1/ETO suppressed promoter activity (Fig. 4B). The inhibitory effect of AML1/ETO is dependent on the presence of the RHD, as the AML1/ETO deletion mutants that lack the middle portion of the RHD have no inhibitory effect on the promoter activity (Fig. 4C). Removal of the C-terminal 174 amino acids from AML1/ETO decreased inhibition to a level similar to that seen with AML1A. Together, these results demonstrate that AML1/ETO inhibits Smad signaling and that inhibition requires the presence of both the RHD and the C-terminal domains in the chimeric protein.

We have demonstrated that the RHD portion of the AML1 protein, which is retained in AML1/ETO, plays a critical role in both its physical association and functional interaction with Smad signaling pathway. This stands in contrast to a recently published report, which indicated that the Smad interacting domain in AML2, a protein quite similar to AML1, maps to the C-terminal portion of the protein (31). On the other hand, Pardali et al. (32) showed that the RHD and the region between
amino acids 371 and 411 in AML1B participate in the AML1B-Smads interaction, supporting our findings. Whether these reported differences result from different methodologies, e.g. comparing in vivo versus in vitro interactions, or relate to differences between the AML1 and AML2 proteins, particularly at their C-terminal portions, remains to be determined. It is possible that the presence of other interacting proteins may affect in vivo interactions between AML proteins and Smads and provide an explanation for at least some of the differences.

AML1B and AML1/ETO differentially regulate target gene activity, with AML1B exerting a stimulatory effect on basal and TGF-β1-stimulated Igα activity and AML1/ETO repressing basal promoter activity and blocking the response to TGF-β1. The different functional effects of the interaction of AML1B and AML1/ETO with Smad proteins is potentially very important in view of the known critical role of TGF-β1 in hematopoiesis as the regulator of the balance between proliferation and differentiation of hematopoietic cells. The activation of target genes by TGF-β1 is highly dependent on the presence or absence of different Smad interacting partners and the regulatory DNA context. Disruption of TGF-β1 signaling by either Smad mutations or functional inactivation of Smads has been associated with malignant transformation of a number of solid tumor malignancies, including colon (36), pancreatic (37), and hepatocellular (38) carcinoma. Our demonstration that AML1/ETO can functionally inactivate the TGF-β1/Smad signaling may provide the basis for some of the malignant features of t(8;21) leukemia. TGF-β1 signaling is apparently impaired by other fusion proteins, such as EWS/FLI-1, which is found in Ewing’s sarcoma and AML1/EVI-1 (39), which is formed as a result of the t(3;21) translocation. EWS/FLI-1 decreases the expression of TGF-β1 receptor type II (40). The AML1/EVI-1 and AML1/MD5/EVI-1 proteins were previously shown to inhibit the effect of TGF-β1 on a TGF-β1-responsive reporter plasmid, 3TP-Lux, which contains AP-1 and Smad binding sites; these fusions also block the growth-inhibitory effects of TGF-β1 on hematopoietic cells (39, 41).

The differential response to AML1B and AML1/ETO could be related to the difference in the recruitment of coactivators and corepressors. Both Smads (42–44) and AML1 (9) were shown to interact with CREB-binding protein/p300. Because AML1 interacts with coactivators via its C-terminal portion, which is lost in AML1/ETO, the chimeric protein not only loses its ability to interact with coactivators but acquires an additional ability to recruit corepressors via its ETO partner (4, 5). Our results indicate that the C-terminal portion of AML1/ETO plays a role in the repression of the transactivation of the promoter activity by TGF-β1, as deletion of this region from AML1/ETO, which can interact with N-CoR and mSin3, decreases its inhibitory effects. Although AML1B itself can recruit corepressor molecules, its interaction with TGF-β1 signaling is a positive or cooperative one (45). Smads can also participate, either directly or indirectly, in the recruitment of corepressors (46–48); whether the interaction of AML1/ETO with Smads affects their ability to interact with corepressors or coactivators and leads to a loss of TGF-β1-regulated growth control is under investigation.

Smad proteins are important suppressors of cell growth; thus the functional inactivation of the Smad signaling pathway by AML1/ETO and other transforming proteins could play a key role in the aberrant behavior of malignant cells.

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