AML1 (CBFα2) Cooperates with B Cell-specific Activating Protein (BSAP/PAX5) in Activation of the B Cell-specific BLK Gene Promoter

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AML1 plays a critical role during hematopoiesis and chromosomal translocations involving AML1 are commonly associated with different forms of leukemia, including pre-B acute lymphoblastic leukemia. To understand the function of AML1 during B cell differentiation, we analyzed regulatory regions of B cell-specific genes for potential AML1-binding sites and have identified a putative AML1-binding site in the promoter of the B cell-specific tyrosine kinase gene, blk. Gel mobility shift assays and transient transfection assays demonstrate that AML1 binds specifically to this site in the blk promoter and this binding site is important for blk promoter activity. Furthermore, in vitro binding analysis revealed that the AML1 runt DNA-binding domain physically interacts with the paired DNA-binding domain of BSAP, a B cell-specific transcription factor. BSAP has been shown previously to be important for B cell-specific regulation of the blk gene. Physical interaction of AML1 with BSAP correlates with functional cooperativity in transfection studies where AML1 and BSAP synergistically activate blk promoter transcription by more than 50-fold. These results demonstrate physical and functional interactions between AML1 and BSAP and suggest that AML1 is an important factor for regulating a critical B cell-specific gene, blk.

AML1 is a member of the PEBP2/CFB family of transcription factors (1, 2). These factors consist of heterodimers between the DNA binding α subunit and the β subunit, CBFβ, which does not bind DNA directly but enhances the binding of the α subunit (3). Multiple α subunit genes, including CBFα1 (AML3), AML1 (CBFα2), and CBFα3 (AML2), as well as alternatively spliced isoforms of the α and β subunits have been detected (4, 5). All of the CBFα proteins have a DNA-binding domain (the runt domain), which is similar to the Drosophila pair-rule gene, runt (6). AML1 and other PEBP2/CFBα proteins are transcription factors whose recognition sequence is required for tissue specific expression of several hematopoietic genes including M-CSF receptor, GM-CSF, IL-3, T cell receptors, immunoglobulin μ heavy chain, defensin NP-3, and myeloperoxidase (7–15). AML1 knockout mice indicate, furthermore, that AML1 is indeed a critical regulator of early hematopoiesis (16, 17). The AML1 gene is frequently associated with chromosomal translocations in different forms of leukemia, including t(8;21), t(3;21), in addition to t(12;21) (1, 18–20). The β subunit of PEBP2/CBF is also involved in a chromosomal inversion, inv (16), associated with FAB M4eo AML (21). Therefore, each of the two chains of the PEBP2/CBF heterodimer is directly implicated in the pathogenesis of leukemia. T(12;21) is a common chromosomal abnormality in childhood pre-B acute lymphoblastic leukemia (20, 22). This translocation generates two chimeric genes, TEL/AML1 and AML1/TEL. Only the TEL/AML1 chimeric gene product is consistently detected in cells with t(12;21) (23). The TEL/AML1 chimeric gene expresses a fusion protein that contains the 333 NH2-terminal amino acids of the TEL protein encoding the Pointed dimerization domain but lacking the Ets DNA-binding domain, and almost the entire AML1 protein, including the AML1 DNA-binding domain. Therefore, the TEL/AML1 fusion protein can interact with AML1 DNA-binding sites and possibly interfere with AML1 function during B cell differentiation. This indicates that AML1 may play an important role in controlling gene expression during normal B cell differentiation.

To study the function of AML1 in B-cell differentiation, we analyzed regulatory regions of B cell-specific genes for the presence of AML1-binding sites. We observed a consensus AML1-binding site in the promoter of the blk gene. BLK, a Src family member, encodes a B cell-specific, 55-kDa protein tyrosine kinase p55<sup>BLK</sup> (24). BLK is associated with the B cell antigen receptor and is involved in signal transduction events (25). The B cell antigen receptor complex is formed by membrane IgM noncovalently associated with heterodimers of B29 and mb-1 (26, 27). Antigen cross-linking to the B cell antigen receptor leads to a rapid and transient increase in tyrosine kinase activity resulting in activation of several signal transduction pathways, including the mitogen-activated protein kinase pathway. The antigen receptor does not itself contain an intrinsic tyrosine kinase domain; therefore, tyrosine kinase activation is due to signal transduction mediated through interaction of two B cell antigen receptor-associated proteins, Igα (mb-1) and Igβ (B29) with the cytoplasmic Src-like kinases, BLK, LYN, and FYN (28). This rapid and transient activation of tyrosine kinases triggers a cascade of downstream events, leading to changes in gene expression and either cell proliferation and differentiation or apoptosis. BLK is exclusively expressed in B cells at the pre-B, pre-B, and mature B cell stages, but not in plasma cells or non-B cells (29). The function of BLK in B cell antigen receptor signaling is not clear, although several studies have implicated the BLK kinase in antigen receptor cross-linking mediated growth arrest and apoptosis (30, 31).
However, transgenic mice expressing a constitutively activated BLK mutant in the B cell lineage develop B lymphoid tumors (32). Therefore, control of blk gene expression could be directly related to B cell development and neoplasia. B cell specificity of blk gene expression is primarily regulated at the transcriptional level and a 320-bp 1 promoter region of the murine blk gene is related to B cell development and neoplasia. B cell specificity of glutathione S-transferase; a.a., amino acid(s).

EXPERIMENTAL PROCEDURES

Electrophoretic Mobility Shift Assay (EMSA)—The probe for the EMSA was a double-stranded oligonucleotide with bp 47 to 28 of the blk upstream sequence (Fig. 1). A 32P-labeled probe was prepared by T4 kinase phosphorylation in the presence of [y-32P]ATP (NEN Life Science Products Inc.). Nuclear protein for the EMSA was prepared from Ba/F3 cells according to the method published by Schreiber et al. (44). Approximately 0.5 ng of probe was incubated with 5 μg of nuclear protein or 1 μl of in vitro translated protein in 20 μl of binding buffer containing 10 mM HEPES, pH 7.9, 30 mM KCl, 5 mM MgCl2, 1 mM dithiothreitol, 1 mM EDTA, 0.4 mM phenylmethylsulfonyl fluoride, 12% glycerol at 4 °C for 20 min (45). All reactions contain 1 μg of poly(dI-dC). Reactions were electrophoresed at 10 V/cm on a 6% polyacrylamide gel (bisacrylamide/acrylamide ratio 5:1) in 0.5 × TBE (45 mM Tris borate, 1 mM EDTA) at 4 °C. For supershift experiments, 1 μl of polyclonal antiserum against AML1 was added to the binding reaction mixture 10 min prior to addition of the probe (46).

Plasmid Construction—A murine blk promoter (bp −191 to +136) DNA fragment was subcloned into the KpnI and XhoI sites of the pXP2 luciferase reporter gene construct to form pBlk-luc (47). Blk-luc (mAML1), which contains a mutation at the AML1 binding was generated using QuickChange Mutagenesis Kit (Stratagene). The AML1 site was changed from TGTGGT to TGCACT. AML1 and CBF family members in the activation of critical B cell developmental genes. Therefore, we searched the regulatory regions of several B cell-specific genes for putative AML1-binding sites and found an AML1 binding consensus sequence “TGTGOT” in the B cell-specific blk promoter at bp −39 to −34, just downstream of the BLK-32P-labeled sequence (Fig. 1). To test whether AML1 can interact with the blk promoter, oligonucleotides containing the AML1 consensus sequence from the blk promoter were used in EMSA experiments with nuclear extracts from the B cell line, Ba/F3, or with in vitro translated AML1. As shown in Fig. 2A, Ba/F3 nuclear extract formed a specific protein-DNA complex with the blk promoter bp 47 to 28 oligonucleotide. This protein-DNA complex was specifically competed by unlabeled self-oligonucleotide or an oligonucleotide encoding the Moloney murine leukemia virus enhancer PEBP2/CFB-binding site (50), but not an unlabeled non-PEBP2/CFB-binding oligonucleotide. These data suggested that this complex contains a PEBP2/CFB related protein. To further characterize this protein, antiserum raised against the NH2-terminal region of one of the PEBP2/ CBF family members, AML1 (46), was used in the EMSA analysis. Upon the addition of the anti-AML1 antiserum, 2

1 The abbreviations used are: bp, base pair(s); BSAP, B cell-specific activating protein; EMSA, electrophoretic mobility shift assay; GST, glutathione S-transferase; a.a., amino acid(s).
specific protein-DNA complex was drastically reduced and a supershifted band was detected indicating that the protein in Ba/F3 cell nuclear extracts interacting with the blk promoter AML1 site is either identical with AML1 or closely related. Additional EMSA analysis demonstrated that in vitro translated AML1 formed a complex with the same radiolabeled blk promoter probe (Fig. 2B). These results clearly demonstrate that PEBP2/CFB transcription factors, such as AML1, can interact specifically with the blk promoter AML1 site.

**AML1 Induces blk Promoter Transcription**—To determine whether the AML1-binding site is functionally relevant for blk promoter activity, the AML1 site was mutated from TGTGGT to TGCACT in the blk promoter luciferase construct to generate pBlk(mAML1)-luc. As shown in Fig. 3A, transient transfection into the B cell line BJAB demonstrated that the blk gene upstream region from bp −191 to +136 has strong promoter activity as previously shown (33). A 25-fold increase in luciferase expression was observed in comparison to the promoter-less luciferase construct pXP2. Mutation of the AML1-binding site in the blk promoter reduced the promoter activity to 67% when compared with the wild type promoter. Furthermore, the same mutation also reduced the promoter activity to a similar level in another B cell line, Ba/F3. This indicates that the AML1-binding site in the blk promoter is a functionally relevant site, although not absolutely essential. To test whether AML1 can indeed transactivate the blk promoter, co-transfection experiments were performed in CV-1 cells with expression vectors for AML1 and its heterodimer partner CBFβ. As shown in Fig. 3B, exogenous AML1 together with CBFβ expression increases transcription of the wild type blk promoter-luciferase construct by 7-fold. Mutation of the AML1-binding site reduced transactivation of the blk promoter by AML1/CFBβ to less than 2-fold. These data together with the results from the DNA-protein interaction studies demonstrate that AML1 or a related
family member plays a significant role in B cell-specific regulation of the \textit{blk} promoter.

**AML1 Physically Interacts with BSAP**—As shown in Fig. 1, the AML1-binding site is relatively close to the transcription initiation site of the \textit{blk} gene. Six base pairs upstream of the AML1 site is a region that has been identified as a BSAP-binding site (33, 34). BSAP is a B cell-specific activating protein, which is a critical transcription factor for B cell-specific gene expression and B cell development. Since AML1 has been shown to interact and cooperate with other transcription factors, and the AML1 runt homology domain is adjacent to the BSAP-binding site, we analyzed whether AML1 can cooperate with BSAP in regulating \textit{blk} gene expression. First, we studied their physical interaction using GST-pull down assays, in which \textit{Escherichia coli} expressing GST fusion proteins immobilized on glutathione-agarose beads were incubated with \textit{in vitro} translated \textit{[^{35}S]}-labeled proteins. As shown in Fig. 4, in vitro translated, \textit{[^{35}S]}-labeled full-length AML1 was directly loaded on the gel (IVT, lane 1), incubated with \textit{E. coli}-produced GST (lane 2), GST-BSAP (lane 3) GST-BSAP (lane 4), or GST-BSAP (lane 5) immobilized on glutathione-agarose beads. Bound proteins were analyzed in SDS-polyacrylamide gels and were visualized by autoradiography.

**AML1 Cooperates with BSAP in Transactivation of the \textit{blk} Promoter**—Both AML1-binding site mutation analysis and transactivation analysis indicate that AML1 is an important transcription factor for \textit{blk} promoter activity (Fig. 3). BSAP has been shown to be a critical regulator of \textit{blk} promoter activity (33, 34) and data in Fig. 4 have demonstrated the physical interaction between these two transcription factors. To determine whether physical interaction between AML1 and BSAP is
AML1 Regulates the blk Promoter

**DISCUSSION**

AML1 is a transcription factor identified by studying t(8;21) associated acute myeloid leukemia (1, 52–54). The function of AML1 in hematopoiesis has been demonstrated by analyzing its role in regulating the expression of critical hematopoietic genes and by studying AML1 null mice (55, 56). AML1 knockout mice die during embryogenesis with the block of definitive hematopoiesis (16, 17). These studies indicate that AML1 plays an important role during early hematopoietic progenitor cell formation. AML1 binds to the regulatory elements of genes specifically expressed in different lineages of hematopoietic cells or of genes important for lineage development. Recently, AML1 has been found to associate with a chromosomal translocation, t(12;21), commonly associated with B-cell lineage differentiation, indicating an important role for AML1 during B cell development (20, 22). Nevertheless, only one potential B cell-specific target gene for AML1, the immunoglobulin heavy chain gene enhancer (57), has been identified to date. Therefore, we decided to search for other potential target genes for AML1 in B cells. We identified a putative AML1-binding site in the promoter region of the B cell-specific blk gene and tested the potential role of AML1 in B-cell specific blk gene regulation. We provide strong evidence here that AML1 directly interacts with a functionally important region of the blk promoter and transactivates the promoter. Furthermore, we demonstrate that AML1 physically interacts with another critical B-cell transcription factor BSAP and synergizes with BSAP in regulating blk promoter activity. There are different alternatively spliced forms of AML1, which have been named as AML1 (AML1a), AML1A (AML1b), and AML1B (AML1c). In this paper, we used “AML1” to include different forms of the AML1 protein. AML1B was used in the transactivation and in vitro transcription reactions.

AML1 is a member of the CBF protein family. All three α subunits of the CBF family contain the highly conserved runt homology domain, which encodes the DNA-binding domain. Therefore, all three α subunits recognize the same “TGTGTT” DNA sequence. Previous studies by others have identified different members of the CBFα protein in B cell lines and a B cell-enriched tissue spleen, using antibodies specifically against three different CBFα subunits. Their results show that AML1 and AML2 are expressed at similar levels in B lineage cells; AML3 is not detectable (58). The AML1 antisera that we used in Fig. 2 is raised against the AML1 NH2-terminal 17 amino acids. The NH2-terminal region is conserved between all three CBFα proteins. Since both AML1 and AML2 are expressed at similar levels in B cells and both AML1 and AML2 have a similar DNA binding activity (58), it is possible that both of them are in the supershifted protein-DNA complex as shown in Fig. 2.

It has been reported previously that CBFβ enhances AML1 binding to DNA (3) and CBFβ-AML1 forms a slower mobility shifted band with DNA than AML1-DNA complex (46). As shown in Fig. 2B, in vitro translated AML1 binds to DNA and addition of CBFβ enhances the binding of AML1 with the DNA. However, we did not detect an obvious slower mobility complex. This is probably due to the lack of a particular modification in the in vitro translation system that is required for forming a stable complex of a heterodimer CBF protein with DNA, or the specific experimental conditions do not favor the formation of the complex during gel electrophoresis.

Mutation of the AML1 site in the blk promoter significantly reduced promoter activity. However, this mutation did not abolish the promoter activity, indicating that AML1 is an important factor for the promoter activity. Other transcription factors that bind to the blk promoter could also contribute to the promoter activity. As shown in Fig. 1, besides the AML1-binding site, there are several other potential transcription factor-binding sites including a binding site for the B cell-specific BSAP transcription factor just upstream of the AML1 site. BSAP has been reported previously to play a crucial role in the regulation of the blk promoter (33, 34). Therefore, we analyzed whether AML1 and BSAP could interact with each other and whether there is any synergy between these two factors in promoter activation. The results from GST-pull down assays demonstrated a strong interaction between these two factors, and the interaction is between the AML1 runt homology domain and the BSAP NH2-terminal paired domain. The original function of the runt homology domain includes direct binding to DNA and formation of heterodimers with CBFβ. There have been reports about AML1 physically interacting with other transcription factors through the runt homology domain, including Ets-1, PU.1, and CAAT enhancer-binding protein family members (14, 48, 49). Similarly, the paired domain of BSAP has been shown to be involved in protein-protein interactions with members of the Ets family (59). The interaction between AML1 and BSAP represents a new class of interaction between AML1 and other transcription factors. Interestingly, it is the DNA-binding domain that is involved in the interaction of both transcription factors. It will be interesting to further study the specific amino acids involved in the different interactions. The interaction and synergy between Ets-1 and AML1 is crucial for the enhancer function of several T cell receptor genes (14, 60);
furthermore, the interactions and synergies between AML1 and PU.1 (49), Myb (61), and CAAT/enhancer-binding protein (48) have been demonstrated to serve critical functions during activation of myeloid specific gene expression. Both AML1 and BSAP are critical regulators of hematopoiesis (16, 17, 62). In addition, both genes have been directly implicated in B cell malignancies (20, 63). The interaction and synergy between AML1 and BSAP could play a significant role in B cell-specific gene expression and disruption of this interaction due to chromosomal translocations or other chromosomal abnormalities could play a critical role in B cell transformation. Further experiments using other B cell-specific gene targets will be extremely interesting.

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