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The LIM domain protein Lmo2 binds to AF6, a translocation partner of the MLL oncogene

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Abstract The LIM only protein Lmo2 plays an important role in hematopoiesis and leukemogenesis. Lmo2 acts as a bridging molecule between components of hematopoietic gene regulatory protein complexes. We used the yeast two-hybrid system to identify novel Lmo2 interacting proteins and found that the AF6 protein binds to Lmo2. AF6 is a recurrent fusion partner of MLL, the human homolog of *Drosophila trithorax* chromatin remodeling protein that is involved in childhood leukemia and mixed lineage leukemia. Our data support the notion that recurrent fusion partners of chimeric MLL proteins recruit hematopoietic gene regulatory complexes. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

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1. Introduction

Proteins with LIM domains play important roles in embryo development and hematopoiesis [1–3]. The LIM signature represents protein interaction domains of approximately 50 amino acids [2]. While many LIM proteins contain various additional domains such as homeodomains or kinase domains, a subfamily that contains LIM domains only (Lmo) is thought to mediate interaction between proteins [1,2].

Lmo2 (also called TTG2; RBTN2) is expressed in myeloid and erythroid precursors of the hematopoietic system and its expression decreases during differentiation [4]. Chromosome translocations affecting both Lmo2 and the related Lmo1 genes were found in a subset of T cell acute lymphoblastic leukemias, and ectopic expression of Lmo2 or Lmo1 induces leukemia (see references in [3]). Disruption of the Lmo2 gene has firmly established its essential role in hematopoiesis [4,5]. Complexes containing Lmo2, GATA1, the helix-loop-helix proteins Tal1/SCL, E47, Ldb1/NLI and the retinoblastoma-binding protein 2 (RBP2) have been identified [6–9]. Accordingly, it has been suggested that bridging between hematopoietic transcription factors is an important function of Lmo2. We set out to identify novel Lmo2 interacting proteins. Here we describe that Lmo2 interacts with the AF6 protein. AF6 is a recurrent fusion partner of MLL, the human homolog of the *Drosophila trithorax* gene that is involved in epigenetic chromatin remodeling events and leukemogenesis [10,11]. Our data raise the possibility that MLL–AF6 fusion proteins interact with Lmo proteins through the AF6 moiety. The results thus suggest a potential connection between hematopoietic transcription factor complexes that play essential roles in progenitor proliferation and differentiation and between MLL–AF6 fusion proteins.

2. Materials and methods

2.1. Plasmid constructs and library screening

A PCR fragment encoding aa 27–158 of the human Lmo2 (ΔN-Lmo2) was used in the yeast two-hybrid screens to screen a rat liver library (Matchmaker, Clontech). Lmo2 mutant cDNAs were constructed by PCR and cloned into pGEX 4T1 (Pharmacia). Rat AF6 (rAF6) cDNA and mutants derived thereof were Flag-tagged and cloned into pcDNA3 (InVitrogen). For the immunoprecipitation experiments, full-length human Lmo2 was HA-tagged and cloned into pcDNA1. rAF6 cDNA was cloned in frame with Gal4–DBD into pcDNA3. Plasmid constructs were sequenced, and protein expression was confirmed. GST pull-down assays were performed as described elsewhere [25].

2.2. Transfection and co-immunoprecipitation experiment

QT6 quail fibroblasts were transfected as previously described [25]. Whole cell lysates were prepared in RIPA buffer, supplemented with protease inhibitors. Lysates (500 μl) were incubated with 3 μg of anti-HA antibodies (Babco) or 5 μg of anti-Gal4 antibodies overnight at 4°C under gentle agitation. Protein G or protein A Sepharose (Pharmacia) was added, incubated for 1 h, and washed extensively with RIPA buffer. Immunoprecipitates were separated by SDS–PAGE, blotted (Millipore) and co-precipitated proteins were revealed with antisera directed against either HA, Gal4, or AF6 (Transduction Laboratories). Immunoblots were developed with ECL (Amersham).

2.3. Reverse transcription-polymerase chain reaction

First strand cDNA was synthesized from total RNA using Superscript™ II (Gibco). RT-PCR was performed with specific primers for MLL–AF6 (250 bp in ML-1 and 400 bp in CTS: forward GTCCAGAGCAGAGCAAACCAG and reverse CTGACATGCACTTCATAGAGTG), Lmo2 (470 bp: forward ATGTCCTCGGCCATCGAA and reverse ATCCCATTGATCTTAGTCCA), Ldb1/NLI (475 bp: forward AGCTAAGAGGGCCCAGC and reverse CACACGGAGCCACTGTGCC), Tal1/SCL (481 bp: forward ACCACCAACAATCGAGTGAAG and reverse GGATGGCAGGATGGAGGCT), GATA2 (163 bp [26]), RBP2 (241 bp: forward TTCTGTGTTGAACGGTTCTTGTCC and reverse CGATAAAGCTGAGCGGATCTGTGA). PCRs were performed by means of 35 cycles (25 cycles for GAPDH) at an annealing temperature of 58°C.

3. Results and discussion

A truncated form of human Lmo2 (aa 27–158; ΔN-Lmo2) was fused to the Gal4 DNA binding domain and used as a bait to identify Lmo2 interacting proteins by yeast two-hybrid

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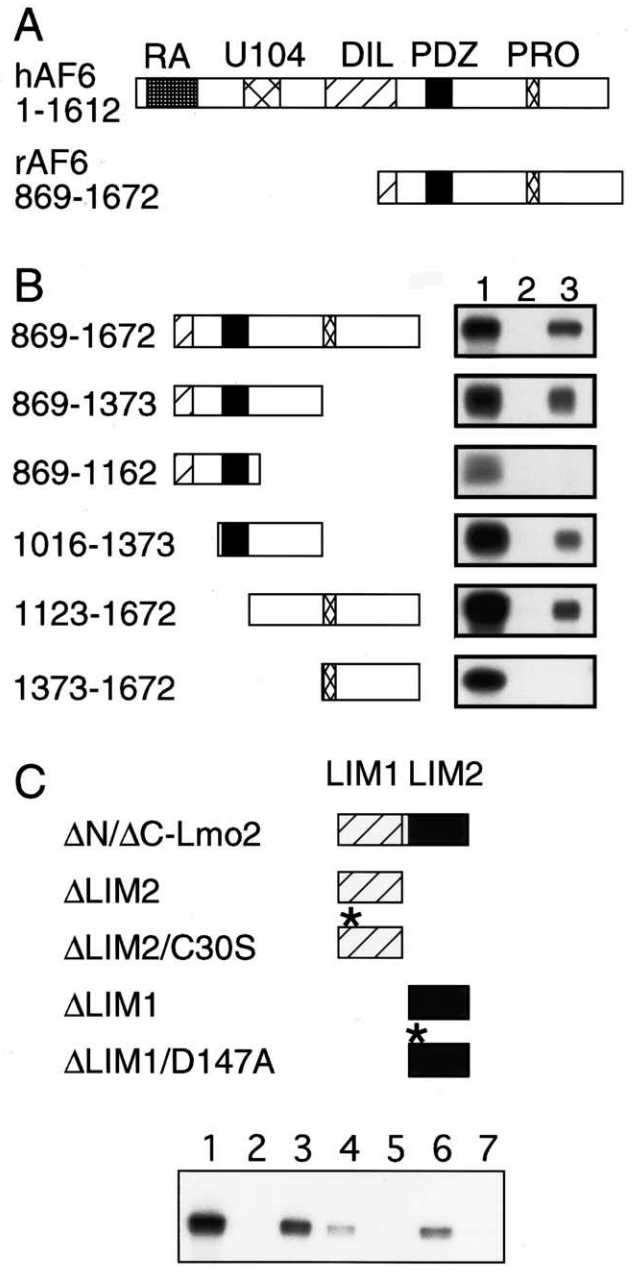

Fig. 1. AF6 interacts with the LIM domains of Lmo2. A: Schematic representation of human AF6 (hAF6) and the rAF6 isolate. hAF6 contains two RAS-binding domains (RA), a U104 and DIL domain as well as a PDZ domain and a proline-rich region (Pro) [27,28]. The rAF6 retains 80 aa from the DIL domain, the PDZ domain (89% homology with hAF6 PDZ) and the Pro domain (83% homology with hAF6 Pro). Alignments were done using the blast search program; hAF6 = NM005936.1. B: Schematic representation of various deletion mutants of rAF6 fused to GST. After in vitro translation and [^{35}S]methionine labeling, rAF6 mutants (lane 1 = input 50%) were incubated with GST (lane 2) or GST–ΔN/ΔC-Lmo2 fusion protein (lane 3). Beads were extensively washed, bound proteins were separated by SDS–PAGE and visualized by autoradiography. C: Schematic representation of deletion mutants of Lmo2 fused to GST. LIM1, 2 (hatched and black box, respectively) represent the two LIM domains of Lmo2. Lmo2 mutants were tested for their ability to bind to ^{35}S-labeled in vitro translated rAF6. Asterisks indicate point mutations introduced in the LIM domains. Lane 1 = input 65%; lane 2 = GST; lane 3 = GST–ΔN/ΔC-Lmo2; lane 4 = ΔLIM2; lane 5 = ΔLIM2/C30S; lane 6 = ΔLIM1; lane 7 = ΔLIM1/D147A.

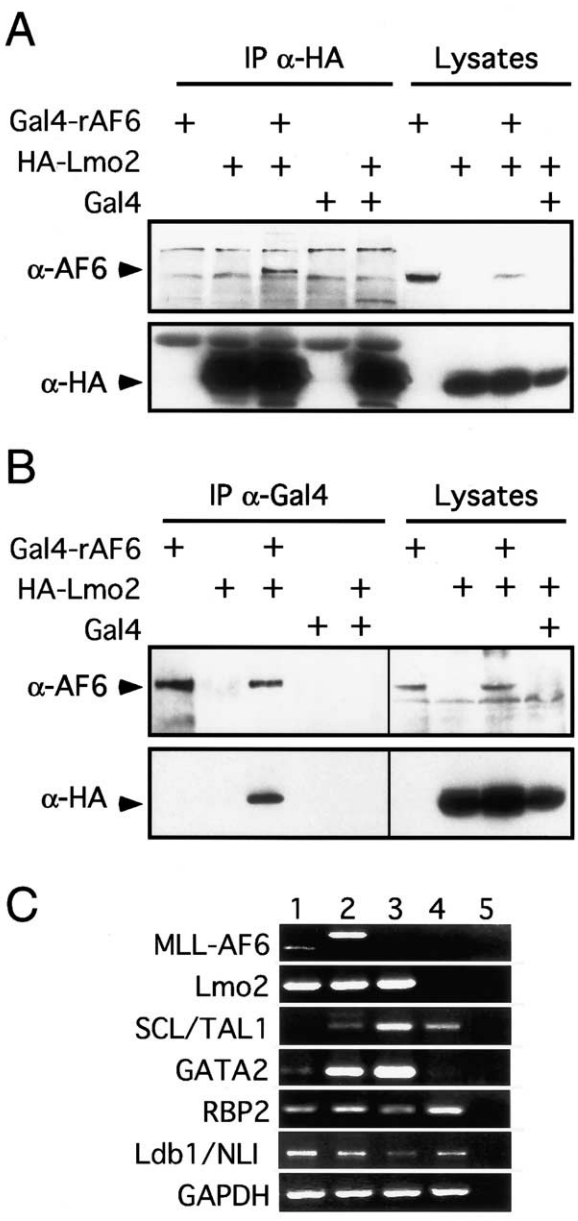

Fig. 2. Interaction of Lmo2 and rAF6 in vertebrate cells and co-expression of Lmo2 interacting proteins in MLL–AF6 leukemic cells. A: QT6 fibroblasts were transfected with HA-tagged Lmo2 (HA-Lmo2) and Gal4 or Gal4–rAF6. Cells were harvested after 24 h and subjected to immunoprecipitation using a HA-tag specific antibody (IP α-HA). Proteins were separated by SDS–PAGE, blotted and probed with antibodies as indicated on the left. The panel on the right shows expression controls of transfected constructs (Lysates). B: QT6 were transfected as described in A. Protein extracts were then subjected to immunoprecipitation using a Gal4-specific antibody. Co-precipitated proteins (IP α-Gal4) were revealed by immunoblotting using antibodies indicated on the left. Expression controls are shown on the right panel (Lysates). C: Co-expression of Lmo2 and transcripts of interacting proteins in MLL–AF6 leukemia cells. Total RNA was isolated from ML-1 (lane 1), CTS (lane 2), HEL (lane 3), and Jurkat cells (lane 4); water control (lane 5). RT-PCR was performed with specific primers for MLL–AF6, Lmo2, Tal1/SCL, GATA2, Ldb1/NLI, RBP2 and GAPDH, as indicated on the left. Positive controls for Lmo2 and for GATA2, HEL cells; for Tal1/SCL, Ldb1/NLI and RBP2, HEL and Jurkat cells. Note the different size of the MLL–AF6 amplified fragments in the two patient cell lines, which is probably due to different breakpoints in the translocations.

screening of rat liver library. The N-terminal part of Lmo2 (aa 1–26) was omitted because it represents a transcriptional activation domain [12,13]. From two million transformed yeast clones, five clones were found to interact specifically with Lmo2. Sequence analysis revealed that a strongly interacting prey construct encoded a C-terminal fragment of the rAF6 protein, also called s-afadin [14,15], shown in Fig. 1A.

The Lmo2 interacting rAF6 clone retained the PDZ domain and a proline-rich C-terminal domain (Fig. 1A). Both types of domains have been previously shown by others to interact with LIM domains [16,17]. Various rAF6 and Lmo2 mutants were generated to confine the interaction between rAF6 and Lmo2. As shown in Fig. 1B, neither the PDZ domain nor the proline-rich domain of rAF6 was required for Lmo2 binding. However, a protein region between amino acid residues 1123 to 1373 of rAF6 was essential for Lmo2 binding. Data base searches with this protein region did not reveal similarities with other known proteins. Nevertheless, residues 1123 to 1373 are 92% identical to their human counterpart, suggesting that the Lmo2 binding region is conserved in the hAF6. Next, deletion mutants of Lmo2 were tested to determine which part of Lmo2 binds to rAF6 (Fig. 1C). Constructs that contain either both LIM domains or a single LIM domain still bound to rAF6 (Fig. 1C, lanes 3, 4 and 6). However, point mutations that disrupt the zinc-finger structure of LIM domains (D147A in ΔLIM1 or C30S in ΔLIM2) entirely abrogated Lmo2–rAF6 interaction (Fig. 1C, lanes 5 and 7). These data indicate that both LIM domains of Lmo2 can bind to rAF6 and that an intact LIM domain structure is indispensable for interaction with rAF6. Interestingly enough, we found that rAF6 also binds to Lmo1 and to RIL in GST pull-down assays (data not shown). Taken together, these results show that AF6 may interact with various LIM proteins.

AF6 is normally expressed at the plasma membrane and locates to the nucleus as an MLL–AF6 fusion protein [18]. An important question therefore was whether interaction between rAF6 and Lmo2 also occurs in the nucleus of vertebrate cells. As we failed to express a full-length tagged version of the MLL–AF6 fusion protein, we generated a Gal4–rAF6 expression construct that also localizes to the nucleus (confirmed by immunohistological staining, data not shown). Immunoprecipitation from transfected fibroblasts revealed that Gal4–rAF6, but not Gal4, co-precipitated along with HA-tagged Lmo2 (Fig. 2A). Similarly, Lmo2 co-immunoprecipitated together with Gal4–rAF6 but not with Gal4 when a Gal4 specific antiserum was used (Fig. 2B). These results show that Lmo2 associates with a rAF6 fusion protein in the nucleus.

Finally, we performed RT-PCR analysis to determine whether Lmo2 interacting partner proteins (see Section 1) are co-expressed with MLL–AF6 translocations. MLL–AF6 transcripts are expressed in the CTS and ML-1 cell lines that were derived from patients with acute myeloid leukemia [18–21]. As shown in Fig. 2C, Lmo2 is expressed in both CTS and ML-1 cells along with GATA2, Ldb1/NLI and RBP2. Moreover, Tal1/SCL that has also been shown to interact with Lmo2 [7,8] was expressed in CTS cells. These data show that various Lmo2 partner proteins are co-expressed in cells that carry the MLL–AF6 translocation. Further investigations are required, however, to show unequivocally that hematopoietic gene regulatory complexes are recruited by MLL–AF6 through Lmo2.

Taken together, our data show that Lmo2 binds to AF6, a recurrent translocation partner of MLL. The MLL gene product is implied in heritable changes in gene expression and has been shown to be involved in more than 40 different recurrent chromosome translocations. Although MLL leukemias are potentially the result of dominant-negative functions of truncated chimeric MLL proteins, a gain of function that determines phenotype and leukemogenic selection is also suggested by the particular fusion partners [10,11]. It is therefore intriguing to see that other MLL-chimeras implicated in leukemogenesis have also been suggested to connect to the hematopoietic transcriptional apparatus [22–24]. The observation that AF6 binds to Lmo2 represents a novel starting point to examine how MLL fusion proteins reprogram hematopoiesis and induce leukemia.

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