Mutations in Multiple Domains of c-Myb Disrupt Interaction with CBP/p300 and Abrogate Myeloid Transforming Ability

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Abstract

The c-myb proto-oncogene is a key regulator of hematopoietic cell proliferation and differentiation. MYB mRNA is expressed at high levels in, and is required for the proliferation of, most human myeloid and acute lymphoid leukemias. Recently, chromosomal translocation and genomic duplications of c-Myb have been identified in human T-cell acute leukemia. The present work focuses on the effects of mutations in different domains of the murine c-Myb protein on its transforming ability as defined by suppression of myelomonocytic differentiation and continued proliferation. Using both a novel myeloid cell line–based assay and a primary hematopoietic cell assay, we have shown that mutation of single residues in the transactivation domain important for CBP/p300 binding leads to complete loss of transforming ability. We also simultaneously mutated residues in the DNA-binding domain and the negative regulatory domain of the protein. These double mutants, but not the corresponding single mutants, show a complete loss of transforming activity. Surprisingly, these double mutants show severely impaired transactivation and are also defective for CBP/p300 binding. Our results imply that multiple Myb domains influence its interaction with CBP/p300, highlight the importance of this interaction for myeloid transformation, and suggest an approach for molecular targeting of Myb in leukemia. (Mol Cancer Res 2009;7(9):1477–86)

Introduction

The c-myb proto-oncogene is the cellular progenitor of the v-myb oncogene carried by the avian myeloblastosis virus (AMV) and E26 retroviruses, which cause acute myeloblastic leukemia or erythroblastosis in chickens (1, 2). c-myb and its product are key regulators of hematopoietic cell proliferation and differentiation. c-myb-null mice display abnormal fetal liver hematopoiesis and die by day 15 of gestation due to severe anemia (3). Moreover, ENU mutagenesis studies in mice revealed point mutations in myb that resulted in hypomorphic alleles and consequent hematopoietic irregularities (4, 5).

Activating lesions in c-myb, mutations, amplification, and truncations of its product, have been described in avian and murine leukemias and lymphomas (6, 7). Although isolated cases of amplification and truncation of MYB have also been described some time ago in human leukemias (8, 9), the use of newer, higher-resolution genomic technologies is suggesting that MYB contributes more frequently to these diseases. Thus, chromosomal translocation and genomic duplications of MYB have been identified in human T-cell acute leukemia (10, 11); more recently, a gain of the MYB locus has been reported in acute myelomonocytic leukemia (12).

Importantly, MYB is expressed at high levels in most human myeloid and acute lymphoid leukemias (13) and is essential for the proliferation of human myeloid leukemia cell lines and primary cell colony formation (14, 15). This widespread involvement of MYB in human leukemia warrants a detailed investigation of the leukemogenic activities of MYB.

The c-myb proto-oncogene encodes a sequence-specific DNA-binding transcription factor (c-Myb; refs. 16, 17). The c-Myb protein contains three functional domains: the DNA-binding domain (DBD), the transactivation domain (TAD), and the negative regulatory domain (NRD; ref. 18). The DBD and TAD are essential for c-Myb function and for transformation (18, 19); hence, it is likely that the protein carries out its normal and transforming functions by regulating the expression of other cellular genes (20). The NRD contains multiple subdomains, including a putative leucine zipper motif/heptad leucine repeat (HLR; refs. 16, 21). Although the mechanism of negative regulation by the NRD is not fully understood, it is likely to act through multiple mechanisms including interaction with other proteins (22-24), intramolecular interactions (25), and effects on protein stability (26).

The potent oncprotein v-Myb<sup>AMV</sup> is a NH<sub>2</sub>- and COOH-terminal truncation mutant of c-Myb. It also harbors 10 point mutations with respect to c-Myb in different domains of the protein (Fig. 1A); together, the truncations and point mutations contribute to its oncogenic activation in avian systems at least (27, 28). There are four DBD point mutations, three of which constitute what we have termed the 3M mutations. These residues and mutations thereof have been implicated in interactions
between Myb and other proteins (23, 29, 30), affect the ability of the protein to induce specific target genes (17, 30), and enhance its transforming activity (29).

Wild-type (WT) c-Myb is able to cause uncontrolled proliferation and impose a block of differentiation in murine hematopoietic cells, typical characteristics of leukemic transformation (31, 32). Truncation of either NH2 or COOH terminal of c-Myb can also lead to oncogenic activation (33, 34). Deletion of the NRD, or even point mutations in the HLR, increase both transactivating and transforming capacities of c-Myb, implying that the NRD is normally involved in repression of c-Myb activity (33, 35, 36). Paradoxically, however, v-MybAMV loses its transforming ability with mutation/truncation of the NRD (37); although the reason for this has been unclear, our present observations (see below) suggest an explanation.

CBP/p300 is a coactivator of c-Myb in an interaction essential for transactivation of at least some promoters (19, 38). Structural studies confirmed interaction between the TAD of c-Myb and the KIX domain of CBP (39), whereas disruption of this interaction by mutation perturbs hematopoiesis in vivo (5). Recent reports have also identified two novel coactivators, Mi-2α and FLASH, which interact with Myb through its DBD and NRD (40, 41). c-Myb also interacts with the corepressors mSin3a, c-Ski, N-CoR, and Tif-1β through the NRD and the R2 and R3 repeats of the DBD (Fig. 1A); correspondingly, mutation/truncation of either NRD or DBD leads to a decrease or loss of some of these corepressor interactions (23). The Myb-binding protein, Mybbp1α (24), also interacts with the HLR and has been reported to act as a coactivator of PGC-1α (42) and Prep1 (43) as well as a coactivator of the dioxin receptor (44).

Transformation-competent forms of Myb retain, and thus may require, at least one WT corepressor interacting domain (either the R2 and R3 repeats of the DBD or the HLR region). Thus, despite harboring mutations in its DBD, v-MybAMV has a high transforming potential possibly due to an intact HLR. Conversely, v-MybAMV loses its transforming ability with mutation/truncation of the NRD (37); although the reason for this has been unclear, our present observations (see below) suggest an explanation.

Results

Generation of Myb Mutants

PCR mutagenesis and domain swapping were used to generate a collection of Myb mutants, which are schematically illustrated in Fig. 1B. The L302A and M303V mutations have been reported previously to interfere with the binding of CBP/p300 to c-Myb (5, 38, 39). The c-Myb 3M mutant has three amino acid substitutions in the DBD (I91N, L106H, and V117D) and has been reported to decrease the interaction of c-Myb with the N-CoR, mSin3a, and c-Ski corepressor molecules (23). c-Myb L3,4P has two point mutations in the HLR, which enhance transactivation and transformation (36), and interferes with co-repressor binding (23). The “double-domain” mutants encompass both the 3M mutations and either a COOH-terminal truncation (c-Myb 3M.CT3) or the L3,4P mutations (c-Myb 3M.L3,4P). The PLT mutants (PLT3 and PLT4) contain substitutions in the DBD and HLR regions, respectively (4). The PLT3.CT3 mutant is a COOH-terminal truncation of PLT3 lacking the NRD.

A Novel Murine Hematopoietic Cell Line Transformation Assay

The FDB-1 murine hematopoietic cell line was used to assay the transforming abilities of the c-Myb mutants. This cell line is factor-dependent, proliferating in the presence of interleukin-3 (IL-3) and differentiating into granulocytes and macrophages in the presence of granulocyte macrophage colony-stimulating factor (GM-CSF) over 7 days (45). It has been reported previously that c-Myb can suppress GM-CSF–induced terminal differentiation of this cell line (46). To use these cells in a transformation assay, cells transduced with the Myb mutants were sorted for green fluorescent protein expression and subsequently used to assay proliferative and differentiation properties. Initial validation of this assay was carried out using WT c-Myb and c-Myb CT3, which conferred continued proliferation in the presence of GM-CSF and imposed a differentiation block. c-Myb CT3-expressing cells exhibited a higher proliferative capacity and a more complete block in differentiation compared with WT c-Myb-expressing cells. Representative data can be seen in Figs. 2 to 4, where WT c-Myb and c-Myb CT3 have been included as positive controls. We then compared the abilities of the mutants to induce proliferation and to block differentiation in cells cultured in GM-CSF. These two properties were quantitated to provide a measure of the transforming ability of the mutants, with differentiation assessed by morphologic classification and surface marker expression. Note that expression of Myb in all transduced FDB-1 cells was confirmed by Western blotting (Supplementary Fig. S2).

Transforming Activity Is Lost in TAD Mutants

As expected, the WT c-Myb-transduced and c-Myb CT3-transduced cells could proliferate continuously in the presence of GM-CSF. In contrast, FDB-1 cells transduced with c-Myb L302A, L302A.CT3, and M303V or, as expected, empty vector all showed only a small increase in number (Fig. 2A) following culture in presence of GM-CSF but then stopped proliferating and underwent differentiation.

To determine the effects of these mutants on the differentiation of FDB-1 cells, two approaches were used. Expression
of Gr-1 and Mac-1, two cell surface markers indicative of myeloid differentiation, were analyzed following culture of transduced cells in GM-CSF for 7 days. Samples were also analyzed by cyto centrifugation, staining with May-Grunwald-Giemsa, and morphologic assessment of differentiation state.
c-Myb CT3-transduced FDB-1 cells displayed a larger number of undifferentiated blast-like cells when compared with WT c-Myb-transduced cells, where cells at intermediate differentiation stages made up >60% of the cells. Cells transduced with the TAD mutants displayed a much higher percentage of differentiated cells compared with WT c-Myb-expressing cells (Fig. 2B).

In the proliferation and differentiation assay, cells transduced with empty vector were employed as negative controls. FDB-1 cells transduced with the TAD mutants also showed a larger increase in expression of the Gr-1 and Mac-1 differentiation markers, similar to that seen with empty vector–transduced cells, in the presence of GM-CSF when compared with positive controls (Myb and CT3Myb), again indicating that these mutants had reduced or no capacity to block differentiation (Supplementary Fig. S1).

DBD/NRD Double Mutants Lose Transforming Ability

As shown in Fig. 3A, the c-Myb 3M variant-transduced FDB-1 cells proliferated at the same rate as WT c-Myb-transduced cells. Not surprisingly in view of previous observations (36), c-Myb L3,4P induced proliferation at rates comparable with that of c-Myb CT3. In contrast, both c-Myb 3M.L3,4P and c-Myb 3M.CT3 double-domain mutants were unable to induce proliferation in the presence of GM-CSF. The c-Myb 3M mutant imposed a partial block in differentiation in GM-CSF, compared with that of WT c-Myb, whereas the c-Myb L3,4P mutant imposed a more complete block, similar to that of c-Myb CT3. Despite their lack of proliferative capacity, the c-Myb 3M.L3,4P and 3M.CT3 mutant cells exhibit a partial block in differentiation (Fig. 3B). These are concurrent with their Gr-1 and Mac-1 expression levels (Supplementary Fig. S1).

c-Myb PLT3CT3 Mutant Loses Ability to Transform

The c-Myb PLT3- and PLT4-transduced FDB-1 cells proliferated at the same rate as WT c-Myb-transduced cells (Fig. 4A). However, the c-Myb PLT3.CT3 double-domain mutant was unable to induce proliferation of FDB-1 cells in the presence of GM-CSF. Interestingly, FDB-1 cells transduced with either of the PLT mutants exhibited a more differentiated phenotype compared with WT c-Myb-transduced cells (Fig. 4B) and a concordant increase in expression levels of Gr-1 and Mac-1 markers (Supplementary Fig. S1).

Primary Cell Transformation by Myb Mutants

To confirm the results obtained from the FDB-1 cell line transformation assays, colony-forming assays were conducted using primary hematopoietic cells from murine fetal liver. This assay system has been used previously to identify and assess the transforming properties of WT, CT3, and L3,4P variants of c-Myb (31, 33, 36). As shown in Fig. 5, WT-Myb and 3M transduced cells formed an average of 20 colonies in methylene blue per 10,000 cells plated, whereas c-Myb CT3- and L3,4P-transduced cells formed almost four times as many, consistent with previous reports. In contrast, L302A, M303V, 3M, L3,4P, and 3M.CT3 completely failed to induce colony formation in this assay. These results confirm the transforming abilities, or lack thereof, of the mutants and are entirely consistent with the results from FDB-1 transformation assays.

TAD Mutants and NRD/DBD Double Mutants Lose Transactivation Ability

To examine the transactivation abilities of the mutants, luciferase assays using the pXPG-6MBS reporter construct (Fig. 6) were carried out. The M303V and L302A mutations have been shown to decrease or abolish the transactivation ability of the protein (5, 38). As expected, c-Myb CT3 and c-Myb L3,4P showed enhanced transactivation abilities compared with WT (36), and we confirmed that the L302A mutation abolishes transactivation by Myb (Fig. 6A). The c-Myb 3M, PLT3, and PLT4 mutants possessed comparable activities with that of the WT in contrast to lower activity from the PLT4 mutant reported by Carpinelli et al. (4). Surprisingly, the double-domain mutants (3M.CT3 and 3M.L3,4P) exhibited minimal transactivation ability, which was substantially less than that of the WT and CT3 Myb forms. A similar result was also obtained with the PLT3.CT3 mutant. These surprising observations—that double mutations outside the TAD abolished transactivation—led us to suspect that these mutations could be affecting interactions of Myb with transcriptional coactivators as well as corepressors.

The ability of a subset of the mutants to activate a natural Myb-responsive promoter, that of the myeloperoxidase (mpo) gene (47), was also tested. WT, 3M, and L3,4P could activate the mpo reporter construct pMPOHTKLUC, whereas transactivation by L302A, M303V, and 3M.L3,4P was severely impaired (Supplementary Fig. S3). Thus, both the TAD and double mutations had similar effects on the ability of Myb to activate a natural promoter to those seen using the pXPG-6MBS reporter.

Transformation- and Transactivation-Deficient Mutants Lose Interaction with CBP/p300

To explore these observations further, GST pull-down assays were carried out to examine the interaction of the various Myb mutants with the KIX domain of CBP. Initially, we observed that the L302A and M303V mutants did not interact with GST-CBP-KIX, which is consistent with that interaction being essential for Myb transformation. To our surprise, we observed that the 3M.L3,4P, 3M.CT3, and PLT3.CT3 double mutants also failed to interact detectably with GST-CBP-KIX, whereas all of the corresponding single-domain mutants did so (Fig. 7). Thus, combined mutation of the DBD and NRD seems to interfere with CBP/p300 binding. Note that this is a distinct effect from the previously reported interaction of CBP with the c-Myb NRD, which is mediated by the CH2 domain of CBP (48), not the KIX domain as seen here.

To confirm that these results also applied to the interaction of full-length CBP/p300 in vivo, coimmunoprecipitation assays with epitope-tagged Myb mutants and p300 in transiently transfected 293T cells were also carried out (Fig. 8). These showed that, as expected, the L302A and M303V mutants do not interact with p300 in vivo. The assays also confirmed the lack of interaction between double-domain mutants and p300 in vivo, in agreement with the results from the GST pull-down assays.
Discussion

Two critical events in leukemogenesis are the acquisition of the ability to proliferate independently of extrinsic growth factors and the disruption of normal differentiation commitment (49). Myb acts at this second level by suppressing the differentiation process. The ability of FDB-1 cells to proliferate in the presence of IL-3, and differentiate in the presence of GM-CSF, enabled us to isolate pure populations of transduced cells expressing the various Myb mutants. These populations were then subsequently assayed for both proliferative and differentiation properties in the presence of GM-CSF. The transforming abilities of WT c-Myb, c-Myb CT3, and c-Myb L3,4P in the FDB-1 transformation assay are concordant with the primary cell transformation data reported previously (36). This shows the suitability of the FDB-1 cell line for this study and establishes it as an appropriate and practical mouse in vitro myeloid transformation assay for future studies.

The c-Myb transcription factor interacts with a large cohort of coactivator and corepressor molecules (illustrated in Fig. 1A). We initially set out, using a series of c-Myb mutants, to investigate which of these interactions would be essential for its transforming potential and, in particular, to define the effects of mutating regions known to bind corepressors as well as coactivators. The L302A mutation has been reported to completely abrogate interaction of c-Myb with the coactivator CBP (39); not surprisingly, our coimmunoprecipitation assays show that the same is true for interaction with the highly homologous p300 protein. This mutant and

FIGURE 2. Transforming properties of the TAD mutants. Proliferation and differentiation of FDB-1 cells transduced with TAD mutants. A. Proliferation in medium containing GM-CSF. For each experiment, 20,000 cells were seeded at day 0; cell counts over a period of 11 d are shown. Bars, SD (n = 3). Experiments were carried out in triplicate and repeated thrice with consistent results. *, P < 0.01. B. Morphologies of cells transduced with empty vector, c-myb CT3, L302A, L302ACT3, or M303V in IL-3 and/or GM-CSF on day 7 showing cells that exhibit an undifferentiated, blast-like morphology (B), “intermediate” cells exhibiting some but incomplete differentiation (I), and cells that have undergone complete monocytic or neutrophilic differentiation (D).
the truncated version, L302A.CT3, were devoid of transforming ability, indicating that interaction of Myb with CBP/p300 is essential for transformation, which is consistent with the requirement for a functional TAD for transformation (18, 19). The M303V mutation has also been reported to alter the ability of c-Myb to recruit the highly homologous p300 coactivator, resulting in decreased transactivation (5). Loss of transforming activity of these mutants is similarly accompanied by severely impaired transactivation abilities as shown by our data and previous reports (5, 38). However, in our system, we observed a complete, rather than partial, loss of transactivation ability by the M303V mutant. This may reflect differences in the promoters used or that Sandberg et al. used Gal4 DBD-fused c-Myb derivatives in their reporter assays. Nevertheless, the viability of c-mybM303V homozygous mice, in contrast to the embryonic lethality of c-myb-null mice (5), does imply that the M303V protein does retain some biological activity.

A c-Myb variant harboring DBD mutations (3M) showed similar transactivating and transforming abilities to WT c-Myb. The 3M mutant contains three point mutations that correspond to those found in the DBD of v-Myb\textsuperscript{AMV}. These mutations have been reported to be activating in v-Myb\textsuperscript{AMV} (29), to prevent interaction of c-Myb with C/EBP\textbeta (50, 51) and histone H3 tails (52), and to interfere with interaction of the DBD of c-Myb with the N-CoR, mSin3a, and c-Ski corepressors (23). However, the 3M mutations did not increase the transactivating or transforming ability of c-Myb in our studies. This could reflect differences

FIGURE 3. Transforming properties of the DBD and NRD mutants. Proliferation and differentiation of FDB-1 cells transduced with DBD/NRD mutants. A. Proliferation in medium containing GM-CSF. For each experiment, 20,000 cells were seeded at day 0; cell counts over a period of 8 d are shown. Bars, SD. Experiments were carried out in triplicate and repeated thrice with consistent results. *, P < 0.01. B. Morphologies of cells transduced with empty vector, WT c-myb, 3M, L3.4P, or 3ML3.4P in IL-3 and/or GM-CSF on day 7 showing cells that exhibit a blast-like morphology (B), intermediate cells (I), and cells that have undergone differentiation (D).

FIGURE 4. Transforming properties of the PLT mutants. Proliferation and differentiation of FDB-1 cells transduced with PLT mutants. A. Proliferation in medium containing GM-CSF. For each experiment, 20,000 cells were seeded at day 0; cell counts over a period of 8 d are shown. Bars, SD. B. Morphologies of cells transduced with empty vector, WT c-myb, PLT3, PLT4, or PLT3.CT3 in IL-3 and/or GM-CSF on day 7 showing cells that exhibit a blast-like morphology (B), intermediate cells (I), and cells that have undergone differentiation (D).
between species (mouse compared to chicken) and/or differences in the transformation assays used in the two systems. Alternatively, the fact that c-Myb has a complete NRD whereas v-Myb AMV has a truncated NRD might affect the degree of negative regulation, preventing activation of the c-Myb protein by the 3M mutations.

The L3,4P mutant showed high transactivation ability in our assays and confirms previous conclusions (36). The transactivation abilities of c-Myb 3M, c-Myb CT3, and c-Myb L3,4P proteins also parallel their transforming properties, once again suggesting a link between the two.

The c-myb plt3 and plt4 hypomorphical alleles that exhibit abnormalities in hematopoiesis harbor mutations in domains important for corepressor binding by the c-Myb protein (DBD and NRD). Although the FDB-1 transformation capacities of the single PLT mutants did not differ significantly from that of the WT, the resultant cells did exhibit a more differentiated phenotype. This may reflect a subtle loss of activity and thus be consistent with the phenotype of the hypomorphic mice.

Surprisingly, all the double-domain mutants (3M, L3,4P, 3M, CT3, and PLT3,CT3), with mutations in both DBD and NRD, completely lost transactivation ability. Our interaction assays show that these double mutants are severely impaired in their ability to interact with CBP/p300. Thus, the simplest explanation for all of our transactivation and transformation data is that interaction with CBP/p300 is central to the ability of c-Myb to transactivate reporter constructs and transform myeloid cells. It is conceivable though that interactions with corepressors and/or other coactivators (see Fig. 1) may be affected by some of the mutations tested here and thus that these also play a role in transformation.

The mechanism by which simultaneous mutation of the DBD and NRD disrupts CBP/p300 binding is not currently clear. Our data suggest that both regions share a redundant function that affects CBP/p300 binding, because the single mutants exhibited WT binding in vitro and in vivo. Note that it is conceivable that some of the single PLT3, PLT4, and 3M mutants have a subtle defect in interaction with CBP/p300 that is not readily detectable in our transactivation or transformation assays. This would be consistent with the recent finding that a mutation in p300 phenocopies the plt3 and plt4 alleles (53). However, Mo et al. (52) have shown that the 3M mutant interacts normally with p300 on at least some endogenous target gene promoters, although it fails to acetylate histone H3 tails and activate transcription from those particular endogenous genes.

One possibility is that these double mutations disrupt the structure or conformation of the protein by preventing interactions with as yet undefined partners or scaffolding proteins. For example, the c-Myb minimal DBD (amino acids 72-192) has been implicated in interaction with Cyp40, which, as a peptidyl-prolyl isomerase, can alter the conformation of substrate proteins (54). The interacting region includes both of the DBD mutations employed in this study (3M and PLT3); importantly, it has been shown that mutations in the DBD encompassing the 3M mutations disrupt the interaction with Cyp40 (54). Recently, Pin1, another peptidyl-prolyl isomerase, has also been reported to interact with NRD of c-Myb in a phosphorylation-dependent manner (55). Disruption of interaction with one peptidyl-prolyl isomerase may not be deleterious, but disruption of interaction with both may be, if they each effect a similar conformational change in Myb, which in turn affects CBP/p300 binding. Such explanations would only be viable if the relevant proteins were also present in the in vitro translation system, that is, in reticulocyte lysates. Alternatively, the double mutations may be affecting intramolecular interactions that stabilize the CBP-binding KIX domain, again in a redundant manner. Further insight into these mechanisms will await three-dimensional structural studies on the entire Myb protein.

In conclusion, our studies point to an essential role for CBP/p300 in Myb-induced transformation. They also reinforce a
strong link between transactivation and transformation (seen in other reports; ref. 35), implying that Myb is likely to control proliferation and differentiation processes through positive regulation of target genes that require coactivation by CBP/p300. This in turn highlights the need for a thorough study of Myb target genes. Finally, as MYB is essential for the proliferation of human myeloid leukemia cell lines (14, 15), it represents a potential therapeutic target in human leukemia and possibly other cancers that are dependent on MYB (56). Our study suggests the possibility of disrupting the interaction between c-Myb and CBP/p300 as an approach to targeting c-Myb. This may be achievable using small molecules or peptides, because disruption of protein-protein interactions with such agents is now considered a feasible strategy for drug development (57, 58).

Materials and Methods

Plasmid Construction and Mutagenesis

c-Myb was obtained from the plasmid pACT-c-myb. c-myb CT3 was obtained by PCR amplification of the first 1,012 bp of the insert from pACT-c-myb. The pACT-c-myb 3M mutant was described previously (59); c-myb L3,4P was obtained from the pACT-c-myb L3,4P plasmid that was generated previously (36). The base changes for the L302A, L302A.CT3, and M303V mutants were generated by PCR-based mutagenesis. The c-myb 3M.L3,4P and c-myb 3M.CT3 constructs were obtained by swapping of domains of the c-myb 3M, c-myb L3,4P, and c-myb CT3 plasmids by restriction digestion and ligation. The PLT3 and PLT4 mutants were a kind gift from Dr. Doug Hilton. The PLT3.CT3 mutant was generated by PCR amplification of the first 1,012 bp of the PLT clone. All generated constructs were subcloned into the pMys-IRES-eGFP retroviral vector (60) with the addition of a HA tag in the COOH terminus. All constructs were verified by sequencing.

Culture of FDB-1 Cells

Retrovirus Production and FDB-1 Infection. HEK 293T cells (2 × 10^6) were cultured in DMEM containing 10% FCS, 2 mmol/L L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin at 37°C in 25 cm² flasks. These cells were transfected with 8 μg total DNA containing 4 μg plasmid of interest (Myb constructs) and 4 μg pEQECO, a murine ecotropic packaging plasmid (61). Lipofectamine 2000 (20 μL; Invitrogen) was added to the transfection cocktail. This mix was incubated with the cells for 8 to 16 h after which medium was changed to antibiotic-free Iscove’s modified Dulbecco’s medium supplemented with 10% FCS and 2 mmol/L L-glutamine. Supernatants containing retrovirus were collected 48 h post-medium change. FDB-1 cells (3 × 10⁵) were transduced with 1 mL virus-containing supernatant for 6 to 16 h. Cells were sorted for green fluorescent protein expression 48 h post-infection using a Mo-Flo Flow cytometer (Becton Dickinson).

FDB-1 Proliferation Assays. FDB-1 cells were cultured in Iscove’s modified Dulbecco’s medium supplemented with 10% FCS and 20 to 40 FDU/mL IL-3. Cells were induced to differentiate when cultured in presence of 50 FDU/mL GM-CSF. Proliferation assays with FDB-1 cells in IL-3 and GM-CSF were carried out with an initial 20,000 cells in each well of a 24-well plate. Cell counts were done on alternate days over a period of 8 to 12 days using a Coulter counter (Beckman Coulter). Assays were done in triplicate and were repeated three times.
**FDB-1 Differentiation Assays.** FDB-1 cells were induced to differentiate over a period of 7 days in GM-CSF. To measure levels of expression of Gr-1 and Mac-1, 1 × 10^6 cells were stained with Gr-1 APC and Mac-1 PE antibodies (BioLegend), washed, and fixed in 10% formalin (Sigma-Aldrich). Stained cells were analyzed on a FACSCalibur flow cytometer (Becton Dickinson). Cytocentrifugation was carried out with 50,000 cells in 200 μL IScove’s modified Dulbecco’s medium containing 20% FCS on a cytocentrifuge (Shandon Southern) at 800 rpm for 3 min. Slides were air-dried, fixed with methanol, and stained with May-Grunwald-Giemsa. Approximately 500 cells per cytospin were scored based on their morphologic characteristics. Counts from at least three different cytospins were averaged.

**Primary Cell Transformation Assays.** Hemopoietic cells (3 × 10^7-5 × 10^7) isolated from the livers of day 14 CBA mouse fetuses were transduced with retroviral supernatants collected from HEK 293T transfections in IScove’s modified Dulbecco’s medium containing 20% FCS on a cytocentrifuge (Shandon Southern) at 800 rpm for 3 min. Slides were air-dried, fixed with methanol, and stained with May-Grunwald-Giemsa. Approximately 500 cells per cytospin were scored based on their morphologic characteristics. Counts from at least three different cytospins were averaged.

**Analysis of Proteins by Western Blotting.** Cells were lysed in 2% SDS-PAGE loading buffer containing a protease inhibitor cocktail tablet (Roche) on ice. Proteins were electrophoresed on 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Millipore). Membranes were incubated with a rat monoclonal anti-HA primary antibody clone 3F10 (Roche). This was followed by washing and incubation with a rabbit anti-rat horseradish peroxidase–conjugated secondary antibody (Sigma-Aldrich) and was visualized by treatment with chemiluminescence reagents (Pierce).

**Luciferase Reporter Assays.** The 6MBS reporter construct contained the SV40 early promoter and six tandem repeats of the Myb-binding site linked to the luciferase gene (48). The pMPOHTKLUC reporter was a kind gift from Dr. Alan Friedman (Johns Hopkins University; ref. 47). HEK 293T cells were cotransfected with a mixture of the 6MBS-Luc reporter (100 ng) and pmo-Luc reporter (100 ng), Renilla luciferase construct (10 ng), and the c-Myb expression plasmid pcDNA3-c-Myb (50-150 ng) or one of the Myb mutants cloned into pcDNA3. The amount of total DNA was adjusted to 250 ng with the empty vector.

Forty-eight hours after transfection, cells were lysed in 100 μL Passive Lysis Buffer (Promega). Luciferase activity was measured using a luminometer (Berthold) and normalized for transfection efficiency using the Renilla luciferase internal control. Each experiment was done in triplicate and repeated with consistency.

**Protein Interaction Assays**

**GST pull-down assays** were done with in vitro translated Myb and Myb mutants present in pcDNA3.1 vectors using the TnT Quick-Coupled transcription/translation kit (Promega). BL21 cells transformed with GST-CBP-KIX were grown up to an absorbance of 0.5 and induced with 1 mmol/L isopropyl-L-thio-β-D-galactopyranoside for 3 to 4 h at 37°C. Preparation of bacterial lysates was carried out as described previously (62). Samples of bacterial lysate were rocked with 100 μL glutathione-agarose beads (Sigma Aldrich) for 3 h at 4°C. Beads were washed five times with 1 mL PBS, once with 1 mL of 0.8 mol/L NaCl, and once with 1 mL binding buffer (19). In vitro translated protein (50-75 μL) was mixed with 500 μL binding buffer and GST or GST-CBP-KIX affinity resin. After rocking overnight at 4°C, resin was washed five times in 1 mL binding buffer and mixed with SDS sample buffer. Proteins were analyzed by SDS-PAGE followed by autoradiography.
Coimmunoprecipitation experiments were carried out by transiently transfecting 293T cells with 4 μg HA-tagged Myb constructs and 4 μg FLAG-tagged p300. Human p300 cDNA was a gift to Dennis Dowhan from David Livingston. The pSG5-KM1F2-hp300 clone has the last 36 amino acids of p300 removed to facilitate cloning and incorporation DNA sequence to produce 2xFLAG epitopes followed by a stop codon. Exact cloning details are available upon request. Forty-eight hours post-transfection, protein lysates were made and immunoprecipitations were done as reported previously (24). FLAG-p300 was precipitated using 1 μg anti-FLAG M2 monoclonal antibody (Sigma Aldrich) per reaction. After SDS-PAGE, membranes were probed with a monoclonal anti-HA antibody clone 3F10 (Roche).

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Mutations in Multiple Domains of c-Myb Disrupt Interaction with CBP/p300 and Abrogate Myeloid Transforming Ability

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