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Enhanced Proliferative Potential of Hematopoietic Cells Expressing Degradation-resistant c-Myb Mutants*[S]

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Francesca Corradini‡§¶, Vincenzo Cesi‡¶, Viviana Bartella‡¶, Elisabetta Pani§¶‖, Rita Bussolari‡§¶, Olivia Candini‡§, and Bruno Calabretta‡**

From the ‡Department of Microbiology and Immunology, Kimmel Cancer Center, Thomas Jefferson Medical College, Philadelphia, Pennsylvania 19107 and the §Department of Medical Sciences, University of Modena and Reggio Emilia, 41100 Modena, Italy

The c-*myb* gene encodes a transcription factor required for proliferation, differentiation, and survival of hematopoietic cells. Expression of c-Myb is often increased in hematological malignancies, but the underlying mechanisms are poorly understood. We show here that c-Myb has a longer half-life (at least 2-fold) in BCR/ABL-expressing than in normal hematopoietic cells. Such enhanced stability was dependent on a phosphatidylinositol 3-kinase (PI-3K)/Akt/GSKIIIβ pathway(s) as indicated by the suppression of c-Myb expression upon treatment with PI-3K inhibitors or co-expression with dominant negative Akt or constitutively active GSKIIIβ. Moreover, inhibition of GSKIIIβ by LiCl enhanced c-Myb expression in parental 32Dcl3 cells. Compared with wild type c-Myb, three mutants (Δ(358–452), Δ(389–418), and L389A/L396A c-Myb) of the leucine zipper domain had increased stability. However, only expression of Δ(358–452) was not affected by inhibition of the PI-3K/Akt pathway and was not enhanced by a proteasome inhibitor, suggesting that leucine zipper-dependent and -independent mechanisms are involved in the regulation of c-Myb stability. Indeed, Δ(389–418) carrying four lysine-to-alanine substitutions (Δ(389–418) K387A/K428A/K442A/K445A) was as stable as Δ(358–452) c-Myb. Compared with full-length c-Myb, constitutive expression of Δ(358–452) and Δ(389–418) c-Myb in Lin-Sca-1$^+$ mouse marrow cells increased cytokine-dependent primary and secondary colony formation. In K562 cells, expression of Δ(358–452), Δ(389–418), and L389A/L396A c-Myb led to enhanced proliferation after STI571 treatment. Thus, enhanced stability of c-Myb by activation of PI-3K-dependent pathway(s) might contribute to the higher proliferative potential of BCR/ABL-expressing and, perhaps, other leukemic cells.

c-Myb, a nuclear phosphoprotein that functions as a sequence-specific transcription factor, is predominantly expressed by primitive hematopoietic progenitor cells and by colorectal and neuroectoderm tumor cells (1, 2). In normal hematopoietic cells, there is compelling evidence in support of an important function of c-Myb, since ablation of c-Myb expression and/or activity by gene knockout or by antisense/dominant negative strategies has demonstrated that it is essential for fetal liver hematopoiesis, erythroid and myeloid bone marrow colony formation, and T-cell development (3–5).

The function of c-Myb in myeloid progenitor cells may depend on its ability to modulate the expression of genes (*i.e. CD34*, c-*kit*, and *flt-3*) required for their proliferation and survival (6–8). A target of c-Myb is also Bcl-2 (9–11), whose expression is important for maintenance of the early progenitor cell pool (12). c-Myb expression is elevated in many cases of acute myeloid and lymphoid leukemia (13–15), but the mechanisms underlying such an increase are unclear. Gene amplification appears to be a rare event (16), and reports of gene truncation are also infrequent (17). Mutations are also uncommon (18), suggesting that this mechanism is not involved in c-Myb overexpression. Knowledge of the mechanisms underlying the enhanced c-Myb expression in hematologic malignancies is important, because numerous studies suggest that the therapeutic potential of targeting c-Myb rests in its differential requirement by normal and leukemic cells (19–21). c-Myb has a short half-life of ~30 min and undergoes proteasome-dependent degradation (22, 23). Since the BCR/ABL oncoproteins of the Philadelphia chromosome have been shown to regulate protein levels by enhancing or suppressing proteasome-dependent degradation (24, 25), we hypothesized that c-Myb levels might be regulated by BCR/ABL-dependent pathways. This would also be consistent with the requirement of c-Myb for the proliferation and survival of CML progenitor cells (20, 21).

In this study, we show that BCR/ABL enhances the stability of c-Myb via PI-3K/Akt/GSKIIIβ–dependent pathway(s). In normal hematopoietic cells, c-Myb mutants without an intact C-terminal leucine zipper and lacking (or carrying a mutation of) four adjacent lysine residues potentially involved in ubiquitination were more stable than wild-type c-Myb, were less down-modulated by suppression of PI-3K[1] activity, and caused markedly increased primary and secondary colony formation.

Expression of the more stable c-Myb mutants led to enhanced proliferation of K562 cells resistant to STI571-induced apoptosis. Together, these data suggest that the enhanced stability of c-Myb may contribute to the increased proliferative potential of BCR/ABL-expressing and, perhaps, of other leukemic cells.

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¶ These authors contributed equally to this work.

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** To whom correspondence should be addressed: Kimmel Cancer Center, Thomas Jefferson University, BLSB, Rm. 630, 233 S. 10th St., Philadelphia, PA 19107. Tel.: 215-503-4522; Fax: 215-923-0249; E-mail: bruno.calabretta@mail.tju.edu.

[1] The abbreviations used are: PI-3K, phosphatidylinositol 3-kinase; IL, interleukin; GFP, green fluorescent protein; KL, Kit ligand; HA, hemagglutinin.

MATERIALS AND METHODS

Plasmids

MigRI Wild Type c-Myb-HA, MigRI Δ(358–452) c-Myb-HA, MigRI Δ(389–418) c-Myb-HA, MigRI Δ(389–418) K442A/K445A c-Myb-HA, MigRI Δ(389–418) K387A/K428A/K442A/K445A c-Myb-HA, and MigRI L389A/L396A c-Myb-HA—MigRI wild type c-Myb was obtained by ligation of the KpnI-XbaI Klenow-filled c-*myb* cDNA from pCMV c-myb-HA into the HpaI-linearized MigRI vector (kind gift of Dr. W. S. Pear, University of Pennsylvania, Philadelphia, PA).

MigRI Δ(358–452) c-myb-HA was cloned by PCR amplification of pCMV c-myb-HA using primers designed to generate 5′ and 3′ c-*myb* fragments lacking the nucleotides corresponding to amino acid 358–452 of human c-Myb. The 5′ and the 3′ primer of the 5′ c-*myb* fragment were as follows: 5′-CCGGTACCATGGCCCGAAG-3′, which includes the underlined KpnI site, and 5′-TGGCAGAGAGATGGAGTGGAGTG-3′, respectively. The 5′ and the 3′ primers of the 3′ c-*myb* fragment were as follows: 5′-GCTATCAAAAGGTCAATCTTAGAA-3′ and 5′-CCTCTAGATTATATCTCGACAGC-3′, which includes part of the HA tag, the stop codon, and the underlined XbaI site), respectively. The 5′ fragment and the 3′ fragment were digested with KpnI and XbaI, respectively, and phosphorylated. Then the fragments were ligated to KpnI-XbaI-digested pcDNA3. After KpnI-XbaI digestion, the released insert was blunt-ended by Klenow and cloned into the HpaI-digested MigRI vector.

MigRI Δ(389–418) c-Myb-HA was cloned by PCR amplification of pCMV c-Myb-HA as described above except that the 3′ primer of the 5′ c-Myb fragment was 5′-AGGTTCTTAACATTATCCAG-3′, whereas the 5′ primer of the 3′ c-Myb fragment was 5′-AACTTCCACCCCCCT-CATT-3′. After KpnI-XbaI digestion, the released insert was blunt-ended by Klenow and cloned into the HpaI-digested MigRI vector.

MigRI Δ(389–418) K442A/K445A c-myb-HA and MigRI Δ(389–418) K387A/K428A/K442A/K445A were generated by site-directed mutagenesis of MigRI Δ(389–418) c-myb-HA.

MigRI L389A/L396A c-Myb-HA was generated by mutating leucine 389 and leucine 396 to alanines by site-directed mutagenesis of MigRI wild type c-Myb-HA.

MigRI ΔR2-R3 c-myb-HA, MigRI ΔR2-R3/del (452–640) c-Myb-HA, and MigRI ΔR2-R3/del (358–640) c-myb-HA—The MigRI ΔR2-R3 c-*myb*-HA plasmid was obtained by PCR amplification from pCMV c-Myb-HA using primers designed to generate a 5′ c-*myb* fragment lacking amino acids 87–189 of c-Myb. The 5′ and the 3′ primers of the 5′ c-*myb* fragment were 5′-CCGGTACCATGGCCCGAAG-3′, which includes the underlined KpnI site, and 5′-GGTCCATGGCCCCTTGA-CAA-3′. This fragment was ligated to a 3′ segment of c-*myb* generated with the following primers: 5′ primer (5′-CGTCGGAAGGTCGAACAG-3′) beginning at nucleotide 565 immediately downstream of the DNA binding domain and a 3′ primer (5′-CCTCTAGATTATATCTCGA-CAGC-3′), which includes part of the HA tag, the stop codon, and the underlined XbaI site). The PCR products were digested with KpnI/XbaI into the pCMV vector. The released KpnI-XbaI c-*myb* was filled by Klenow and ligated into the dephosphorylated HpaI-digested MigRI vector.

The MigRI ΔR2-R3/del (358–640) c-myb-HA plasmid was obtained by PCR amplification from pCMV ΔR2-R3 c-myb-HA using the KpnI/c-Myb 5′ primer (see above) and a 3′ primer designed to terminate at amino acid 357 of c-Myb and including the HA tag, a stop codon, and the XbaI restriction site. The PCR fragment was KpnI-XbaI-digested and ligated to a KpnI-XbaI-digested pcDNA3. After KpnI-XbaI digestion, the c-*myb* fragment was blunt-ended by Klenow and subcloned into a dephosphorylated HpaI-digested MigRI. The orientation was determined by nucleotide sequence.

The MigRI ΔR2-R3/del (452–640) c-myb-HA plasmid was obtained by PCR amplification from pCMV ΔR2-R3 c-myb-HA using the KpnI/c-*myb* 5′ primer and a 3′ primer (5′-TGGGGTTCTAAAAACAGTATTTTCC-′), which ends in correspondence of amino acid 452 of c-*myb* and includes the HA tag, the stop codon, and the XbaI restriction site. The PCR product was KpnI-XbaI-digested and ligated into the KpnI-XbaI-digested pcDNA3 vector. After KpnI-XbaI digestion, the c-*myb* fragment was blunt-ended by Klenow and cloned into HpaI-linearized MigRI.

MigRI GSKIIIβ-S9A-HA—To generate this plasmid, pCR 3.1 S9A GSKIIIβ-HA (kind gift of Dr. P. Klein, University of Pennsylvania) was digested by the EcoRI restriction enzyme, and the released fragment was subcloned into a dephosphorylated EcoRI-digested MigRI. The orientation of mutant S9A GSKIIIβ-HA was established by sequence analysis.

pCMV-S9A GSKIIIβ—This plasmid was obtained by ligating the EcoRI fragment of pCR 3.1 S9A GSKIIIβ-HA at the EcoRI site of pcDNA3.

pCMV c-Myb-HA—This plasmid was obtained from pSV c-myb as described (26).

pCMV Akt K179M-HA—This plasmid was the kind gift of Dr. Philip Tsichlis (Tufts University, Boston, MA)

Cells

The murine IL-3-dependent 32Dcl3 myeloid precursor cell line (27) was maintained at 37 °C in Iscove's modified Dulbecco's medium, supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, penicillin/streptomycin (100 μg/ml each), and 10% WEHI-conditioned medium as a source of IL-3 (28). Clones of 32Dcl3 cells expressing $p210^{BCR/ABL}$ were generated in our laboratory and maintained as described (29). 32Dcl3 and 32D-BCR/ABL cells expressing HA-tagged wild-type or mutant c-Myb were established by retrovirus infection and isolation of GFP-positive cells by flow cytometry activated cell sorting.

CD34+ cells from leukophoresis of a CML-AP patient with roughly 20% double Philadelphia[1] chromosome metaphases or from cord blood mononuclear cells were purified using the CD34 Multisort Kit (Milteny Biotec, Auburn, CA). Cells were kept overnight in Iscove's modified Dulbecco's medium supplemented with 20% fetal bovine serum, 2 mM glutamine, and human recombinant IL-3 (20 ng/ml), IL-6 (20 ng/ml), Flt-3 ligand (100 ng/ml), and Kit ligand (KL) (100 ng/ml) (Stem Cell Technologies, Inc., Vancouver, Canada) prior to cycloheximide treatment.

Normal murine marrow cells were obtained from the femurs of C57BL/6 mice after hypotonic lysis and enriched for lineage-negative (Lin−) hematopoietic precursors using the standard StemSep protocol (Stem Cell Technologies, Vancouver, Canada). Lin-Sca-1^+ cells were obtained by flow cytometry-activated cell sorting using a phycoerythrin-conjugated anti-Sca-1 antibody (Pharmingen).

K562 cells, a human cell line derived from a patient with CML blast crisis (30), were maintained in culture in Iscove's modified Dulbecco's medium supplemented with 10% fetal bovine serum.

Transfection and Retroviral Infection

For transient transfection, 293T cells were grown for 16–18 h to 70% confluence and transfected with 30 μg of plasmid DNA by calcium phosphate precipitation using the ProFection System (Promega). The empty pCMV plasmid was used to normalize for equal amounts of transfected DNA.

32Dcl3, 32D-BCR/ABL, and K562 cells expressing HA-tagged wild-type and mutant c-Myb in the MigRI vector (31), a derivative of MSCV2.2 (32), were established by retroviral infection as described (33). Briefly, infectious supernatants from transiently transfected Phoenix cells (kind gift of Dr. G. P. Nolan, Washington University, St. Louis, MO) were collected 48 h after transfection and used to infect normal or BCR/ABL-expressing 32Dcl3 cells; 24 h later, infected cells were sorted for GFP positivity and cultured as described above.

Lin-Sca-1^+ mouse marrow cells were cultured 24–48 h in the presence of IL-3 (2 ng/ml), KL (10 ng/ml), and Flt-3 ligand (5 ng/ml) before infection with the control or the c-*myb* retrovirus as described (31) and flow cytometry isolation of GFP-positive cells.

Western Blot, c-Myb Half-life, and Ni^{2+}-Nitrilotriacetic Acid-mediated Nickel Chromatography

Western blots were performed using anti-c-Myb monoclonal antibody (clone 1-1; Upstate Biotechnology, Inc., Lake Placid, NY), anti-HA antibody (Bapco), and anti-HSP90 antibody.

Cells were harvested, washed twice with ice-cold phosphate-buffered saline, and lysed (10^7 cells/100 μl of lysis buffer) in HEPES buffer (10 mM HEPES (pH 7.5), 150 mM NaCl, 10% (v/v) glycerol, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 25 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 5 mM benzamidine, 1 mM Na_3Vo_4, 50 mM NaF, 10 mM β-glycerol phosphate) containing 1% (v/v) Nonidet P-40. Lysates were obtained and processed for Western blotting as described (34).

After densitometric scanning of the band corresponding to c-Myb, the half-life of wild-type and mutant c-Myb was calculated using the formula $t_{½} = (0.693xt)/\ln(Nt/No)$ as described (22).

Ni^{2+}-nitrilotriacetic acid-mediated nickel chromatography (Qiagen, Inc., Valencia, CA) was performed under denaturing conditions as suggested by the manufacturer.

Northern Blot Analysis

Total RNA was extracted using Tri-Reagent (Molecular Research Center, Inc.). For Northern blot analysis, RNA (15 μg) was fractionated

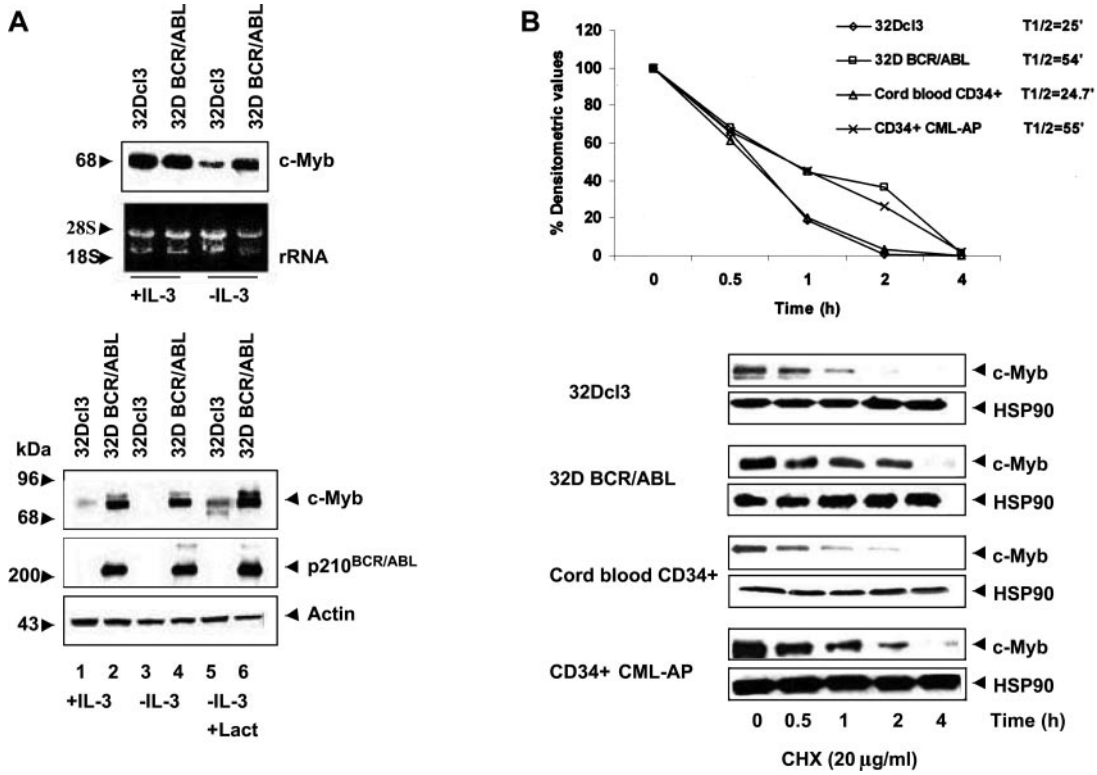

FIG. 1. **Expression and half-life of c-Myb in normal and BCR/ABL-expressing cells.** *A*, c-*myb* mRNA (*upper part*) and protein (*lower part*) levels in exponentially growing parental and BCR/ABL-expressing 32Dcl3 cells (*lanes 1* and *2*) and in IL-3-starved (8 h) cells, untreated (*lanes 3* and *4*) or treated with lactacystin (10 μM) (*lanes 5* and *6*). *B*, c-Myb half-life in cycloheximide-treated cells. Levels of HSP90 were measured as loading control. Results are representative of three different experiments.

onto denaturing 1% agarose, 6.6% formaldehyde gels, transferred to a nylon membrane (Amersham Biosciences), and hybridized to a radiolabeled c-*myb* cDNA fragment.

RESULTS

Enhanced Stability of c-Myb in BCR/ABL-expressing Cells—To assess whether the BCR/ABL oncogene has an effect on c-Myb expression, we compared mRNA and protein levels of c-Myb in parental myeloid precursor 32Dcl3 cells and in cells expressing $p210^{BCR/ABL}$ (Fig. 1). By Northern blot, c-*myb* levels were essentially similar in cells grown in the presence of IL-3; upon IL-3 deprivation (8 h), c-*myb* mRNA levels decreased in parental but not in BCR/ABL-expressing cells. Compared with BCR/ABL-expressing cells, levels of c-Myb protein were lower in parental cells grown in the presence of IL-3 and became essentially undetectable after an 8-h starvation (Fig. 1*A*). Expression of c-Myb was restored by treatment of IL-3-starved 32Dcl3 cells with the proteasome inhibitor lactacystin (Fig. 1*A*), suggesting that IL-3 starvation promotes the degradation of c-Myb primarily via proteasome-dependent mechanisms.

Together, these data suggest that the increased expression of c-Myb in 32D-BCR/ABL cells reflects, in part, enhanced stability. Thus, levels of c-Myb were assessed by Western blotting in cells treated with cycloheximide to block protein synthesis. In parental 32Dcl3 cells, c-Myb expression was rapidly down-modulated and was essentially undetectable after a 2-h treatment (Fig. 1*B*); levels of c-Myb were clearly more stable in 32D-BCR/ABL cells in which they were almost unchanged at 2 h and still detectable after a 4-h treatment (Fig. 1*B*). The half-life of c-Myb was also measured in CD34+ cells from a patient in CML-AP with a double Philadelphia chromosome in roughly 20% metaphases; the kinetics of c-Myb expression was more similar to that of BCR/ABL-expressing than parental 32Dcl3 cells (Fig. 1*B*). Instead, the half-life of c-Myb from cord blood CD34+ cells was similar to that of 32Dcl3 cells (Fig. 1*B*). By densitometry analysis, the half-life of c-Myb was ~25 min in normal cells and 55 min in BCR/ABL-expressing cells.

The Stability of c-Myb Is Regulated by a PI-3K-dependent Pathway—To investigate pathways potentially involved in the regulation of c-Myb stability, parental and BCR/ABL-expressing cells were treated with inhibitors of the PI-3K and mitogen-activated protein kinase pathway and assessed for c-Myb expression at different times after treatment. The PI-3K inhibitor LY294002 markedly suppressed c-Myb expression in parental and BCR/ABL-expressing 32Dcl3 cells (Fig. 2*A*), whereas the mitogen-activated protein kinase/extracellular signal-regulated kinase kinase inhibitor PD98059 (36) had no effect (Fig. 2*B*) at a concentration preventing extracellular signal-regulated kinase phosphorylation in IL-3-treated 32Dcl3 cells (Supplemental Fig. 1). The PI-3K inhibitor wortmannin also down-modulated c-Myb levels (not shown), confirming the involvement of a PI-3K-dependent pathway.

GSKIIIβ is negatively regulated by PI-3K via Akt, and phosphorylation of GSKIIIβ recognition motifs (*i.e.* the GSKIIIβ motif of β-catenin) may promote substrate recognition and proteasome-dependent degradation (37). Accordingly, we tested whether GSKIIIβ might be involved in the regulation of c-Myb stability by measuring c-Myb levels in parental and BCR/ABL-expressing 32Dcl3 cells treated with the GSKIIIβ inhibitor LiCl (38). Fig. 2*C* shows that c-Myb expression is up-modulated in LiCl-treated parental 32Dcl3 cells, whereas no effect was detected in BCR/ABL-expressing cells, probably reflecting suppression of GSKIIIβ activity induced by the BCR/ABL-dependent constitutive activation of PI-3K/Akt.

The involvement of the PI-3K/Akt/GSKIIIβ pathway in regulating c-Myb stability was also tested in cells ectopically expressing dominant negative Akt or constitutively active GSKIIIβ (39). 293T cells (which do not express endogenous c-Myb) were co-transfected with HA-tagged c-Myb and a dom-

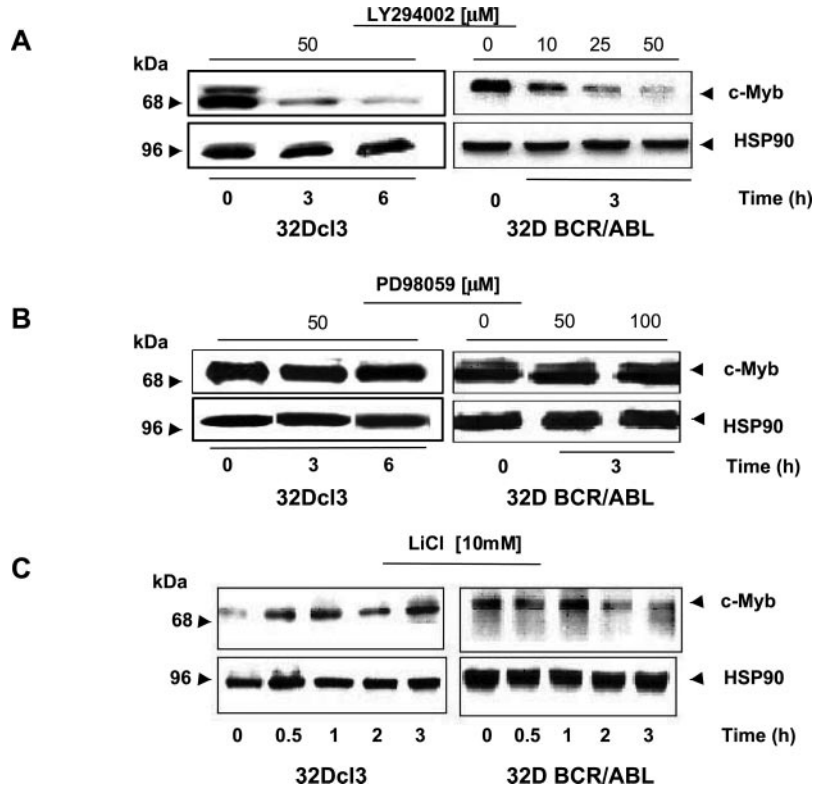

FIG. 2. **Effect of LY294002 (*A*), PD98059 (*B*), and LiCl (*C*) on c-Myb expression.** Western blot shows c-Myb levels in parental or BCR/ABL-expressing cells treated with PI-3K, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase, or GSKIIIβ inhibitors. As a control, phosphorylation of extracellular signal-regulated kinase was inhibited by PD098059 treatment of IL-3-stimulated 32Dcl3 cells (data not shown). Results are representative of three different experiments.

inant negative Akt plasmid (K179M) or a constitutively active (S9A) GSKIIIβ mutant (39). 36 h post-transfection, levels of c-Myb were measured in untreated cells and in cells treated with cycloheximide (3 h) to suppress protein synthesis. As indicated by the anti-c-Myb-HA Western blot (Fig. 3*A*), expression of dominant negative K179M Akt or the constitutively active S9A GSKIIIβ markedly down-modulated expression of HA-tagged c-Myb. As expected, the effect was even more apparent after cycloheximide treatment (Fig. 3*A*). The effect of constitutively active GSKIIIβ on endogenous c-Myb was assessed in GFP-sorted 32D-BCR/ABL cells transduced with the MigRI bicistronic retrovirus expressing S9A GSKIIIβ and GFP. Compared with cells transduced with the empty MigRI retrovirus, cells expressing GSKIIIβ S9A had markedly lower levels of endogenous c-Myb (Fig. 3*B*), consistent with the involvement of GSKIIIβ in regulating c-Myb stability.

The Leucine Zipper Domain and Lysines 387 and 428 Are Involved in c-Myb Degradation—To identify the region of c-Myb that may be required for protein degradation, we utilized two approaches: ubiquitination assays of c-Myb mutants in 293T cells and analysis of mutant c-Myb expression in retrovirus-transduced hematopoietic cells. The DNA binding domain of c-Myb is heavily ubiquitinated and appears to be a target of the p53-regulated SIAH-1 ubiquitin ligase (40). Thus, we generated c-Myb mutants lacking repeat 2 and repeat 3 of the DNA binding domain and with C-terminal truncation of increasing length (see Supplemental Fig. 1). In 293T cells, ΔR2-R3 c-Myb and ΔR2-R3/del (452–640) c-Myb were ubiquitinated, but ΔR2-R3/del (358–640) c-Myb was not (Fig. 4*A*). Thus, ΔR2-R3/del (452–640) and ΔR2-R3/del (358–640) c-Myb were cloned in the MigRI bicistronic retrovirus and transduced in 32Dcl3 cells. Levels of the encoded truncated proteins were investigated by anti-HA Western blotting in cycloheximide-treated GFP-sorted cells. Expression of ΔR2-R3/del (452–640) c-Myb was much more rapidly down-modulated than that of ΔR2-R3/del (358–640) c-Myb (Fig. 4*B*), suggesting that the segment of c-Myb corresponding to amino acids 358–452 is involved in c-Myb turnover in normal 32Dcl3 cells. Thus, an internally deleted c-Myb cDNA lacking the nucleotides corresponding to amino acids 358–452 (Δ(358–452) c-Myb-HA) (see Supplemental Fig. 1) was cloned in the MigRI retrovirus and transduced in parental and BCR/ABL-expressing 32Dcl3 cells. GFP-positive cells were sorted and assessed for c-Myb-HA expression at different times after cycloheximide treatment. In 32Dcl3 cells, expression of wild-type c-Myb decreased early after cycloheximide treatment and was essentially undetectable at 1 h, whereas that of Δ(358–452) c-Myb-HA was readily detectable (only a 2–3-fold decrease at 1 and 2 h) (Fig. 4*C*), indicating that the leucine zipper/negative regulation domain of c-Myb is involved in regulation of its stability.

Since amino acids 358–452 of c-Myb do not correspond only to the leucine zipper, two other mutants (Δ(389–418) c-Myb and L389A/L396A c-Myb) (see Supplemental Fig. 1) were generated to test more directly the involvement of this domain. The kinetics of expression of these two mutants in cycloheximide-treated 32Dcl3 cells was more similar to that of Δ(358–452) than wild-type c-Myb. However, expression of these two mutants was barely detectable at 2 h, a time point at which Δ(358–452) c-Myb was decreased only 3-fold compared with the untreated cells. Together, these data indicate that the leucine zipper of c-Myb is involved in protein degradation but suggest that other amino acids in the 358–452 segment play some role in further destabilizing c-Myb. Consistent with this interpretation, treatment with the proteasome inhibitor MG-132 enhanced HA c-Myb levels in 32Dcl3 cells expressing the full-length c-*myb* (~13-fold) or the Δ(389–418) and L389A/L396A c-*myb* mutants (3–5-fold) but had virtually no effect (1.5-fold increase) in cells expressing the Δ(389–452) c-*myb* (Fig. 4*D*). Thus, two additional mutants of Δ(389–418) in which 2 or 4 lysine residues were replaced by alanine (Δ(389–418) K442A/K445A and Δ(389–418) K387A/K428A/K442A/K445A) were generated and retrovirally transduced in 32Dcl3 cells. The stability of Δ(389–418) K387A/K428A/K422A/K445A c-Myb was essentially identical to that of Δ(358–452) c-Myb (Fig.

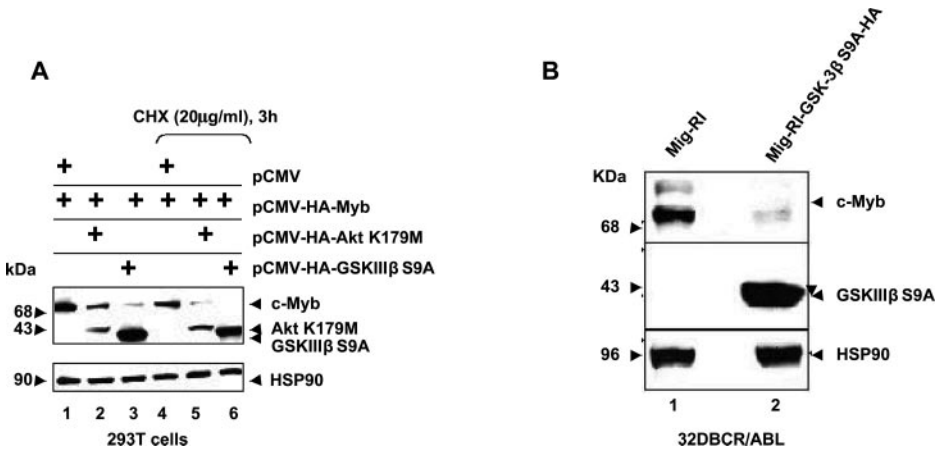

FIG. 3. **Role of Akt and GSKIIIβ in c-Myb stability.** *A*, Western blot shows c-Myb levels in 293T cells co-transfected with HA-tagged c-Myb and dominant negative Akt or constitutively active GSKIIIβ, untreated or treated with cycloheximide. Ectopically expressed proteins were detected by anti-HA Western blotting. HSP90 levels were measured as control of equal loading. *B*, Western blot shows levels of endogenous c-Myb in GFP-sorted 32D-BCR/ABL cells after infection with the empty retrovirus (MIG-RI) or a constitutively active GSKIIIβ (Mig-RI-GSK-3β S9A-HA). HSP90 levels were measured as control of equal loading. Results are representative of three independent experiments.

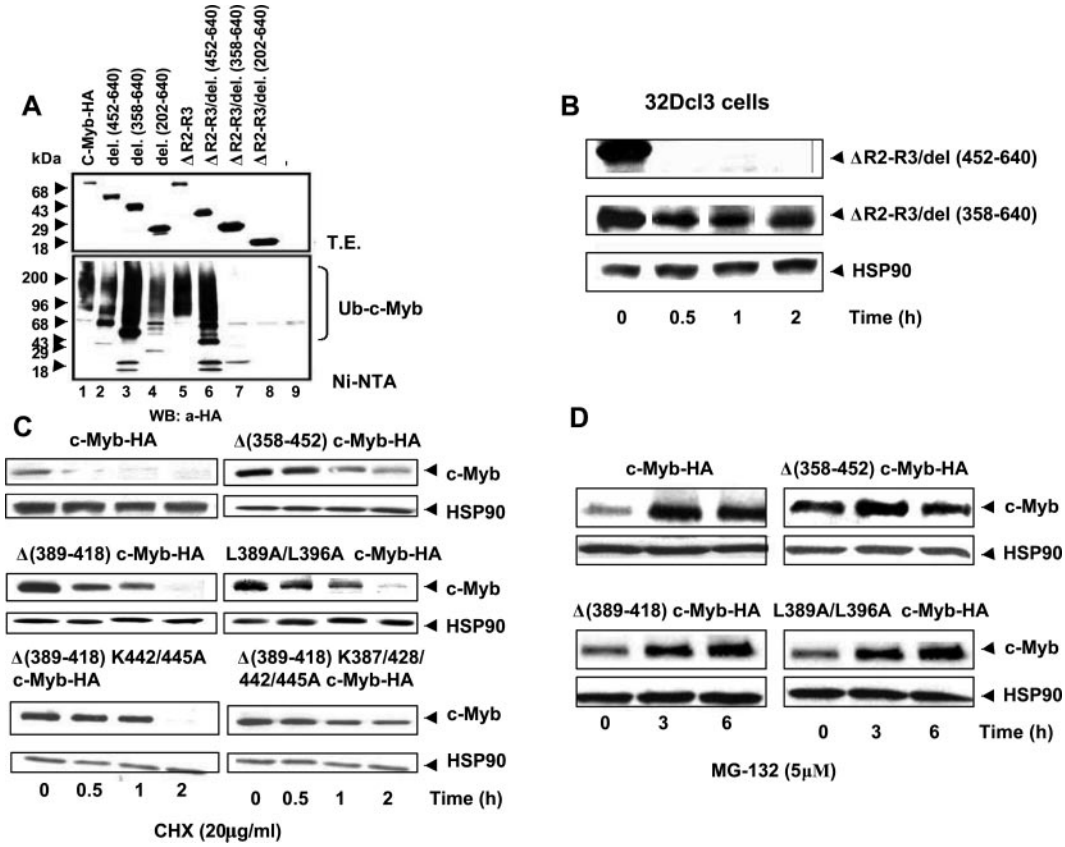

FIG. 4. **Polyubiquitination and stability of mutant c-Myb.** *A*, ubiquitination of wild-type and mutant HA-tagged c-Myb in 293T cells co-transfected with His_6-ubiquitin. The Western blot shows HA-tagged c-Myb in total extracts (*upper panel*) or in Ni^{2+}-nitrilotriacetic acid-purified His-tagged proteins (*lower panel*). *B*, expression of HA-tagged internally deleted and C terminus-truncated c-Myb in cycloheximide-treated 32Dcl3 cells. *C*, expression of HA-tagged wild-type and mutant c-Myb in cycloheximide-treated 32Dcl3 cells. *D*, expression of HA-tagged c-Myb in MG-132-treated 32Dcl3 cells. 32Dcl3 cells were retrovirally transduced with the corresponding wild-type or mutant *c-myb* plasmid.

4*C*), suggesting that the leucine zipper and additional lysine residues in the amino acid region 358–452 are both required for c-Myb degradation. As expected, the kinetics of wild-type and Δ(358–452) c-Myb expression was essentially undistinguishable in cycloheximide-treated 32D-BCR/ABL cells (not shown).

Mechanisms of Enhanced Stability of Δ(358–482) c-Myb—To investigate the mechanism(s) underlying the enhanced stability of Δ(358–452) c-Myb, expression of HA-tagged wild-type and mutant c-Myb was assessed in 32Dcl3 and K562 cells before and after treatment with the PI-3K inhibitor LY294002. In 32Dcl3 cells, a 6-h treatment with LY294002 induced approximately a 10-fold decrease in the levels of HA-tagged wild-type c-Myb (Fig. 5*A*); by contrast, the effect of LY294002 on mutant c-Myb was less evident, with the levels of Δ(358–452) c-Myb being reduced less than 2-fold and those of Δ(389–418) and L389A/L396A c-Myb being reduced ~4-fold (Fig. 5*A*). Treatment of HA-c-Myb-expressing K562 cells with LY294002 also led to down-modulation of the full-length, but not the Δ(358–452), c-Myb (Fig. 5*B*); the effect was specific because expression of wild-type or Δ(358–452) c-Myb was not affected by treatment with the mitogen-activated protein kinase inhibitor PD98059 (not shown). Down-modulation of wild-type c-Myb in LY294002-treated cells was reversed by incubation with the proteasome inhibitor MG-132 (data not shown), consistent with the possibility that PI-3K-dependent protein-protein interaction(s) at amino acids 358–452 is involved in proteasome-dependent degradation of c-Myb.

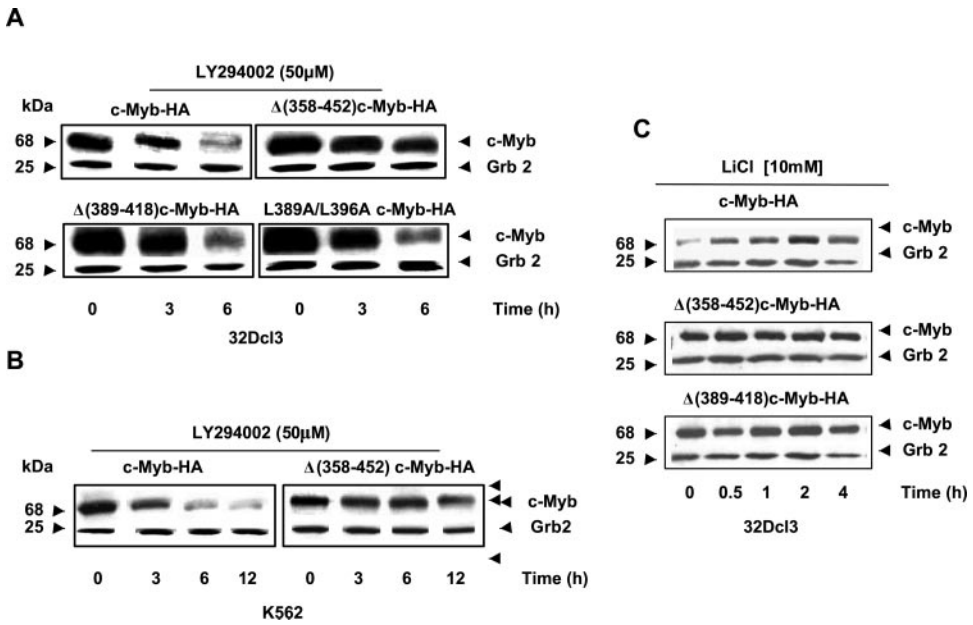

FIG. 5. **Expression of wild-type and mutant c-Myb in retrovirally transduced cells treated with the PI-3K inhibitor LY294002 and the GSKIIIβ inhibitor LiCl.** *A*, Western blot shows levels of HA-tagged c-Myb (wild-type and mutant) in 32Dcl3 cells treated with LY294002; *B*, Western blot shows levels of HA-tagged c-Myb (wild-type and Δ(358–452)) in K562 cells treated with LY294002. *C*, Western blot shows levels of HA-tagged c-Myb (wild-type and mutant) in LiCl-treated 32Dcl3 cells. Grb2 levels were measured as loading control.

We further investigated the role of the PI-3K/Akt/GSKIIIβ pathway in c-Myb stability by assessing whether inhibition of GSKIIIβ by LiCl would induce an increase in the expression of the leucine zipper c-Myb mutants. LiCl treatment of 32Dcl3 cells ectopically expressing HA-tagged c-Myb induced enhanced expression of wild-type c-Myb (Fig. 5*C*) but not of Δ(358–452) and Δ(389–418) c-Myb (Fig. 5*C*). These results, together with the effects of LY294002 (Fig. 5*A*) and MG-132 (Fig. 4*D*) on wild-type and mutant c-Myb, are best explained by two indirect mechanisms (one PI-3K/Akt- and one GSKIIIβ-dependent) whereby a protein or a protein complex binds to the leucine zipper and adjacent lysines (probably 387 and 428) to promote c-Myb degradation. In BCR/ABL-expressing cells, constitutive activation of the PI-3K/Akt pathway and inhibition of GSKIIIβ activity enhances c-Myb stability.

Effects of Stable c-Myb Mutants on Normal and BCR/ABL-expressing Cells—The biological effects of degradation-resistant c-Myb mutants were tested in retrovirally transduced normal myeloid precursors and K562 cells. Lineage-negative mouse marrow cells were transduced with the MigRI empty vector, the full-length c-Myb, the Δ(358–452) c-Myb, or the Δ(389–418) c-Myb. After GFP sorting, an equal number of cells were plated in methylcellulose in the presence of IL-3, KL, and Flt-3 ligand or in the presence of IL-3 alone, and colonies were scored 7 days later.

In a typical experiment in which 2×10^4 GFP-positive cells were cultured in the presence of IL-3, KL, and Flt-3 ligand, cells transduced with the MigRI retrovirus generated 150 ± 50 colonies; by contrast, cells transduced with the full-length c-*myb* retrovirus generated 350 ± 50 colonies, and cells transduced with Δ(358–452) or Δ(389–418) c-*myb* yielded an excess of 500 colonies. Increased colony formation by cells transduced with Δ(358–452) or Δ(389–418) c-*myb* was also noted in methylcellulose plates supplemented with IL-3. In the presence of a high concentration of IL-3 (10 units/ml), the number of colonies from cells transduced with either mutant was 3–4-fold higher than that from wild-type c-*myb*-transduced cells. In the presence of suboptimal concentrations of IL-3 (1 unit/ml), such a difference was even higher.

Colony assays were also performed using retrovirally transduced Lin-Sca-1$^+$ cells. Lin-Sca-1$^+$ marrow cells expressing Δ(358–452) or Δ(389–418) c-Myb were markedly more clonogenic than cells expressing the full-length c-Myb (Fig. 6*A*).

Secondary colony formation was also tested by replating cells obtained after solubilization of methylcellulose. Cells transduced with Δ(358–452) or Δ(389–418) c-*myb* formed 3–10-fold more colonies than cells transduced with wild-type c-*myb* (Fig. 6*B*). By contrast, cells transduced with the MigRI empty vector were unable to form secondary colonies. After three replating, assays, cells expressing wild-type c-Myb no longer formed colonies, whereas Δ(358–452) or Δ(389–418) c-Myb-expressing cells continued to form colonies in the presence of IL-3, KL, and Flt-3 ligand (not shown). Moreover, Δ(358–452) c-Myb-expressing cells grew in liquid culture for more than 6 months.

Upon culture with individual cytokines, proliferation was only in part supported by KL, whereas the cells died rapidly when cultured with Flt-3 ligand. Interestingly, Δ(358–452) and Δ(389–418) c-Myb-expressing cells proliferated vigorously in cultures supplemented with IL-3 only, suggesting that mutant c-Myb expression leads to enhanced IL-3R expression. Indeed, a 2-fold increase in IL-3Rα expression was detected by immunofluorescence in Δ(358–452) c-Myb compared with wild-type c-Myb expressing Lin-Sca-1$^+$ cells (Fig. 6*C*).

To determine whether the biological effects of full-length and mutant c-Myb could be correlated with protein stability, c-Myb-expressing Lin-Sca-1$^+$ cells were treated with the protein synthesis inhibitor cycloheximide, and levels of ectopic c-Myb were measured by anti-HA Western blotting. As expected, expression of wild-type c-Myb was rapidly down-modulated and was barely detectable after 1 h (Fig. 6*D*). By contrast, levels of Δ(358–452) and Δ(389–418) c-Myb were more abundant than those of wild-type c-Myb and showed approximately a 4-fold decrease after a 2-h cycloheximide treatment (Fig. 6*D*). Of interest, steady state levels (time 0) of wild-type c-Myb were less than levels of mutant c-Myb (Fig. 6*D*).

The effect of wild-type and mutant c-Myb was also tested in K562 cells. We reasoned that ectopic expression of mutant c-Myb may render these cells more resistant to apoptosis in-

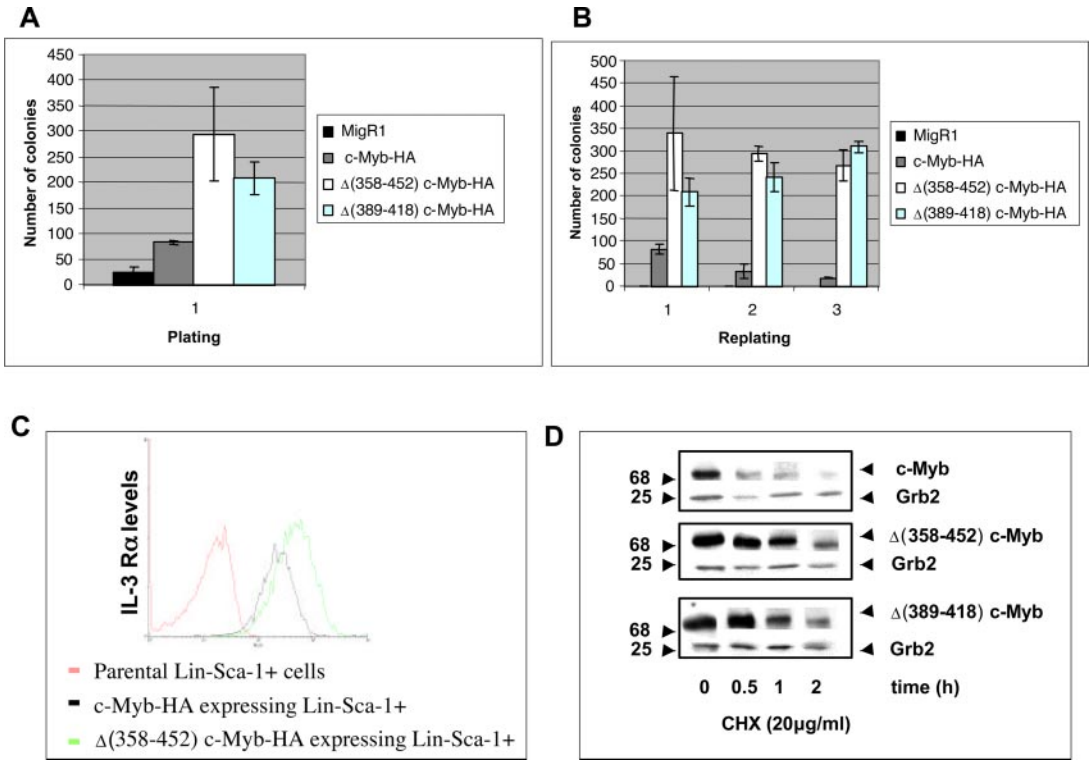

FIG. 6. **Effects of degradation-resistant c-Myb mutants on Lin-Sca-1$^+$ mouse marrow cells.** *A*, colony formation of c-Myb-transduced Lin-Sca-1$^+$ cells; *B*, replating assays of c-Myb-transduced Lin-Sca-1$^+$ cells; *C*, levels of IL-3Rα detected by flow cytometry; *D*, levels of HA-tagged c-Myb (wild-type or mutant) in cycloheximide-treated Lin-Sca-1$^+$ cells. Grb2 levels were measured as loading control.

duced by treatment with STI571 or enhance the proliferation of cells surviving after STI571 treatment. Thus, the number of K562 cells transduced with the MigRI empty vector or expressing full-length, Δ(358–452), Δ(389–418), or L389A/L396A c-Myb was assessed at 24, 48, and 72 h after treatment with STI571 (2 μM added at 0, 24, and 48 h). In STI571-treated cultures, the number of cells expressing wild-type c-Myb decreased less than that of parental cells. Moreover, K562 cells expressing mutant c-Myb exhibited a slower decrease than wild-type c-Myb-expressing cells (Fig. 7*A*); among the mutants, Δ(358–452) c-Myb was the more potent in maintaining viable cells after STI571 treatment. To assess whether the higher number of c-Myb mutant-expressing K562 cells was caused by reduced susceptibility to STI571-induced apoptosis, we measured hypodiploid DNA content by flow cytometry-activated cell sorting analysis and cell clone outgrowth by limiting dilution assays after STI571 treatment of parental and c-Myb-expressing K562 cells. Both assays did not reveal differences in apoptosis susceptibility between wild-type and mutant c-Myb-expressing cells (not shown), suggesting that the increased number of mutant c-Myb-expressing cells reflected enhanced proliferation of cells escaping STI571-induced apoptosis. The effects of c-Myb proteins in K562 cells were correlated with their levels of expression before and after STI571 treatment. As shown in Fig. 7*B*, the steady state levels of HA-tagged wild-type c-Myb were lower than those of mutant c-Myb, especially Δ(358–452) c-Myb, and STI571 treatment induced a more rapid down-modulation of wild-type than mutant c-Myb. Interestingly, Δ(358–452) c-Myb was more resistant than the other mutants to STI571-induced down-modulation (Fig. 7*B*).

DISCUSSION

The c-*myb* gene is required for normal hematopoiesis, as indicated by the results of *in vivo* and *in vitro* studies utilizing various strategies for suppression of c-Myb expression/activity (3–5). Moreover, disruption of c-Myb function in leukemic cells leads to inhibition of proliferation and/or survival and suppression of leukemogenesis *in vivo* (19, 20, 41). Despite the importance of c-Myb as regulator of leukemic cell proliferation and survival, the molecular mechanisms responsible for enhanced c-Myb activity in leukemic cells are poorly understood.

In this study, we investigated (i) if enhanced c-Myb stability is a mechanism of activation in BCR/ABL-expressing cells; (ii) the signal transduction pathways involved; and (iii) the biological consequences of expressing more stable forms of c-Myb in normal and leukemic cells. In 32D BCR/ABL-expressing cells and in CD34+ cells from a CML-BC sample, c-Myb has a longer half-life than in normal cells. Since the stability of c-Myb is regulated by ubiquitination and proteasome-dependent degradation (40, 42), we undertook experiments to compare the pattern of ubiquitination and stability of several c-Myb mutants in normal and BCR/ABL-expressing cells. This analysis was complicated because c-Myb is heavily ubiquitinated at multiple lysine residues in the DNA binding domain (repeat 2 (R2) and repeat 3 (R3)) and in its C terminus. Thus, we generated c-Myb mutants lacking the R2 and R3 of the DNA binding domain and with deletion of increasing length in the C terminus, tested their ubiquitination and stability, and found that amino acids 358–452 are required for the rapid turnover of c-Myb in normal cells. The stability of two additional mutants, Δ(389–418) c-Myb, which only lacks the putative leucine zipper of c-Myb, and L389A/L396A c-Myb, in which two leucines of the leucine zipper domain were replaced by alanines, was similar but not identical to that of Δ(358–452) c-Myb, suggesting that critical residues regulating c-Myb turnover reside in the leucine zipper, but additional amino acids in the 358–452 region might also play a role in destabilizing c-Myb. Indeed, mutation of 4 lysine residues outside of amino acids 389–418 led a to further increase of c-Myb stability essentially identical to that of Δ(358–452) c-Myb. Since mutation of lysines 442 and 445 did

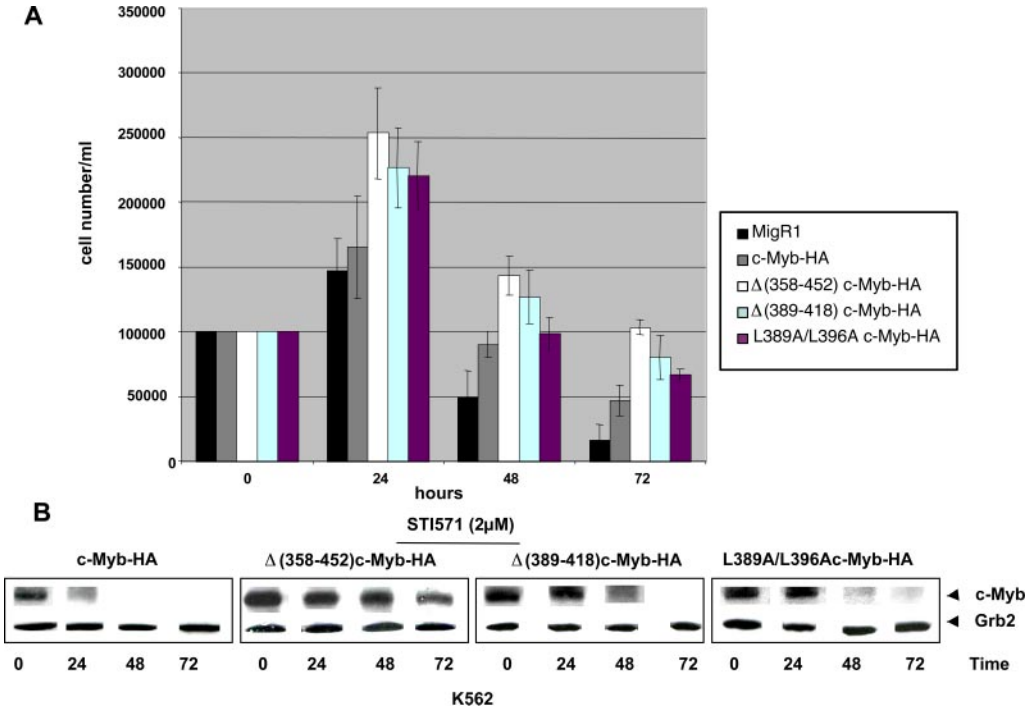

FIG. 7. **Effects of degradation-resistant c-Myb mutants on viability of STI571-treated K562 cells.** *A*, number of c-Myb-transduced K562 cells after STI571 treatment; *B*, levels of HA-tagged c-Myb (wild type and mutant) in STI571-treated K562 cells. Grb2 levels were measured as loading control.

not enhance further the stability of Δ(389–418) c-Myb, it is likely that the critical residue is lysine 387 or 428 or both.

Of interest, the increased stability of Δ(358–452)-, Δ(389–418)-, and Δ(389–418) Lys to Ala c-Myb mutants is reminiscent of murine Δ(372–417) c-Myb previously shown to be more stable than wild-type c-Myb (43). However, in that study, the authors neither distinguished the separate role of the leucine zipper and adjacent sequences nor addressed the involvement of PI-3K-dependent pathways in c-Myb stability (see below).

Wild-type c-Myb was down-regulated by inhibitors of the PI-3K/Akt pathway, whereas it was up-regulated by treatment with the GSKIIIβ inhibitor LiCl. Conversely, the PI-3K/Akt inhibitor LY294002 had no effect on the expression of Δ(358–452) c-Myb and only a modest effect on the other two leucine zipper mutants. Likewise, LiCl treatment did not induce an increase in the expression of mutant c-Myb. All together, these findings suggest that the effect of the PI-3K/Akt pathway could be indirect (*i.e.* by modulating the ability of interacting protein(s) to bind the c-Myb leucine zipper and perhaps other adjacent regions) and, in part, mediated via GSKIIIβ, the Akt substrate whose activity is inhibited by phosphorylation. Proteins interacting with c-Myb at the leucine zipper were previously identified (43–46), but it is unclear whether they were involved in c-Myb degradation. Amino acids 458–462 of c-Myb correspond to a potential GSKIIIβ phosphorylation site, which could be involved in substrate recognition by a ubiquitin ligase-containing complex and proteasome-dependent degradation (37, 47, 48). Thus, we generated the Δ458–462 c-Myb mutant and tested its stability in 32Dcl3 cells. The half-life of this mutant was undistinguishable from that of wild-type c-Myb (not shown), indicating that direct phosphorylation by GSKIIIβ in a site adjacent to the leucine zipper/negative regulatory region is not involved in c-Myb degradation.

Ectopic expression of degradation-resistant mutant c-Myb had profound effects in normal hematopoietic cells and in the Philadelphia[1] K562 cells. The effects of mutant c-Myb on normal marrow cells were similar to those induced by leucine zipper mutants of c-Myb in mouse fetal liver cells (49, 50). Those studies were the first to demonstrate a negative role of the leucine zipper for the biological effects of c-Myb but did not establish a correlation with the stability of c-Myb.

Compared with full-length c-Myb-expressing cells, mutant c-Myb-expressing Lin-Sca-1$^+$ cells had markedly enhanced clonogenic potential in primary and secondary methylcellulose assays, and Δ(358–452) c-Myb-expressing cells were continuously proliferating in culture for more than 6 months. A partial immunophenotyping of these cells revealed that they were 100% Sca-1$^+$ but entirely negative for the expression of the Gr-1 differentiation marker.

A complete characterization of c-Myb-expressing Lin-Sca-1$^+$ marrow cells in terms of surface marker expression, growth factor requirement for proliferation, and differentiation potential *in vitro* and *in vivo* is now in progress.

Expression of Δ(358–452)-, Δ(389–418)-, and L389A/L396A c-Myb markedly increased the number of cells surviving after STI571 treatment. By comparison, the effect of wild-type c-Myb was more modest.

The possibility that c-Myb-expressing cells were more resistant than parental cells to STI571-induced apoptosis seems unlikely, since hypodiploid DNA content and cell clone outgrowth was not affected by expression of mutant c-Myb. Thus, an explanation for the higher number of mutant c-Myb-expressing K562 cells may rest in the enhanced proliferative potential of cells escaping STI571-induced apoptosis. Whereas these findings may not be relevant to explain the STI571-resistant phenotype of CML-blast crisis cells, the enhanced stability of c-Myb in leukemic cells with constitutively active BCR/ABL kinase activity may favor the clonal expansion of STI571-resistant cells.

The biological effects of the c-Myb mutants utilized here are probably due to enhanced transcription activation. Indeed, in transient expression assays in 293T cells, Δ(358–452) c-Myb was 4–5-fold more potent than full-length c-Myb in transactivating a luciferase reporter gene driven by a minimal promoter and Myb binding sites (not shown). Moreover, the increased expression of IL-3Rα in mutant c-Myb-expressing cells may be

due to a transcriptional mechanism, since the mouse IL-3Rα gene contains Myb binding sites in its promoter (51).

The effects of Δ(358–452) c-Myb are similar to those induced by the p89 alternatively spliced form of c-Myb (51). This form is translated from a c-*myb* mRNA variant, which contains an insertion of 363 bases between exons 9 and 10 leading to disruption of the leucine zipper. This variant is a more potent transactivator than c-Myb, and 32Dcl3 cells expressing p89 c-Myb are considerably more resistant than parental or wild-type c-Myb-expressing cells to apoptosis induced by growth factor deprivation (52). A 2-amino acid leucine zipper mutant of wild-type c-Myb was not as effective as p89 c-Myb (51), suggesting that other modifications/interactions of the region that includes the leucine zipper domain may be necessary to enhance the effects of c-Myb. This finding is consistent with our data indicating that the effects of Δ(358–452) c-Myb in K562 cells are stronger than those of L389A/L396A c-Myb.

The biological effects of mutant c-Myb could also be explained by loss of a site of interaction with a c-Myb inhibitor. Indeed, c-Myb itself may function as an inhibitor; there is evidence that it homodimerizes through the leucine zipper and that in this form it is a less potent transactivator because of defective DNA binding (53).

Enhanced stability and failure to interact with an inhibitor(s) are not necessarily mutually exclusive mechanisms to explain the potent biological effects of mutant c-Myb. As in the case of the p53-MDM2 interaction, where MDM2 functionally inactivates p53 and promotes its degradation (54, 55), a c-Myb-interacting protein may inhibit the transactivation activity of c-Myb and promote its degradation. In K562 cells, such an inhibitor may be expressed at low levels or may be unable to interact with a c-Myb protein not subjected to the post-translational modification(s) necessary for recognition as a substrate. Thus, the identification and characterization of novel c-Myb-interacting proteins or a reassessment of the biological properties of those previously reported (44, 46) seems necessary for a better understanding of the regulation of c-Myb in normal cells and of its altered function in leukemic cells.

A pathway activated by Wnt-1 signaling has been reported to promote c-Myb degradation via Nemo-like kinase phosphorylation at multiple sites (56), some of which may be included in the amino acid region 358–452 investigated here. The biological significance of these findings remains unclear, since Wnt signaling is known to promote the expansion of hematopoietic progenitor cells (35), a situation that might be expected to be associated with high levels of c-Myb expression. This raises the possibility that c-Myb stability is finely regulated by multiple pathways, one of which may predominate depending on the stage of differentiation, cell cycle activity, or transformation.

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