The cell cycle-regulated B-Myb transcription factor is required for early embryonic development and is implicated in regulating cell growth and differentiation. In addition to its transcriptional regulatory properties, recent data indicate that B-Myb can release active cyclin/Cdk2 activity from the retinoblastoma-related p107 protein by directly interacting with the p107 N terminus. As this p107 domain has homology to the cyclin-binding domains of the p21Waf1/Cip1 family of cyclin-dependent kinase inhibitors (CKIs), we investigated in this study whether B-Myb could also interact with these CKIs. No in vivo interaction was found with either p21Waf1/Cip1 or p27Kip1, however, binding to p57Kip2 was readily detectable in both in vivo and in vitro assays. The B-Myb-interacting region of p57Kip2 mapped to the cyclin-binding domain. Consistent with this, B-Myb competed with cyclin A2 for binding to p57Kip2, resulting in release of active cyclin/Cdk2 kinase. Moreover, B-Myb partially overcame the ability of p57Kip2 to induce G1 arrest in Saos-2 cells. Despite similarities with previous p107 studies, the B-Myb domains required for interaction with p57Kip2 were quite different from those implicated for p107. Thus, it is evident that B-Myb may promote cell proliferation by a non-transcriptional mechanism that involves release of active cyclin/Cdk2 from p57Kip2 as well as p107.

The vertebrate Myb transcription factors comprise a small family of regulatory proteins with critical roles in cell proliferation and differentiation. The prototype of this family, c-Myb, is required for hematopoietic cell development (1), whereas A-Myb is necessary for spermatogenesis and breast development (2). In contrast to c-Myb and A-Myb, which are expressed in restricted tissue lineages, B-Myb is expressed throughout embryonic development and in all tissue lineages (3). Consistent with this expression pattern, B-Myb is produced in S phase (6). In addition to this level of regulation, the B-Myb protein undergoes a post-translational modification specifically during S phase resulting from phosphorylation by the cyclin A2/Cdk2 protein kinase (6–10). The major cyclin A2/Cdk2 phosphorylation sites lie within the C-terminal half of B-Myb (11–13) and map within or adjacent to a negative-regulatory domain (NRD) that has been implicated in restricting B-Myb DNA-binding and transactivation activities (8–10). Significantly, cyclin A2 kinase activity appears to counteract the inhibitory effect of the NRD and results in markedly enhanced B-Myb transactivation activity (7–10).

Current evidence indicates that transcriptional and post-translational mechanisms combine to direct high levels of hyperactivated B-Myb protein to S phase, strongly suggesting that B-Myb plays a critical role at this stage of the cell cycle. Consistent with this notion, B-Myb antisense oligonucleotides inhibit proliferation of certain myeloid, lymphoid, glioblastoma, fibroblast, and neuroblastoma cell lines (14–17), whereas, conversely, constitutive expression of B-myb prevents cell cycle arrest in serum-deprived BALB/c 3T3 fibroblasts (15) and interleukin-6-induced M1 myeloid leukemia cells (18). Altogether, these studies indicate an essential role for B-Myb in cell growth, and it is therefore of interest to determine precisely what functions B-Myb provides and at what stage of the cell cycle these are required.

Deployment of a dominant interfering Myb protein, engineered to competitively inhibit transactivation at Myb-regulated promoters, provided evidence that the transcriptional regulatory activity of B-Myb contributes to G1/S transition in embryonal stem (ES) cells (19). Nonetheless, there is surprisingly little indication to date of the identity of critical target genes that could account for this requirement. Thus, recent microarray screens identified a panel of potential B-Myb-regulated genes (20), however, it is not immediately apparent how their products could impinge upon cell cycle regulation. Other studies suggest that B-Myb influences cell proliferation by interacting with cell cycle regulatory proteins in a manner that is independent of the transcriptional activity of B-Myb. Thus, ectopic expression of B-myb can promote transition from the G1 to the S phase of the cell cycle in cells in which a G1 checkpoint has been activated by p53 or p107 (16, 21). Notably, a transcriptionally inactive B-Myb mutant (MUT-7) was partially active in overcoming p53-induced growth arrest in Saos-2 cells (16), moreover, using an extensive panel of B-Myb mutants, we
recently found that the ability of M-Myb to overcome p107-induced G1 arrest was dependent upon a direct physical interaction between these proteins but did not correlate with transactivation function (22, 23).

M-Myb binds to an N-terminal region of p107 that contains a cyclin-binding motif required for interaction with, and inhibition of, cyclin A2/Cdk2 and cyclin E/Cdk2 kinase activities (23). This finding suggested that M-Myb binding could sequester the p107 cyclin-binding motif and thereby block p107 from acting as a Cdk inhibitor (CKI). Consistent with this notion, we found that co-expression of M-Myb rescued cyclin E/Cdk2 activity in cells ectopically expressing p107 (23), although a similar activity could not be demonstrated for cyclin A2/Cdk2. Mutation or deletion of the p107 cyclin-binding motif (VACRKSV; amino acids 65–71) drastically reduced binding to M-Myb, suggesting that this motif is directly involved in M-Myb recognition. Notably, the p21WAF1, p27KIP1, and p57KIP2 CKIs contain related cyclin-binding motifs in which the central ACR core is conserved (24). We therefore investigated whether M-Myb was able to interact with these other CKIs and by so doing release active Cdk activity.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—The pCMVp21, pCMVp27, and pDNA3.9E10p57 plasmids were obtained from Dr. Xin Lu. The pT81luc 3xA, pCMVgal, pCMVcycA2, and pCMVcK62 plasmids were described previously (8). Expression vectors (pCMX) encoding Myc-epitope-tagged cyclin A2 and cyclin E were obtained from Dr. Jonathon Pines. The pcDNA3 expression vector encoding a Myc-epitope-tagged RB C792 and cyclin E were obtained from Dr. Jonathon Pines. The pcDNA3 expression vector encoding Myc-epitope-tagged RB C792 and cyclin E were obtained from Dr. Jonathon Pines. Expression vectors (pCMX) encoding Myc-epitope-tagged cyclin A2 and cyclin E were described previously (8). Two days after transfection, cell extracts were prepared for luciferase assays as described (5) or for IP/Western blot analysis. 

**RESULTS**

**Complex Formation between B-Myb and p57KIP2**—It has been shown previously that B-Myb is able to overcome a G1 block imposed by either p53 or p107 (16, 21). To understand the molecular basis for these effects, we have studied in some detail the functional and physical interactions between B-Myb and p107 (20, 23). We found that the ability of B-Myb to counter p107-mediated G1 arrest depends on a direct interaction with an N-terminal domain of p107. This p107 domain overlaps with a larger domain required for cyclin/Cdk2 binding, and it is notable that mutation of the N-terminal cyclin-binding motif in p107 abrogated binding to B-Myb. Because this motif is partially conserved in CKIs of the Cip/Kip family (Fig. 1A), it is plausible that co-immunoprecipitation analysis. p57KIP2 was successfully co-immunoprecipitated with a specific B-Myb antibody, whereas p21Cip1/Waf1 was co-immunoprecipitated to a much lesser extent and p27KIP1 was not brought down at all (Fig. 1B). The molecular basis for these effects, we have studied in some detail the functional and physical interactions between B-Myb and p107 (20, 23). We found that the ability of B-Myb to counter p107-mediated G1 arrest depends on a direct interaction with an N-terminal domain of p107. This p107 domain overlaps with a larger domain required for cyclin/Cdk2 binding, and it is notable that mutation of the N-terminal cyclin-binding motif in p107 abrogated binding to B-Myb. Because this motif is partially conserved in CKIs of the Cip/Kip family (Fig. 1A), it is plausible that co-immunoprecipitation analysis. p57KIP2 was successfully co-immunoprecipitated with a specific B-Myb antibody, whereas p21Cip1/Waf1 was co-immunoprecipitated to a much lesser extent and p27KIP1 was not brought down at all (Fig. 1B). 

**Cell Cycle Analysis—Saos-2 cells seeded at 50% confluency on 10-cm dishes were co-transfected with 15 μg of pDNA3/B-Myb, the indicated amounts of pDNA3.9E10p57 and 2 μg of pCMV.CD20 (encoding the cell surface marker CD20). The total amount of transfected DNA was kept at 30 μg by adding the appropriate amount of pDNA3 empty vector. Cells were detached 2 days post-transfection by washing with 3 mM EDTA in PBS and then stained in 100 μl of PBS with 20 μl of fluorescein isothiocyanate-labeled CD20 antibodies (BD Biosciences). After overnight fixation at 4 °C with 70% ethanol, cells were stained in PBS containing 18 μg/ml propidium iodide and 8 μg/ml RNase A for 30 min at room temperature. The proportion of transfected cells together with plasmids encoding p21Cip1/Waf1, p27KIP1, and p57KIP2 were determined by flow cytometry with a BD Biosciences FACSort using the FL1 channel, and the cell cycle profiles were obtained and quantified in the FL2A channel using CellQuest and FlowJo software.**
We were also interested in knowing if the ability of B-Myb to interact with p57KIP2 was shared with other members of the Myb family. To address this question, Saos-2 cells were co-transfected with either B-Myb or c-Myb expression plasmids together with a plasmid encoding Myc epitope-tagged p57KIP2. A polyclonal Myc antibody was used to immunoprecipitate p57KIP2, and the presence of B-Myb and c-Myb in the co-immunoprecipitate was assessed on a Western blot using specific antibodies. This indicated that p57KIP2 was immunoprecitated by B-Myb considerably better than c-Myb, as evidenced by a comparison of the relative intensities of the input and co-immunoprecipitated signals (Fig. 1D).

B-Myb Interacts with the Cyclin/Cdk-binding Domain of p57KIP2—To identify the p57KIP2 region involved in B-Myb binding, we generated p57KIP2 mutants truncated at the C and N termini (Fig. 2A). Mutant proteins were subsequently 35S-labeled using a rabbit reticulocyte in vitro transcription/translation system and mixed individually with baculovirus-expressed B-Myb or cyclin A2. The experiments were then subjected to immunoprecipitation with antibodies raised against B-Myb and cyclin A2. The inputs and immunoprecipitates were analyzed on a SDS-PAGE gel, and the labeled proteins were visualized by autoradiography. Western blot analysis was also carried out to monitor that equal amounts of proteins had been immunoprecipitated. These experiments showed that both cyclin A2 and B-Myb bound to an N-terminal fragment of p57KIP2 (amino acids 1–129), which contains the cyclin/Cdk-binding domain (Fig. 2B). In contrast, minimal binding of B-Myb and cyclin A2 was observed to C-terminal fragments (amino acids 122–316 or 240–316). These results indicate that B-Myb binds to a region of p57KIP2 that overlaps with its cyclin/Cdk-binding domain, and this situation is reminiscent of the interaction of B-Myb with the p107 N-terminal cyclin-binding domain (23).

The p57KIP2 cyclin-binding domain is composed of three different motifs that are involved in cyclin/Cdk binding and inhibition: (i) the cyclin binding region (CBP1), (ii) the Cdk binding region (KB), and (iii) the 310-helix region (Fig. 3A). In addition, this domain contains a putative α-helical region, linking the CBP1 and the KB motifs, which has been reported to be essential for binding the muscle-specific transcription factor MyoD (26). To characterize further the N-terminal regions of p57KIP2 required for binding to B-Myb, small deletions and point mutations were introduced into this region. It was of particular interest to investigate the role of the CBP1 motif in this interaction, because this motif is homologous to the N-terminal p107 cyclin-binding motif (ACRK), which was found to contribute to B-Myb binding (23). Therefore, a 6-amino acid deletion (Δ29–34) was introduced into the CBP1 motif. Additionally, point mutations were introduced into the α-helical region and the 310-helix (R44L and F90A/Y91A: Fig. 3A). Equivalent mutations in mouse p57KIP2 were found to reduce association with MyoD and cyclin A2, respectively (26, 27).

To assess the effects of these mutations on the inhibition of cyclinA2/Cdk2 activity, we monitored the phosphorylation levels of a C-terminal fragment of pRb (Rb C792–928) in co-transfected Saos-2 cells using a phosphospecific antibody on Western blots. In this assay, wt p57KIP2 inhibited cyclin A/Cdk2-mediated RB phosphorylation almost entirely, and similar levels of CKI activity was observed with the R44L mutant in which the α-helical region was mutated (Fig. 3B). In contrast, deletion within the CBP1 motif (Δ29–34) and point mutation of the 310-helix region (F90A/Y91A), or a combination
of these two mutations, abolished the CKI activity as expected (Fig. 3B). We then compared the ability of these mutants to interact with cyclin A2 and B-Myb. WT and mutant p57KIP2 proteins were 35S-labeled in a rabbit reticulocyte in vitro transcription/translation system and mixed individually with baculovirus-expressed B-Myb or cyclin A2. Following co-immunoprecipitation with B-Myb and cyclin A2 antibodies, the labeled p57KIP2 proteins were displayed on SDS-PAGE gels and quantitated by phosphorimaging. Taking the binding to wt p57KIP2 as 100%, it is evident that the R44L mutation had no effect on the ability of this protein to interact with either cyclin A2 or B-Myb (Fig. 3C). This finding suggests that, in contrast to interactions with MyoD, this region of p57KIP2 does not contribute significantly to binding B-Myb. Mutation of either the central proline/alanine-rich region or the 310-helix severely reduced binding to cyclin A2 (Fig. 3C), however, in each instance binding to B-Myb was reduced by only approximately half. B-Myb binding was further reduced to 25% in a p57KIP2 mutant harboring mutations in both these motifs (Fig. 3C). These results indicate that, although the p57KIP2 motifs required for cyclin A2/Cdk2 association contribute to B-Myb binding, they do not have the same overarching significance for this interaction as they do for binding cyclins A2/Cdk2. It is clear overall, however, that the region of p57KIP2 that interacts with B-Myb overlaps with the N-terminal cyclin/Cdk inhibitory domain. In this respect, the interaction of B-Myb with p57KIP2 has parallels with its interaction with p107.

**B-Myb Counteracts Cyclin/Cdk Inhibitory Activity of p57KIP2**

Fig. 2. B-Myb interacts with the N terminus of p57KIP2. A, schematic diagram of the p57KIP2 protein showing the N-terminal cyclin/Cdk-binding domain, the central proline/alanine-rich region (PAPA repeats) and the C-terminal QT domain. Shown below are the p57KIP2 mutants used in this study. B, a coupled rabbit reticulocyte transcription/translation system was used to make [35S]methionine-labeled wt and mutant p57KIP2 proteins in vitro. Unprogrammed mock lysates and the p57KIP2 lysates were then mixed with either baculovirus-expressed B-Myb or cyclin A2. 10% of each mixture was retained (Input), and the remainder was subjected to immunoprecipitation with rabbit polyclonal B-Myb or cyclin A2 antibodies. Radiolabeled p57KIP2 proteins in the inputs and immunoprecipitates were detected on Western blots by autoradiography, whereas B-Myb and cyclin A2 were detected by ECL.
B-Myb Counteracts Cyclin/Cdk Inhibitory Activity of p57KIP2

**Figure 3.** The cyclin binding region and the 310-helix region of p57
KIP2 contribute to B-Myb interactions. A, alignment of the N-terminal mouse and human p57KIP2 protein sequences showing the motifs involved in cyclin/Cdk inhibition. The cyclin binding region (CBPI), Cdk binding region (KB), and the 310-helix region are highlighted in boxes. The dots in the human sequence (hg57KIP2) indicate amino acid identities with the mouse protein (mp57KIP2). The mutated amino acids (D29–34, R44L, and F90A/Y91A) present in p57mut mutants used in the current study are underlined and indicated below. An arrow indicates the N-terminal start of mutant P1 illustrated in Fig. 2 A, B, Saos-2 cells were transfected with pcDNA3.RB C792–928 (4 μg), pCMVcycA2 (2 μg), and pCMVcdk2 (2 μg) as indicated together with 1 μg of pcDNA3.9E10p57 or plasmids expressing the p57mut mutants R44L, Δ29–34, F90A/Y91A, and Δ29–34/F90A/Y91A. Cell extracts were made 2 days post-transfection and subjected to Western blot analysis with rabbit polyclonal antibodies specific for phospho-RB (S807/S811), cyclin A2 and Cdk2, and the 9E10 monoclonal antibody to detect Myc-tagged p57KIP2 proteins. C, a coupled rabbit reticulocyte transcription/translation system was used to make [35S]methionine-labeled wt and mutant p57KIP2 proteins in vitro. Unprogrammed mock lysate and equivalent radiolabeled amounts of the p57KIP2 lysates were mixed with either baculovirus-expressed B-Myb or cyclin A2, and the mixtures were subjected to immunoprecipitation with rabbit polyclonal B-Myb or cyclin A2 antibodies. Radiolabeled p57KIP2 proteins were detected on dried SDS-PAGE gels by phosphorimaging, and were quantified using ImageQuaNT software. The binding of mutant p57KIP2 proteins to cyclin A2 and B-Myb was calculated in three independent experiments, and the average is shown taking the binding to wt p57KIP2 as 100%.

**B-Myb and Cyclins Compete for Binding to p57KIP2**—Because the B-Myb and cyclin A binding sites on p57KIP2 overlap, it seemed likely that expression of B-Myb would compete for binding to this N-terminal region and potentially prevent inhibition of cyclin/Cdk2 by p57KIP2. To test this notion, we first determined whether B-Myb and cyclin A2 formed mutually exclusive complexes with p57KIP2. Expression plasmids encoding wt B-Myb, cyclin A2, and p57KIP2 were co-transfected into Saos-2 cells, and lysates were made 2 days post-transfection. Proteins co-immunoprecipitating with either B-Myb or cyclin A2 were then detected on Western blots. It is clear from this experiment that p57KIP2, but not B-Myb, was co-immunoprecipitated using cyclin A2 antibodies. In the converse experiment, B-Myb antibodies also efficiently co-immunoprecipitated p57KIP2, however, no cyclin A2 was brought down (Fig. 6A). To provide further evidence that B-Myb and cyclin A2 actually compete for binding p57KIP2, fixed amounts of cyclin A2 and p57KIP2 expression vectors were co-transfected into Saos-2 cells together with increasing amounts of a B-Myb plasmid. Western blot analysis of proteins co-immunoprecipitated with p57KIP2, clearly showed that the amount of cyclin A2 associated with p57KIP2 is titrated out with increased input of B-Myb.

To assess whether competition by B-Myb for the p57KIP2 N-terminus prevented inhibition of cyclin/Cdk2 kinase activity by this domain, we monitored kinase activity in co-transfected Saos-2 cells using Rb C792–928 as a substrate. As expected, increasing the amount of transfected p57KIP2 resulted in progressive inhibition of cyclin A2/Cdk2 kinase activity (Fig. 6C, lanes 2–4). Notably, inhibition of cyclin A2/Cdk2 kinase activity was overcome partially by co-expressing B-Myb (compare lanes 5 versus 3 and 6 versus 4 in Fig. 6C). Furthermore, this effect was not exclusive for cyclin A2/Cdk2, because B-Myb was also able to rescue p57KIP2-mediated inhibition of cyclin E/Cdk2 kinase activity (Fig. 6D). Altogether, our results clearly demonstrate that B-Myb binds a region of p57KIP2, which overlaps with its cyclin/Cdk inhibitory domain.

**B-Myb Partially Overcomes a G1 Block Imposed by p57KIP2**—It has been suggested that the Cdk2-inhibiting activity of p57KIP2 is crucial for its growth suppression function (27). The fact that B-Myb can overcome p57KIP2-mediated inhibition of cyclin A2/Cdk2 and cyclin E/Cdk2 kinase activities therefore raised the question whether B-Myb would be able to overcome a G1 block arrest imposed by p57KIP2. To address this question we first titrated the amount of p57KIP2 plasmid required to induce G1 arrest in Saos-2 cells. The percentage of cells in G1 was assessed by flow cytometry using a CD20 marker to discriminate the transfected cells. The increase in the G1 population...
tion directly correlated with the amount of the p57KIP2 plasmid transfected, and at the maximum input of 2 μg of pcDNA3.9E10p57 almost a 50% increase in the G1 population was observed (Fig. 7A). The increase in the G1 population was mirrored by reductions in both the S and G2/M compartments. Notably, ectopic expression of B-Myb partially overcame the p57KIP2-induced G1 block and substantially increased the proportion of cells in S phase (Fig. 7B). Transfection of a higher amount of a B-Myb expression vector did not result in any further reversal of the p57KIP2-mediated G1 block (data not shown), suggesting that p57KIP2 may in part induce cell arrest by mechanisms other than cyclin/Cdk2 inhibition. For example, it has been reported that the C-terminal QT domain can also induce G1 arrest in Saos-2 cells through interacting with and inhibiting PCNA-dependent DNA elongation (28), and it is not expected that B-Myb would affect this activity.

**DISCUSSION**

We have shown in this study that B-Myb binds to a region of p57KIP2 that comprises its cyclin/Cdk inhibitory domain. In this respect, these findings are reminiscent of previous studies, in
which we showed that B-Myb bound to the N-terminal cyclin-binding domain of p107, which has some homology to p57\textsuperscript{KIP2}, and prevented p107 from inhibiting cyclin E/Cdk2 activity (22, 23). Using a range of B-Myb mutants, it was found unexpectedly that the B-Myb domains involved in binding p107 and p57\textsuperscript{KIP2} are quite distinct, and indeed B-Myb is able to interact simultaneously with p107 and p57\textsuperscript{KIP2} (Fig. 8A).

Interactions between B-Myb and p57\textsuperscript{KIP2} resulted in mutual inhibition of their functions. The ability of B-Myb to inhibit p57\textsuperscript{KIP2} function can readily be explained by competition between B-Myb and cyclin/Cdk2 complexes for binding to the inhibitory p57\textsuperscript{KIP2} cyclin/Cdk-binding domain (Fig. 6). In part, inhibition of B-Myb transactivation activity by p57\textsuperscript{KIP2} resulted from suppression of cyclin A2/Cdk2 kinase activity, which is responsible for phosphorylating B-Myb during S phase and plays a critical role in enhancing the transcriptional properties of B-Myb. In addition, it was evident that p57\textsuperscript{KIP2} directly inhibited B-Myb transactivation function, because a constitutively active B-Myb mutant (B-Myb\textsuperscript{H1100A}561) whose activity does not depend upon cyclin A-mediated hyperphosphorylation could also be inhibited to a significant extent by p57\textsuperscript{KIP2} (Fig. 5B). Presumably, this latter effect results from direct binding of p57\textsuperscript{KIP2} to B-Myb\textsuperscript{H1100A}561. Because p57\textsuperscript{KIP2} interacts with the N-terminal region of B-Myb that is required for DNA binding, we investigated whether this activity was impaired in the presence of p57\textsuperscript{KIP2}. Using extracts prepared from co-transfected cells in electrophoretic mobility shift assays, however, we found no evidence that the DNA-binding activity of B-Myb was affected by p57\textsuperscript{KIP2} (24). It is therefore presumed that p57\textsuperscript{KIP2} binding inhibits B-Myb transactivation activity through a different mechanism, for example, by potentially blocking the interaction of B-Myb with co-activators such as CBP/p300 or PARP (25, 29, 30).

The p57\textsuperscript{KIP2} gene displays a restricted pattern of expression in tissues and is expressed predominantly in terminally differentiated cells. The p57\textsuperscript{KIP2} protein preferentially associates with and inhibits G\textsubscript{1} Cdk's, and this activity can result in G\textsubscript{1} arrest (31, 32). Evidence obtained from gene knockout studies indicates that p57\textsuperscript{KIP2} has a specific role during mouse development that cannot be compensated for by other CKIs (33, 34). Loss of p57\textsuperscript{KIP2} function results in increased cell proliferation and a concomitant reduction in cell differentiation in certain tissues such as skeletal muscle and cartilage. The pattern of expression of p57\textsuperscript{KIP2} is almost diametrically opposed to that of B-myb, whose expression is restricted to proliferating tissues (3). It is possible, therefore, that B-Myb and p57\textsuperscript{KIP2} play antagonistic roles in regulation of development and cell differen-

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\footnote{M. Joaquin and R. J. Watson, unpublished data.}
Although data that bear on this possibility are quite limited, it has been demonstrated that ectopically expressed B-Myb prevents late events during myeloid differentiation, including the loss of proliferative capacity associated with terminal differentiation (18, 35, 36). Although expression of p57
KIP2
has not been analyzed in the myeloid cell lines used in the experiments.

**FIG. 6.** B-Myb and cyclin A2 form mutually exclusive complexes with p57
KIP2
and compete to bind p57
KIP2
. A, Saos-2 cells were co-transfected with pCDNA3/B-myb (10 µg), pCMVcycA2 (2 µg), and pCDNA3.9E10p57 (2 µg) as indicated. Control cells received empty vectors. Cell extracts were made 2 days post-transfection, and 10% of the lysates were retained (Input). The remainders were subjected to immunoprecipitation with a rabbit polyclonal B-Myb antibody. Inputs and immunoprecipitates were run on the same SDS-PAGE gel, and Western blots were probed with the 9E10 monoclonal antibody to detect Myc-tagged p57
KIP2
and rabbit polyclonal B-Myb and cyclin A2 antibodies. B, Saos-2 cells were transfected with increasing amounts of pCDNA3/B-myb (5, 10, and 15 µg) as indicated by the gradient, pCMVcycA2 (2 µg) and pCDNA3.9E10p57 (2 µg). Cell extracts were made 2 days post-transfection and 10% of the lysates were retained (Input). The remainders were subjected to immunoprecipitation with a rabbit polyclonal 9E10 antibody to pull-down Myc-tagged p57
KIP2
. Inputs and immunoprecipitates were run on the same SDS-PAGE gel, and Western blots were probed with the 9E10 monoclonal antibody to detect p57
KIP2
and rabbit polyclonal B-Myb and cyclin A2 antibodies.

**FIG. 7.** B-Myb partially overcomes a G1 block imposed by p57
KIP2
. A, Saos-2 cells were co-transfected with pCDNA3.9E10p57 (0.5, 1, and 2 µg) and pCMV.CD20 (2 µg). Cells were subjected to fluorescence-activated cell sorting analysis 2 days post-transfection. Transfected cells were discriminated by staining for CD20, and the average percentage increase of cells in G1 phase of three independent experiments is presented as a bar chart. B, Saos-2 cells were co-transfected with pCDNA3.9E10p57 (2 µg), pCDNA3/B-myb (15 µg), and pCMV.CD20 (2 µg) and subjected to fluorescence-activated cell sorting analysis 2 days post-transfection. Percentage changes of cell populations in G1, S, and G2/M phases for three independent experiments are shown in the bar chart.
these studies, p57KIP2 is known to be expressed in chronic myelogenous leukemias, and notably its expression is downregulated during the evolution to more immature cells that occurs in blast crisis (37). It may be anticipated, therefore, that one consequence of ectopically expressing B-myb during myeloid differentiation would be to counteract the ability of p57KIP2 to sequester and inhibit cyclin/Cdk activity, thereby maintaining the cell in cycle. Presumably, B-Myb could have similar antagonistic effects on p57KIP2 function in other cell types, perhaps counteracting inhibition of cyclin/Cdk activity until such time during development and differentiation when B-Myb transcription diminishes in response to the induction of repressive E2F complexes.

This study was precipitated by the finding that B-Myb bound to the cyclin-binding domain of p107 (23), a property that depends in part on an N-terminal p107 motif that is homologous to cyclin-binding motifs in the p21Waf1/Cip1, p27KIP1, and p57KIP2 proteins (24). We found, however, that mutation of this sequence (CBP1) in p57KIP2 had relatively little impact on B-Myb binding (Fig. 3), moreover, p21Waf1/Cip1 and p27KIP1 proteins did not interact with B-Myb in vivo (Fig. 1C). Previous studies have also shown that B-Myb bound only weakly to p130 (23), which also contains a cyclin-binding motif with homology to p107 and p57KIP2. It can therefore be concluded that the cyclin-binding motif is not a primary determinant for B-Myb interactions with p107 and p57KIP2. Rather B-Myb binds to a region of these CKIs, which overlaps with their cyclin-binding motifs. It is also evident that different regions of B-Myb are required for its interactions with p107 and p57KIP2. Despite these differences, it is intriguing that B-Myb has a similar ability to suppress inhibition of CKI activity specified by these proteins. This suggests that B-Myb influences the cell cycle in ways other than simply at the gene transcriptional level expected of a transcription factor, that is by blocking the cyclin-dependent kinase inhibitory activities of p107 and p57KIP2. It may be relevant that the Drosophila Myb protein, DMyb, has also been proposed to have multiple functions in the cell cycle, acting both at the transcriptional level and directly in site-specific DNA replication (38).

The p57KIP2 protein has previously been reported to bind to two other transcription factor, MyoD and p53 (26, 39). Interactions with MyoD were found to involve a putative alpha-helical domain that links the p57KIP2 cyclin and Cdk binding sites (26). Thus, the R33L mutation in murine p57KIP2, which is predicted to have a major influence on helicity in this region, was found to dramatically reduce binding of MyoD without affecting cyclin/Cdk interactions (26). Our study showed, however, that the equivalent mutation in human p57KIP2, R44L, had no effect on B-Myb binding (Fig. 3). Because the R44L mutation in human p57KIP2 is predicted to substantially increase helicity in this region, as does the R33L mutation in the mouse protein (26), it is suggested that the p57KIP2 domain requirements for binding B-Myb and MyoD are quite different. It is also notable that B-Myb is not directly competed with cyclin D1 for binding to p57KIP2, suggesting that these binding sites overlap, whereas the binding of MyoD to p57KIP2 was competed by the cyclin D1/Cdk4 complex but not by cyclin D1 alone. Again, this evidence is consistent with different p57KIP2 domain requirements for interactions with B-Myb and MyoD. It is also notable that the effects of p57KIP2 binding on B-Myb and MyoD function are distinct: this interaction enhancing transactivation by MyoD (26) while diminishing transactivation by B-Myb (Fig. 5). Thus, it is anticipated that p57KIP2 would work cooperatively with MyoD in myogenic differentiation, whereas it would have an antagonistic effect with B-Myb in cellular differentiation.

Gene knock-out studies have shown that B-Myb plays a critical role in early embryogenesis. Although the only function yet ascribed to B-Myb during development is a requirement for growth of the inner cell mass of the embryo, it is unlikely that this effect involves p57KIP2, because expression of this CKI could not be detected in ES cells, which are thought to be derived from this embryonic tissue. The B-Myb gene is expressed ubiquitously in tissues within proliferating cells throughout embryonic development and adulthood, and it is anticipated that the B-Myb protein will play a significant role in regulating cell proliferation and differentiation. The development of conditional knock-out and RNA interference technologies to ablate B-Myb expression will be essential to dissect the role of B-Myb in later embryonic development, and it will be of interest to determine the involvement of interactions with p57KIP2 and p107 in this activity.

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