Mip/LIN-9 Regulates the Expression of B-Myb and the Induction of Cyclin A, Cyclin B, and CDK1*

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Members of the novel family of proteins that include Drosophila Mip130, Caenorhabditis elegans LIN-9, and mammalian LIN-9 intervene in different cellular functions such as regulation of transcription, differentiation, transformation, and cell cycle progression. Here we demonstrate that LIN-9, designated as Mip/LIN-9, interacts with B-Myb but not with c-Myb or A-Myb. Mip/LIN-9 regulates the expression of B-Myb in a post-transcriptional manner, and its depletion not only decreases the level of the B-Myb protein but also affects the expression of S phase and mitotic genes (i.e. cyclin A, CDK1, and cyclin B). The critical role of Mip/LIN-9 on the expression of S and G2/M genes is further supported by the finding that coexpression of Mip/LIN-9 and B-Myb results in the activation of cyclin A and cyclin B promoter-luciferase reporters, and both proteins are detected on the cyclin A and B promoters. Interestingly, although Mip/LIN-9 promoter occupancy peaks earlier than B-Myb, the highest levels of expression of cyclins A and B correlate with the maximum binding of B-Myb to these promoters. These data support the concept that Mip/LIN-9 is required for the expression of B-Myb, and both proteins collaborate in the control of the cell cycle progression via the regulation of S phase and mitotic cyclins.

Mammalian LIN-9 (1) is a member of the Mip/LIN-9 family, which also includes Caenorhabditis elegans LIN-9, the first member described (2, 3), Drosophila Mip130 and Aly (4–8), and Arabidopsis thaliana Always Early (9). Drosophila Mip130 and C. elegans LIN-9 are in the same pathway as the pocket proteins. Similarly, it has been proposed that the mammalian homolog of the Mip/LIN-9 family collaborates with pRB in tumor suppression (1). We independently cloned mammalian members of the Mip/LIN-9 family (10). We identified two human forms encoding proteins with predicted molecular masses of ~62 and 54 kDa, whereas in mouse cells only cDNAs encoding the 62-kDa form were isolated (10). A mutant form of the mouse gene that lacks the first 84 amino acids corrects the CDK4 null phenotype and restores the expression of E2F-regulated genes, indicating that mammalian Mip/LIN-9 functions in G1 downstream of CDK4 (10).

One of the Drosophila members of the Mip/LIN-9 family, Mip130, is a part of a group of proteins designated as Mip (Myb-interacting proteins), which has a dual role regulating DNA replication at specific foci and transcription of specific genes (4–6). Mip130 is part of a large complex termed dREAM (Drosophila RB, E2F, and Myb), which also includes other Myb-interacting proteins such as Mip40 and Mip120. Interestingly, the expression of some proteins in the complex is required for the stability of other components (4–6). For example, depletion of Mip130 abrogates the expression of the transcription factor Dm-myb. Mip130 is epistatic to Dm-myb because its deletion rescues the lethal phenotype produced by the knock-out of Dm-myb (5). Therefore, these authors proposed that this lethal effect is because of unchecked repressor activity of Mip130.

Unlike Drosophila, there are three mammalian Myb proteins: A-, B-, and c-Myb. The retroviral oncogene v-Myb is also a member of this family (for recent reviews see Refs. 11–15). B-Myb is ubiquitously expressed, whereas A- and c-Myb are tissue-specific. As in the case of Dm-myb, the absence of B-Myb in mice produces an early embryonic lethal phenotype underscoring the role of B-Myb in development (16). The B-Myb protein plays an important role in the regulation of cell cycle progression by controlling the transcription of genes necessary for S and M phases (reviewed in Refs. 11–15, 17). As in the case of other proteins required for cell cycle progression, the activity of B-Myb is regulated by cyclin-driven phosphorylation, in particular by CDK2-cyclin A and CDK2-cyclin E complexes (18–25). B-Myb contains 15 sites that are targets for cyclin-dependent kinases, and the mutation of most, if not all, of these sites is required to decrease B-Myb-driven transcriptional activation via the Myb-binding site (MBS)3 (24, 25). Interestingly, B-Myb-induced transcription does not require the presence of an MBS on the target promoters in all cases, suggesting that B-Myb can regulate the activity of other transcription factors (25–29). It has also been reported that B-Myb can interact with cyclin D (30, 31), whereas c-Myb interacts with CDK4, -6, and cyclin D

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3 The abbreviations used are: MBS, Myb-binding site; mAb, monoclonal antibody; pAb, polyclonal antibody; ChIP, chromatin immunoprecipitation; DMEM, Dulbecco’s modified Eagle’s medium; MEF, mouse embryonic fibroblast; FACS, fluorescence-activated cell sorter.
Mip/LIN-9 Is Required for B-Myb Activity

(32). However, CDK4, -6, and cyclin D complexes do not phosphorylate or activate B-Myb but rather inhibit its activity (30–33). One possible interpretation is that the CDK4, -6, and cyclin D complex may be responsible for impeding the early activation of B-Myb. Additionally, several lines of evidence also indicate that B-Myb functions downstream of p53. First, overexpression of B-Myb overcomes the growth arrest induced by p53 even in the presence of high levels of p21 and negligible CDK2/cyclin E kinase activity (34). Second, RAS and B-Myb are transforming in MEFs in the presence of normal p53 and ARF (35). Interestingly, the deletion of B-Myb in DT40 cells impairs the response to DNA-damaging agents (36).

Our present knowledge regarding the function of mammalian members of the Mip/LIN-9 family is limited to its role as a collaborator of pRB in tumor suppression and a downstream target of CDK4/cyclin D (1, 10). Because Drosophila Mip130 interacts with Dm-myb (4–6), we tested the hypothesis that mammalian Mip/LIN-9 could also associate with B-Myb and that this complex would be an important driver of S phase and mitosis. Our results indicate that indeed, human and mouse forms of Mip/LIN-9 associate with B-Myb. The presence of mammalian Mip/LIN-9 is pivotal for the expression of the B-Myb protein because its depletion reduces the expression of B-Myb and its target genes cyclin A, cyclin B, and CDK1. The role of Mip/LIN-9 in cell proliferation was further demonstrated by the finding that in its absence p53−/− MEFS become contact-inhibited at lower densities. Moreover, Mip/LIN-9 participates in the regulation of S and G2/M genes as supported by the finding that coexpression with B-Myb produces a significant increase in luciferase activity of cyclin A- and B-promoter constructs. This effect is further increased by coexpression of a known activator of B-Myb, cyclin A (18–25). The regulation of the expression of cyclins A and B by B-Myb and Mip/LIN-9 is at the promoter levels because both proteins associate with these promoters in ChIP assays. Surprisingly, although maximum promoter occupancy by Mip/LIN-9 precedes the peak in B-Myb binding, the latter has a closer temporal relationship with the highest level of protein expression for these cyclins. Altogether, these data support the concept that Mip/LIN-9 and B-Myb collaborate in the regulation of cell cycle progression by controlling the induction of S phase and mitotic cyclins.

MATERIALS AND METHODS

Tissue Culture, Constructs, and Transfections—U2OS, T98G, B-JAB, HL-60, NIH3T3, and p53−/− MEFS were grown in DMEM supplemented with 10% fetal bovine serum, glutamine, and nonessential amino acids. MEFS were seeded at a density of 3 × 105 on 60-mm dishes 24 h before infection with the indicated retroviruses. Retroviruses (pBabe-puro and pBabe-puro-Mip/LIN-9 antisense) were produced in an ecotropic packaging cell line as described previously (37). Forty-eight hours after infection, cells were selected in complete growth medium supplemented with 1 μg/ml puromycin for 14 days.

Immunoprecipitations and Immunoblotting—Western blot analysis was performed as described previously (10). Mip/LIN-9 mouse monoclonal (mAb) and rabbit polyclonal antibodies (pAb) against LIN-9 fusion proteins and peptides were developed in our laboratory (10). The following antibodies were also used in these studies: anti-B-Myb (N-19; sc-724); -cyclin B (sc-245), A-Myb (sc-28686) and -cyclin A (sc-751) were from Santa Cruz Biotechnology. The anti-CDK1 antibody (610038) was from BD Transduction Laboratories; the anti-acetylated histone 4 and c-Myb were from Upstate Biotechnology, Inc.; and the anti-tubulin antibody (ab3194) was from Abcam. Additionally, an anti-B-Myb serum was kindly provided by Dr. Robert Lewis. Densitometric analysis was performed using the computer program NIH Image version 1.62.

Cell Cycle Synchronization—NIH3T3 cells or p53−/− MEFS were plated at 105 per 100-mm dish and 24 h later were washed with phosphate-buffered saline and cultured in serum-free DMEM for 48–72 h. Cells were released by changing medium to DMEM supplemented with 10% fetal bovine serum. Cells were harvested at the indicated time points following release. Synchronization was measured by FACS analysis of propidium iodine-stained cells.

B-Myb Gene Expression Using Real Time Reverse Transcription-PCR—Total RNA (0.3 μg) was reverse-transcribed in a 20-μl reaction using the Reverse-it™ 1st strand synthesis kit from Abgene Biotechnologies Ltd. Real time quantitative PCR was achieved by using a cDNA equivalent of 30 ng of total RNA. The reaction was performed in 25 μl using SYBR® Green PCR master mix (Applied Biosystems) according to the manufacturer’s instructions. PCR was developed with the ABI PRISM 7900HT sequence detection system (Applied Biosystems). Amplification was performed using a 2-min step at 50 °C and a 10-min denaturation step at 95 °C, followed by 40 cycles of 15 s of denaturation at 95 °C, 1 min of primer annealing, and a polymerization step at 60 °C. Each sample was processed in triplicate, and the data are expressed as the mean threshold cycle (CT) ± S.D.

Promoter and ChIP Assays—The luciferase assay system from Promega was used for promoter assays. Transfections were carried out with Lipofectamine 2000 according to the manufacturer’s instructions. T98G and U2OS cells were plated in triplicate at 2.5 × 105 per well in 12-well dishes 24 h before transfection with a total of 2.1 μg of plasmid DNA (0.6 μg of each construct and 0.3 μg of the reporter) (38). Control enhanced green fluorescent protein and pCMV vectors were used to maintain the total amount of DNA at 2.1 μg. Twenty four hours post-transfection, cells were washed with phosphate-buffered saline, lysed, and read on a Victor 2 plate reader.

ChIP assays were performed using the EZ ChIP (Upstate Biotechnology, Inc., Lake Placid, NY) following the manufacturer’s instructions with some modifications. Briefly, 107 cells per immunoprecipitation were cross-linked with 1% formaldehyde and sonicated to produce chromatin fragments between 500 and 1,000 bp. Chromatin was subsequently incubated with 2 μg each of preimmune mouse IgG (Sigma), Mip/LIN-9 mAb, B-Myb pAb (N-19; Santa Cruz Biotechnology), or acetylhistone H4 antiserum (Upstate Biotechnology, Inc.). Immunoprecipitated samples were treated as described by the manufacturer’s instructions, and purified DNA from the ChIP samples was assayed by semiquantitative PCR using primers for the E2F-regulated promoter sequences of mouse cyclin A2 and cyclin B1 as described previously (17, 39). PCR amplicons were separated
Mip/LIN-9 Is Required for B-Myb Activity

FIGURE 1. Mip/LIN-9 interacts with B-Myb. A and B, MEFs null for p53 (A) and human U2OS cells (B) were used for coimmunoprecipitation of endogenous Mip/LIN-9 and B-Myb. Immunoprecipitations (IP) were performed with a specific anti-B-Myb rabbit pAb and anti-Mip/LIN-9 mAb. Thirty micrograms of total lysate (lysate) was used as control input. Immunoblotting (WB) was sequentially performed with the same anti-B-Myb (upper panels) and anti-Mip/LIN-9 (lower panels) antibodies used for immunoprecipitation. The asterisk indicates a background band, which migrates slightly faster than the specific band for B-Myb, only detected in immunoprecipitations with the normal rabbit serum (NR) in human cells. NM, normal mouse serum. The open circle indicates the migration of the IgG heavy chain (B, lower panel, lane 3). L and S show the migration of Mip/LIN-9 long (62 kDa) and short (54 kDa), respectively. C and D, HL60 or B-JAB lysates were used to determine whether Mip/LIN-9 interacts with c-Myb and A-Myb, respectively. Immunoprecipitations were performed as described in A and B. E, mapping of the regions of Mip/LIN-9 that intervene in the interaction with B-Myb. 293T cells were transfected with B-Myb and green fluorescent protein (GFP)-tagged constructs encoding full-length Mip/LIN-9 (1–542), truncations 1–147, or 148–542. Immunoprecipitations were performed with an anti-B-Myb antibody followed by immunoblotting with an anti-green fluorescent protein and -B-Myb antibodies. The right panel shows a direct immunoblotting to assess the expression of the transfected green fluorescent protein fusion proteins (100 µg/lane). The difference in resolution between the left and right panels is because of the use of a higher percentage acrylamide gel in the latter.

on 2% agarose gels and subsequently visualized by ethidium bromide staining.

RESULTS

LIN-9 and B-Myb Form a Complex in Mammalian Cells—Because Drosophila Mip130 and Dm-Myb are part of the same protein complex, we sought to determine whether their mammalian homologs, Mip/LIN-9 and B-Myb, are also associated in human and mouse cells. To address this question, we performed coupled immunoprecipitation/Western blot using anti-B-Myb and anti-Mip/LIN-9 antibodies and protein lysates obtained from mouse and human cell lines. Fig. 1 shows that the anti-Mip/LIN-9 antibody (Fig. 1A, upper panel, lane 2) coprecipitates a protein that comigrates with endogenous B-Myb (lane 3) in p53−/− MEFs and human U2OS (Fig. 1B, upper panel, lane 4). In some human cells such as U2OS, a background band that migrates faster than the specific band for B-Myb is present in immunoprecipitations with the normal rabbit serum used as control for B-Myb (Fig. 1B, compare lanes 1 and 2). Importantly, the reciprocal experiment shows that endogenous Mip/LIN-9 is coprecipitated by the anti-B-Myb pAb (Fig. 1, A and B, lower panels). The data obtained with human cells, which unlike mouse cells express both Mip/LIN-9 and B-Myb, long and short, further indicate that B-Myb interacts with both forms of Mip/LIN-9 (Fig. 1B, lower panel, lane 2). These results demonstrate that endogenous Mip/LIN-9 and B-Myb, similarly to Mip130 and Dm-Myb in Drosophila, are part of the same protein complex.

Because there are extensive areas of homology among Myb family members, we next tested if Mip/LIN-9 was also able to interact with c-Myb and A-Myb. To address this issue, HL-60 and B-JAB cells, were used for coimmunoprecipitation experiments. Fig. 1C shows that anti-Mip/LIN-9 and c-Myb mAbs fail to coprecipitate c-Myb and Mip/LIN-9, respectively. Similarly, the Mip/LIN-9 and A-Myb mAbs do not coprecipitate A-Myb or Mip/LIN-9 (Fig. 1D). These results demonstrate that Mip/LIN-9 specifically interacts with B-Myb.

To determine the region of Mip/LIN-9 involved in the interaction with B-Myb, 293 cells were transfected with enhanced green fluorescent protein fusion constructs encoding different regions of Mip/LIN-9. Fig. 1E shows that B-Myb interacts with the full-length form of Mip/LIN-9 and a truncation encoding the region 148–542 suggesting that either the B-Myb-binding domain extends beyond amino acid 147 or another region of the Mip/LIN-9 interacts with B-Myb.

Depletion of Mip/LIN-9 Decreases the Expression of B-Myb and Its Transcriptional Targets Cyclins A2 and B1 and CDK1—We sought to determine the role of Mip/LIN-9 in cell cycle regulation using a knockdown approach. For these experiments, we selected p53−/− MEFs because they are immortal and contain wild type pRB, and previous reports suggested that B-Myb acts downstream of p53 (34, 35). After several failed attempts to produce a knockdown using short interfering RNAs against the mouse gene (data not shown), we successfully reduced the expression of Mip/LIN-9 using a retrovirus carrying this gene in the antisense orientation. It quickly became evident that the puromycin-resistant clones carrying the antisense construct proliferated slower than a pool of p53−/− MEFs infected with empty vector (see below). Clones were expanded and tested for the expression of Mip/LIN-9. Fig. 2 (A and B, upper and lower panels) shows that the antisense construct reduced the expression of Mip/LIN-9 by 60–75% of control cells (empty vector) after normalization by the expression of tubulin. Similar results were obtained with other clones obtained from two independent antisense infections of p53−/− MEFs (data not shown). Immunoblotting of asynchronously growing MEFs with an anti-B-Myb pAb (Fig. 2B, upper panel) revealed that B-Myb was also decreased in all three clones. Moreover, the decrease in Mip/LIN-9 also affected the expression of B-Myb-regulated genes (17) such as cyclin A, cyclin B, and CDK1 (Fig. 2B, lower panel).

To determine whether the decrease in B-Myb was because of transcriptional or post-transcriptional mechanisms, we per-
Mip/LIN-9 Is Required for B-Myb Activity

FIGURE 2. Antisense (AS) depletion of Mip/LIN-9 is accompanied by a reduction in the expression of B-Myb. The expression of Mip/LIN-9 (A and B), B-Myb (B), as well as other cell cycle regulators (B) in a pool of cells infected with empty retroviral vector (pBP) or clones (23, 31, 33) carrying antisense for Mip/LIN-9 was determined by Western blotting (IB) with specific antibodies. The lower panel in A is a graphic representation of the expression of Mip/LIN-9 in each clone normalized by the level of tubulin and expressed as percentage of vector control cells (pBP). Densitometric analysis was performed using the program NIH Image. C, expression of B-Myb mRNA in cells transfected with the Mip/LIN-9 antisense construct (closed bars) or empty vector (open bars). Real-time PCR was performed as described under “Materials and Methods” using total RNA from asynchronous cells. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as control. D, depletion of Mip/LIN-9 affects proliferation of p53 AS cells and controls, indicating that the decrease in B-Myb expression is because of post-transcriptional mechanisms. Experiments intended to determine whether the mechanism involved proteosomal degradation or translational regulation did not provide conclusive results (data not shown). For instance, treatment with the proteosomal inhibitor MG132 increased the expression of Mip/LIN-9 in control cells with no clear effect on B-Myb.

Cell proliferation studies also demonstrated that the absence of Mip/LIN-9 and the decrease in B-Myb and its regulated genes affected the pattern of proliferation of p53-/- MEFs, because clones expressing the antisense construct plateau at lower densities than cells expressing empty vector (Fig. 2D). Moreover, cells carrying the antisense construct remained flat and were not transformed by oncogenic RAS, whereas control cells were spontaneously transformed in culture after a few passages (data not shown). These results suggest that Mip/LIN-9 regulates the expression of B-Myb, as well as S and G2/M genes required for cell proliferation.

Mip/LIN-9 Collaborates with B-Myb in the Induction of Cyclin A and Cyclin B—We next determined the effect of the knockdown of Mip/LIN-9 on cell cycle re-entry of serum-starved cells after being released in growth medium. Although p53-/- MEFs expressing control vector showed limited synchronization, likely due to the known fact that the lack of p53 leads to the acquisition of additional mutations in culture and spontaneous transformation, the induction of cyclin A occurs at 12 h when cells start entering S phase. Similarly, cyclin B is low at 12 and 16 h and increases at 20 h when the number of cells in G2/M doubles. In contrast, cells expressing antisense for Mip/LIN-9 reached S phase with a 4–8-h delay compared with control cells (Fig. 3, bottom). Interestingly, the levels of B-Myb in p53AS cells increased 24 h after release following the increase in Mip/LIN-9. The expression of B-Myb target genes such as cyclin A, cyclin B, and CDK1 (17) was also delayed in cells with depletion of Mip/LIN-9, likely reflecting the low levels of B-Myb expressed in these cells. The expression of cyclin A at 20 h after release, when B-Myb is not detectable, likely reflects the regulation by B-Myb-independent pathways. Importantly, the expression of CDK4, CDK2, or tubulin, which are not regulated by B-Myb, remain basically unchanged demonstrating that depletion of Mip/LIN-9 specifically affects these B-Myb-dependent genes.

One important issue to address is whether the Mip/LIN-9 knockdown directly decreases B-Myb expression resulting in lower levels of B-Myb target genes and slower cell proliferation, or the down-regulation of B-Myb and its transcriptional targets is the consequence of a decrease in cell proliferation produced by Mip/LIN-9 knockdown by other mechanisms. If the first scenario were true, it would be expected that overexpression of Mip/LIN-9 would collaborate with B-Myb in the transcription of B-Myb target genes. To address this question, we studied the activation of two B-Myb-regulated genes (cyclin A and B) by Mip/LIN-9 and B-MYB using promoter-luciferase constructs. We selected for these experiments T98G cells because they lack the CDK4 and -6 inhibitor p16 (40). In this genetic context, the
unchecked activity of CDK4 in T98G inactivates the pRB pathway and eliminates the effect that Mip/LIN-9 has in collaboration with this pocket protein as reported previously (1).

FIGURE 4. B-Myb and Mip/LIN-9 are required for the activation of the cyclin A and B promoters. T98G cells were transfected with the cyclin A (A) or cyclin B (B and C) promoter-luciferase constructs and the indicated combinations of expression vectors encoding B-Myb, Mip/LIN-9, and control as described under “Materials and Methods.” Luciferase activity was assessed 48 h later. C, similar experiments as in B but including the expression of cyclin A. The lower level of luciferase activity detected after cotransfection of Mip/LIN-9 and B-Myb is because of the use of more genes (cyclin A) in these experiments.

pronounced augment in cyclin B promoter-luciferase activity of ~27-fold. Similar results were obtained when these experiments were performed in U2OS cells that also lack p16 (data not shown). Altogether, these data demonstrate that Mip/LIN-9 and B-Myb collaborate in the transcriptional activation of the cyclin A and B promoters and that the activity of this complex is further increased by cyclin A/CDK2.

It has been reported that the cyclin B promoter has an MBS and an activating E2F-response element (17). A closer analysis of the promoter sequence also revealed that there is an additional putative E2F site upstream to the elements reported previously (Fig. 5A, E2Fd). Mutation of the MBS produced between 50 and 75% decrease in promoter activation by Mip/LIN-9 and B-Myb (Fig. 5B). Additionally, the mutation of the previously reported proximal E2F site (Fig. 5B), as well as the putative E2F distal site (data not shown), also reduced promoter activation by 75–80%. These data indicate that the cyclin B promoter requires the integrity of the MBS and E2F sites for efficient activation. Therefore, promoter mutation does not allow us to determine whether the effect of Mip/LIN-9 on transcriptional activation of the cyclin B promoter relies exclusively on the MBS. The requirement of E2F- and Myb-binding sites for transcriptional activation of the cyclin B promoter resembles the scenario previously described for the CDK1 promoter, which is also regulated by E2F and B-Myb (17).

Mip/LIN-9 Occupies the Cyclin A and B Promoters—A previous report demonstrated that B-Myb occupies the cyclin A promoter (17). ChIP analysis of the cyclin A promoter (Fig. 6A) shows that although low levels of B-Myb are associated with this promoter in G0, the maximum promoter binding is
Mip/LIN-9 Is Required for B-Myb Activity

Previous reports indicated that mammalian Mip/LIN-9 collaborates with pRB in transformation (1), and it regulates G1 progression downstream of CDK4/cyclin D (10). The finding that the Drosophila ortholog, Mip130, forms a repressor complex with Dm-myb raised the possibility that in mammalian cells Mip/LIN-9 could also interact with B-Myb. However, it is well established that mammalian B-Myb in concert with activating forms of E2F functions as a transcriptional activator responsible for the induction of genes required for S phase and G2/M (17), thus suggesting a different function for the mammalian Mip/LIN-9-B-Myb complex. Our results demonstrate that endogenous Mip/LIN-9 and B-Myb form a complex in mouse and human cells. B-Myb not only interacts with the 62-kDa form Mip/LIN-9 or long form, which is expressed in mouse and human cells, but also with the 54-kDa splice variant or short form, which lacks exon 4 and is mainly detected in human cells. The finding that significant amounts of Mip/LIN-9 and B-Myb are coprecipitated by anti-B-Myb and Mip/LIN-9 antibodies, respectively, suggests that a significant proportion of both proteins participate in this complex. Importantly, Mip/LIN-9 specifically interacts with B-Myb because no association with c-Myb or A-Myb was observed.

Interestingly, depletion of Mip/LIN-9 in p53−/− MEFs produced a concomitant decrease in the B-Myb protein. The mechanism that leads to the decrease in B-Myb expression is post-transcriptional because no differences in the levels of B-Myb mRNA were observed between control and cells in which Mip/LIN-9 was depleted using an antisense approach. The decrease in Mip/LIN-9 and B-Myb translated into a marked reduction in the expression of S and G2/M genes regulated by E2F and B-Myb such as cyclin A, cyclin B, and CDK1. These changes are responsible for a delay in cell cycle progression and a decrease in cell proliferation characterized by the development of contact inhibition at low densities. Importantly, the coexpression of Mip/LIN-9 and B-Myb, but not either gene alone, was required for a significant transcriptional activation of cyclin A and B promoters indicating that both proteins play a role in the activation of these S and G2/M genes. Moreover, the requirement of both proteins for the induction of cyclin A, cyclin B, and CDK1 explains why these genes were not induced in microarray studies after overexpression of B-Myb alone (15, 41).

The finding that coexpression of cyclin A with Mip/LIN-9 and B-Myb produced an even more dramatic increase in the luciferase activity agrees with previous reports (18–25) indicating that phosphorylation by CDK2/cyclin A plays a major role in the activation of B-Myb-driven transcription. We do not think that CDK2/cyclin A also phosphorylates Mip/LIN-9 because all attempts to show phosphorylation by this cyclin-dependent kinase or even CDK4 have been unsuccessful (data not shown). Thus, it is likely that only B-Myb is the target of CDK2/cyclin A, and the activity of this kinase is essential for the transcriptional activation of the complex because cotransfection of dominant-negative CDK2 completely abrogated transcription observed in S phase and mitosis (Fig. 6A, 16–20 h). Interestingly, Mip/LIN-9 showed a different pattern of promoter occupancy; it associated with the cyclin A promoter in G0 when the levels of this cyclin are very low and then it became undetectable once cells re-enter the cell cycle and finally re-associated with this promoter in S phase (Fig. 6A, 12–16-h time points). Importantly, the highest levels of Mip/LIN-9 promoter occupancy preceded the peak of B-Myb binding and coincided with the initial expression of cyclin A (Fig. 6D, 12 h). However, the highest level of cyclin A protein expression correlates with the peak in B-Myb binding to the cyclin A promoter at the end of S phase (20 h). Concurrently, acetylation of histone 4 associated with the cyclin A promoter increased in S phase (Fig. 6A, 12 h) and remained high until the end of S phase (20 h).

A similar study of the cyclin B promoter (Fig. 6B) shows that very low levels of B-Myb can be detected at early stages of the cell cycle; however, binding increases significantly at the end of S phase as reported previously (17). Surprisingly, occupancy of the cyclin B promoter by Mip/LIN-9 is evident in S phase and barely detectable when maximum binding of B-Myb is observed. As in the case of cyclin A, binding of B-Myb to the cyclin B promoter closely precedes the peak in cyclin B protein expression (Fig. 6D). Importantly, although high levels of acetylated histone 4 are detected in this promoter from late G1 to the end of S phase, the association of Mip/LIN-9 coincides with the peak of promoter-associated histone 4 acetylation. The binding of Mip/LIN-9 and B-Myb to the cyclin A and B promoters is specific because they cannot be detected on an irrelevant promoter such as actin.

Altogether, the data obtained with promoter-luciferase and ChIP assays indicate that Mip/LIN-9 and B-Myb collaborate in the regulation of the cyclin A and B promoters. However, the mechanisms that lead to transcription of cyclins A and B are likely more intricate and may not be explained by a simple model involving the simultaneous binding of an Mip/LIN-9-Myb complex to activate transcription (see “Discussion”).

**FIGURE 6.** Binding of Mip/LIN-9 and B-Myb to the cyclin A and cyclin B promoters. NIH3T3 cells were synchronized by serum starvation for 3 days, released in growth medium, and harvested at the indicated time points. Cell cycle distribution was assessed by FACS (D, bottom), and chromatin was extracted for immunoprecipitation with anti-Mip/LIN-9, anti-B-Myb, or -acetylated histone 4 (ac.H4) antibodies (normal IgG was used as control). ChIP analysis of the cyclin A (A), cyclin B (B), and actin (C) promoters were performed as described under “Materials and Methods.” D, expression of cyclins A, B, and CDK (control) by lysates of synchronized cells was determined by Western blotting with specific antibodies. The cell cycle distribution corresponding to each time point is shown at the bottom. cyc, cyclin; WB, Western blot.
of the cyclin B-luciferase reporter (data not shown), as reported previously for other B-Myb-activated promoters (18–25). The study of the requirement of the MBS for the activation of the cyclin B promoter by Mip/LIN-9 was inconclusive because not only the mutation of this site but also of the two E2F sites severely impairs promoter activation by B-Myb and Mip/LIN-9. This scenario is similar to that reported for the CDK1 promoter, which also requires intact MBS and E2F sites for efficient transcriptional activation (17). However, it is worth mentioning that B-Myb can also activate transcription of promoters that lack an MBS (24, 25) such as the cyclin A promoter (17). The finding that Mip/LIN-9 and B-Myb can occupy the cyclin A promoter suggests that they directly participate in promoter activation via a novel MBS or through the association with other transcriptional regulators. We are currently mapping the elements required for activation of this promoter by Mip/LIN-9 and B-Myb to elucidate the mechanism of transcriptional activation employed by these proteins.

The analysis of the cyclin A and B promoters using ChIP assays revealed interesting differences in the pattern of promoter occupancy by Mip/LIN-9 and B-Myb. Although low levels of B-Myb were detected on the cyclin A and B promoters in G0 and G1, the highest level of promoter binding correlated with the maximum expression of the corresponding proteins in S phase and mitosis. On the other hand, although Mip/LIN-9 was clearly detected on the cyclin A promoter, and to a much lesser extent on the cyclin B promoter, in G0, the association was no longer present once the cells re-enter the cell cycle. The interaction with these promoters reappeared and reached its peak in S phase, followed by a progressive decline as the cells approach mitosis. The association with these promoters in G0 and the lack of promoter occupancy in early G1 is not surprising because Mip/LIN-9 may participate in the repression of E2F-regulated genes in G0/G1, which is negatively regulated by CDK4 (10). However, the finding that binding of Mip/LIN-9 to the cyclin A and B promoters peaked before B-Myb was unexpected given the strong promoter activation produced by overexpression of both proteins. Moreover, in the case of the cyclin B promoter, there is almost no simultaneous association of both proteins with the promoter. These results suggest that the binding of a Mip/LIN-9-B-Myb complex to the promoter to activate transcription may not be sufficient to explain promoter activation.

Different scenarios can explain the sequential association of Mip/LIN-9 and B-Myb to the cyclin A and B promoters. First, Mip/LIN-9 may bind to B-Myb on the cyclin A promoter at the beginning of S phase (see Fig. 6A) to stabilize B-Myb and trigger the initial induction of cyclin A. This first stage is followed by phosphorylation of B-Myb by CDK2/cyclin A leading to stabilization of B-Myb independent of Mip/LIN-9. In this scenario, the activation of cyclin B at a later stage (mitosis) does not depend directly on a Mip/LIN-9-B-Myb complex but rather on the previous induction of cyclin A and B-Myb stabilization. This would explain why B-Myb and Mip/LIN-9 do not occupy the cyclin B promoter simultaneously. An alternative explanation is that Mip/LIN-9 has the dual function of repressing these promoters in early S phase and stabilizing B-Myb. The repression would be released by activation of B-Myb via CDK2 phosphorylation.

This model would imply that mammalian Mip/LIN-9 functions in a similar manner as Drosophila Mip130 where it has been proposed that Dm-Myb releases the repressor effect of Mip130. This model is supported by the finding that the mutation of Mip130 rescues the phenotype produced by the lack of Dm-Myb (18–25), and the B-Myb-binding site may reside within the region missing in the mutation Δ84 of Mip/LIN-9 (10). Finally, a third possibility is that the earlier binding of Mip/LIN-9 to the cyclin A and B promoters produces changes in chromatin structure that facilitate the binding or the activation of transcription by B-Myb. These possibilities are currently under investigation.

In summary, the data presented here demonstrate the following. 1) Mip/LIN-9 is critical for the expression of the B-Myb protein, which is likely through a post-transcriptional mechanism. 2) Both proteins are required for the induction of cyclins A and B and cell cycle progression. 3) The mechanism involves binding of Mip/LIN-9 and B-Myb to the target promoters.

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