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The Retinoblastoma Family Member p107 Binds to B-MYB and Suppresses Its Autoregulatory Activity*

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Arturo Sala‡§, Antonio De Luca‡¶‖, Antonio Giordano‡¶‖, and Cesare Peschle‡ ‡‡**

From the ‡Kimmel Cancer Institute, Department of Microbiology-Immunology and ¶Pathology, Anatomy & Cell Biology, Jefferson Medical College, Philadelphia, Pennsylvania 19107 and the ‡‡Laboratorio di Ematologia Oncologia Istituto Superiore di Sanità, Rome, Italy

It was recently reported that B-MYB can overcome p107-induced growth arrest. Here we show that B-MYB autoregulation of its own transcription is specifically suppressed by p107 and transient transfection assays with p107 deletion constructs determined that the carboxyl terminus of the protein, containing the major pocket region, was associated with inhibition of B-MYB-dependent transactivation. Consistent with these results, co-immunoprecipitation studies showed that p107 interacted *in vivo* with B-MYB through its pocket and carboxyl terminus domain. Thus, B-MYB-dependent promotion of cell proliferation and gene transactivation might be specifically repressed by the growth suppressor p107 through direct interaction with B-MYB.

A growing body of evidence indicates that the product of the B-*myb* gene is directly involved in the control of mammalian cell growth and differentiation (1–5), and a recent study in the developing embryo has revealed that B-*myb* expression is ubiquitous and tightly associated with proliferating tissues (6). However, it is still unclear how B-*myb* exerts its functions, and, although B-MYB is able to bind to promoters containing *myb*-binding sequences, based on its homology to the protooncogene c-*myb*, there is controversial evidence concerning the ability of B-MYB to transactivate the bound promoters. Some investigators proposed that B-MYB is a negative factor, based on experiments showing that B-MYB can suppress c-*myb*-induced transactivation of the MIM-1 promoter or inhibit collagen type 1 expression, through direct binding to *myb* sites located in the promoters (7, 8). It was suggested that B-MYB transactivation is cell type-dependent, requiring the presence of a specific co-factor (9). In a recent study, we have demonstrated that overexpression of a human B-*myb* cDNA promotes DNA synthesis of T98G human glioblastoma cells and that B-MYB can rescue the G1 block imposed by the growth suppressor p107 (10). We have also found that p107 represses transcription of a B-*myb* promoter construct linked to luciferase, suggesting that p107 and B-MYB are functional antagonists (10). These results were consistent with previous studies in which was demonstrated that B-*myb* levels are negatively regulated by growth suppressors, such as p53 and transforming growth factor-β (11, 12). In this report, we provide evidence that B-MYB, similarly to other protooncogenes, is able to transactivate its own promoter; this finding gave us the opportunity to further investigate the functional interaction occurring between B-MYB and p107.

MATERIALS AND METHODS

Plasmids—The plasmids pCMV-B-MYB, pCMVP107, pCMVPRB, pCMVN385, pCMVC768, PGLHBMYB(mut), and PHEBO-B-MYB were all described elsewhere (11, 13–15).

Cell Lines and Transfections—SAOS2 cells and T98G were obtained from ATCC and were passaged and maintained as described (10). T98G cells were stably transfected, according to the calcium phosphate precipitation method, with the vector PHEBO-B-MYB or the parental vector PHEBO, containing the metallothionein promoter. 12 days after transfection, a cell line was established that showed inducible B-*myb* expression upon stimulation with 100 μM $ZnS0_4$. Plain T98G cells or transfected cell lines were cultured in growing medium with 10% FCS[1] or in medium containing 0.1% FCS, that cause the cells to arrest in G0 with down-regulation of endogenous B-*myb* transcription.

Immunoprecipitation and Western Blot—About 1.5×10^6 SAOS2 cells were transfected with 5 μg of CMV-B-MYB and CMVp107 plasmids, and, after 36 h, the cells were lysed in a buffer containing 0.1% Triton-X, 250 mM NaCl, 50 mM Tris, pH 7.4, 1 mM EDTA, protease, and phosphatase inhibitors. Lysates were clarified by centrifugation and incubation with 50 μl of Protein A-Sepharose beads for 30 min at 4 °C. B-MYB antiserum (3) or SD9 (Santa Cruz) antibody was added at a 1:1000 or 1 μg/ml dilution, respectively, and, after 1 h at 4 °C, 30 μl of Protein A-Sepharose was added, and the samples were rocked for an extra hour at 4 °C. Immune complexes were resolved onto a 7.5% Protean mini gel (Bio-Rad) and blotted onto nitrocellulose. Western blots were performed with B-MYB antiserum or SD9 antibody used at a concentration of 1:1000 or 1 μg/ml, respectively, in Blotto (5% dry-milk in phosphate-buffered saline).

RESULTS AND DISCUSSION

In an attempt to delineate genes specifically responsive to B-MYB activity, we transfected T98G human glioblastoma cells with a vector containing the metallothionein promoter driving a human B-*myb* cDNA, and we were able to establish a cell line that presented inducible expression of exogenous B-*myb*. T98G cells are highly dependent on the presence of serum for their growth and when these cells are serum-starved they arrest in G0. In these conditions, expression of growth-regulated genes, such as B-*myb*, is turned off, and we soon discovered that overexpression of the exogenous B-*myb* cDNA resulted in higher levels of endogenous B-*myb* mRNA. This effect was better detected in low serum conditions, when the endogenous B-*myb* message is almost undetectable in the control vector-transfected and plain T98G cell lines (Fig. 1, *A* and *B*). Transient transfection assays with B-*myb* promoter constructs driving the luciferase gene indicated that B-MYB was indeed able to transactivate its own promoter through a DNA binding-independent mechanism that involves direct interaction with

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§ To whom correspondence should be addressed: Kimmel Cancer Institute, Thomas Jefferson University, BLSB Building, Rm. 528, 233 S. 10th St., Philadelphia, PA 19107. Tel.: 215-503-1763; Fax: 215-923-0249.

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[1] The abbreviations used are: FCS, fetal calf serum; CMV, cytomegalovirus.

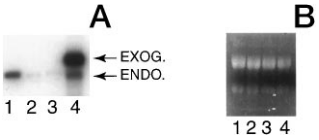

FIG. 1. **Endogenous B-*myb* transcript is up-regulated in T98G cells conditionally expressing a human B-*myb* cDNA.** Total cellular RNA was extracted with the guanidine-phenol method, run onto a 1% agarose gel, and blotted onto nitrocellulose. The whole human B-*myb* cDNA, labeled with ^{32}P, was used as a probe. *Lane 1*, T98G cells in growing medium; *2*, T98G cells in medium with 0.1% FCS; *3*, T98G transfected with empty vector in 0.1% FCS containing 100 μM $ZnSO_4$; *4*, T98G cells transfected with PHEBO-B-MYB vector in 0.1% FCS containing 100μM $ZnSO_4$. Endogenous and exogenous B-*myb* transcripts are indicated by the *arrows*. *B*, ethidium bromide staining of the gel shown in *A* to demonstrate equal loading of the lanes.

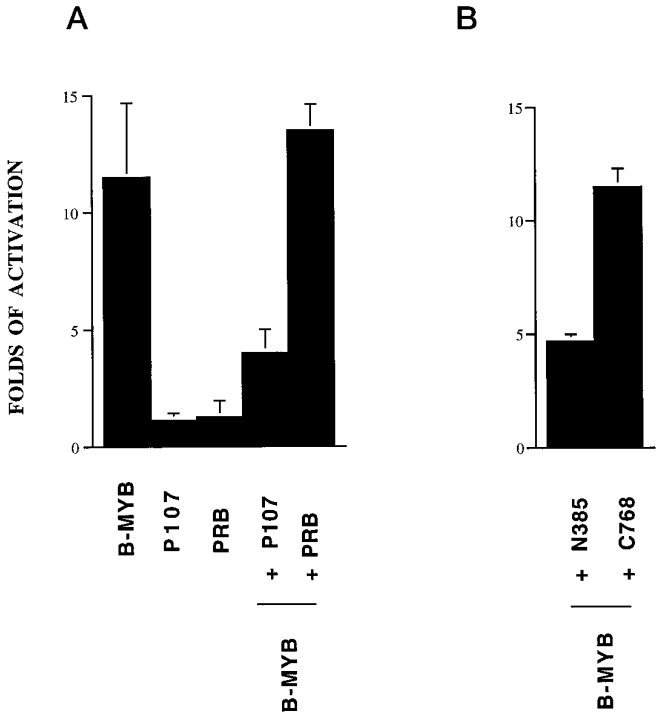

FIG. 2. **p107 suppresses B-MYB-dependent transactivation of the B-*myb* promoter.** *A*, 1 μg of pGLBMYB(mut) vector was cotransfected with 1 μg of CMV-B-MYB with or without 1 μg of CMV-pRb or CMV-p107 in SAOS2 cells. *B*, 1 μg of pGLBMYB(mut) vector was cotransfected with 1 μg of CMV-B-MYB with or without 1 μg of CMV-N385 or CMV-C768 in SAOS2 cells. The counts/min corresponding to the promoter plus empty CMV vector were assumed to be 1, and values were expressed as folds of activation over this arbitrary value. Transfections in *A* and *B* are representative of the same set of experiments; therefore, the data are directly comparable. Transfections were performed by the calcium phosphate method in 35-mm wells, and luciferase assay was performed according to the manufacturer's instructions (Promega). Each set of experiments was performed in triplicate and repeated six times. Standard deviations are indicated by the *error bars*.

other factors.[2] Because in previous experiments it was determined that p107 can functionally antagonize B-MYB activity (10), we investigated whether the B-*myb* autoregulatory mechanism was affected by abnormal levels of p107. p107, but not pRb, was able to inhibit B-MYB-induced transactivation of a B-*myb* promoter construct harboring a mutated E2F site (Fig. 2*A*). The mutant promoter was used to avoid inhibition through the E2F pathway (10), and SAOS2 cells were used because it was reported that T98G cells are refractory to p107 activity (13, 16). Mutant N-385, that lacks the amino terminus but contains the major pocket region of p107 protein, suppressed B-MYB

[2] A. Sala and C. Peschle, manuscript in preparation.

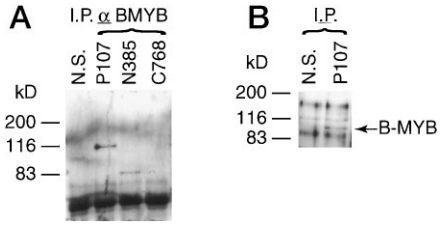

FIG. 3. **B-MYB protein binds to the pocket region of p107.** *A*, cells were transfected with p107 or p107-deletion mutants, and immunoprecipitation was carried out with anti-B-MYB antibody as described under "Materials and Methods." Lanes corresponding to the different transfections are indicated on the *top* of the gel. *N.S.* indicates extracts from cells transfected with p107 and incubated with normal rabbit serum. Wild-type and mutant p107 proteins were evidenced by Western blotting with SD9 antibody, purchased from Santa Cruz. *B*, cells were transfected with p107 and lysates were prepared as described in *A*. Immunoprecipitation (*I.P.*) was performed with anti-p107 antibody (SD9) or control antibody (indicated by *N.S.*) followed by Western blot with anti-B-*myb* antibody.

transactivation, similarly to its wild-type counterpart; in contrast, mutant C-768, that lacks the carboxyl terminus and part of the pocket, lost the suppressive activity (Fig. 2*B*). These results might suggest that p107 directly interacted with B-MYB through its pocket domain. In fact, co-immunoprecipitation studies performed with transiently transfected SAOS2 cells confirmed that p107 interacted directly with B-MYB through the carboxyl terminus and pocket region, as shown by the ability of B-MYB to bind wild type p107 and mutant N-385, but not mutant C-768 (Fig. 3*A*). Ectopic expression of mutant C-768 was readily detected by Western blotting transfected SAOS2 cell lysates with the anti-p107 antibody SD9 (not shown). These results were confirmed by the ability of SD9 antibody to pull down B-MYB protein (Fig. 3*B*). We were able to detect an interaction in SAOS2 cells only by overexpressing both p107 and B-MYB, probably due to the very low endogenous protein levels in these cells. Further experiments with other cell lines will be necessary to detect an interaction in conditions that do not require protein overexpression.

In a previous study we reported that p107 inhibits wild-type B-MYB promoter transcription (10). These results were consistent with studies conducted on the human B-*myb* promoter showing that an E2F site, containing p107, conferred growth-regulated activity to the promoter (14). In the light of the present data, we speculate that, in addition to mechanisms involving E2F members, p107 might control B-*myb* promoter transcription via direct binding to B-MYB protein, resulting in suppression of B-*myb* promoter autoregulation. The direct binding of B-MYB to p107 would also suggest a mechanism by which B-MYB rescues the block of cell proliferation induced by p107. This model is reminiscent of the interactions occurring between p107 and E2F, and it is of interest to note that, similarly to E2F, B-MYB binds to the pocket region of p107. Several studies have demonstrated that B-MYB is required for the growth and survival of mammalian cells. This study further supports the notion that B-MYB must be subjected to the stringent control of growth suppressor genes, such as p107, indicating that their negative effects on cell proliferation might occur, at least in part, through inhibition of B-MYB activity.

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