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

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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-myc 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-myc expression is ubiquitous and tightly associated with proliferating tissues (6). However, it is still unclear how B-myc 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-myc, 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-myc-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-myc cDNA promotes DNA syn-

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MATERIALS AND METHODS

Plasmids—The plasmids pCMV-B-MYB, pCMVP107, pCMVPBR, pCMVNS85, 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-myc expression upon stimulation with 100 μM ZnSO4. Plain T98G cells or transfected cell lines were cultured in growing medium with 10% FCS or in medium containing 0.1% FCS, that cause the cells to arrest in G0 with down-regulation of endogenous B-myc transcription.

Immunoprecipitation and Western Blot—About 1.5 × 106 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. T98G anti-serum (3) or SD9 (Santa Cruz) antibody was added at a concentration of 1:1000 or 1:50 for 1 h at 4 °C, 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% SDS-polyacrylamide gel and electrotransferred to nitrocellulose. Western blots were performed with B-MYB antisera 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-myc cDNA, and we were able to establish a cell line that presented inducible expression of exogenous B-myc. 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-myc, is turned off, and we soon discovered that overexpression of the exogenous B-myc cDNA resulted in higher levels of endogenous B-myc mRNA. This effect was better detected in low serum conditions, when the endogenous B-myc message is almost undetectable in the control vector-transfected and plain T98G cell lines (Fig. 1, A and B). Transient transfection assays with B-myc 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...
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 32P, was used as a probe. Lane 1, T98G cells in growing medium; 2, T98G cells in medium with 0.1% FCS, 3, T98G cells transfected with empty vector in 0.1% FCS containing 100 μM ZnSO4; 4, T98G cells transfected with PHEBO-B-MYB vector in 0.1% FCS containing 100 μM ZnSO4. Endogenous and exogenous B-myb transcripts are indicated by the arrows.

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-RB or CMV-p107 into 293T cells. B, 1 μg of pGLBMYB(mut) vector was cotransfected with 1 μg of CMV-B-MYB with or without 1 μg of CMV-RB or CMV-N385 or CMV-C768 into 293T cells. The counts/min corresponding to the promoter plus empty 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.

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. 2B). 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. 3A). 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. 3B). 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 regulation through 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 autoregulation. The direct interaction between p107 and B-MYB was shown by protein pull-down assays and by competitive binding assays.

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