

B-*myb* proto-oncogene products interact in vivo with each other via the carboxy-terminal conserved region

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Abstract Using the yeast two-hybrid assay and in vivo binding assay, we investigated whether B-*myb* oncogene products (B-*myb*) can associate with each other. Specificity tests of the yeast two-hybrid system showed a self-association of B-*myb* proteins in yeast. Cotransfection experiments demonstrated that B-*myb* proteins form a complex in vivo. Deletion analysis revealed that this binding was sufficiently mediated by the carboxy-terminal conserved region of B-*myb*. In addition, the B-*myb* self-association is directly dependent on the amount of expressed B-*myb* in cells and slightly increased by the dephosphorylation state. These results suggested that B-*myb* could form a complex and influence its transcriptional activity.

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Key words: B-*myb* oncogene; Self-association; Conserved region

1. Introduction

B-*myb* is a member of the *myb* family of transcription factors including A-*myb* and c-*myb* [1]. A number of lines of evidence indicate that B-*myb* is important in controlling cell proliferation and differentiation [2–5]. In contrast to the restricted expression of c-*myc*, the most characterized member of the family, B-*myb* expression is more ubiquitous and is cell cycle-dependent [1,6]. Expression of B-*myb* is down-regulated in resting cells but is significantly induced during G₁/S transition and the S phase of the cell cycle. B-*myb* expression is decreased during terminal differentiation of promyelocytic and neuroblastoma cell lines [4,7,8], and the proliferation of human leukemic cells is inhibited by introduction of antisense B-*myb* oligonucleotides [7]. Consistent with these observations, overexpression of B-*myb* was found to overcome G₁ blocks mediated by checkpoint regulators such as p53 and p107 [5,9]. In addition, recent studies suggested that B-*myb* function is associated with the presence of cofactors such as cyclin A and cyclin E and is dependent on its phosphorylation [10,11]. These data strongly indicate that B-*myb*, like c-*myb*, plays a fundamental role in cellular proliferation.

B-*myb* has three functional domains for transactivation [12]: a DNA binding domain, an acidic region for transcriptional activation, and a carboxy-terminal conserved region which is a proline-rich domain and shows significant homology with other members of the *myb* gene family such as A-*myb* and c-*myb*. The mechanism of B-*myb* transcriptional activity has not been elucidated, but a recent study apparently

indicated that the conserved region is critical for transactivation by B-*myb*, presumably via the binding of some cofactors that may be involved in the modification and activation of B-*myb* [13]. The conserved region was suggested to be a protein binding domain and a putative phosphorylation site. Using an affinity resin, analysis of the metabolically labeled CV-1, HeLa and/or NIH3T3 cells showed that several cellular proteins were bound to the conserved region in a cell type-specific manner [13]. In addition, recent studies showed that the cyclin A-cdk2 complex could induce phosphorylation of B-*myb* and potentiate the transcriptional activity of B-*myb* [10,11,14]. In view of the multiplicity of protein-protein interactions in many transcription factors containing basic helix-loop-helix (b-HLH) and leucine zipper (LZ) motifs, the possibility of investigating the dimerization and/or oligomerization of B-*myb* in vivo is attractive since the biological importance of and structural similarities in the carboxy-terminus of the *myb* family suggest that the conserved region containing many proline residues and a putative ankyrin repeat-like motif may function for protein-protein interactions.

In this study, we report a self-association of B-*myb* in vivo. Deletion studies demonstrate that the carboxy-terminal conserved region is critical for B-*myb* self-association. Moreover, the B-*myb* self-association is regulated by both the degree of expression and the phosphorylation state, at least in part, of B-*myb*. These results suggest that the self-association is critical for modulation of the transactivation by B-*myb*.

2. Materials and methods

2.1. Cell lines and reagents

293T cells, a derivative of human kidney embryonal fibroblast containing SV40 T antigen, were obtained from Dr. E.S. Jun (Korea Research Institute of Science and Technology, Taejeon, South Korea) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin-streptomycin and 1 mM glutamine in an atmosphere of 5% CO₂ at 37°C. The anti-FLAG (M2) antibody, dithiothreitol (DTT), aprotinin, and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma (St. Louis, MO, USA). The mouse hybridoma cell line producing anti-GST antibody was generously provided by Dr. D.S. Im (Korea Research Institute of Science and Technology, Taejeon, South Korea). Polyvinylidene difluoride (PVDF) membrane was purchased from Millipore Corp. (Bedford, MA, USA). Oligonucleotides were synthesized from Bioneer Corp. (Cheongwon, Chungbuk, South Korea).

2.2. Cloning of a full-length human B-*myb* cDNA

A partial B-*myb* cDNA (pJG4-5-B-*myb*) obtained from a HeLa cell cDNA library was kindly provided by Dr. Y. Yang (Korea Research Institute of Science and Technology, Taejeon, South Korea). A human keratinocyte cDNA library [15] was screened using pJG4-5-B-*myb* as a probe. Positive clones, contained within the pBluescript SK[−] phagemid, were excised in vivo from the Uni-ZAP XR vector using Ex-Assist helper phage system (Stratagene, La Jolla, CA, USA), following the protocol recommended by the manufacturer. The largest clone

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(~2.8 kb) containing the full-length cDNA for human B-myb (accession number X13293) was chosen and sequenced on both strands.

2.3. Expression constructs

A partial B-myb cDNA (pJG4-5-B-myb) was subcloned as an *EcoRI/XhoI* fragment into the *EcoRI/SalI* site of pFLAG-CMV-2. The eukaryotic glutathione *S*-transferase (GST) expression vector (pEBG) was kindly supplied by Dr. E.S. Jun. pEBG-B-myb, an amino-terminally truncated version containing part of the acidic region and a complete conserved region, and pEBG-WT-B-myb containing a full-length B-myb cDNA were constructed in several steps. We first cloned the *EcoRI/XhoI* fragment of B-myb cloned in pJG4-5 into pBacPAK9 (Clontech, Palo Alto, CA, USA), digested it with *EcoRI* plus *NotI* and subcloned it into pBluescript KS (Stratagene, La Jolla, CA, USA). Finally, the *ClaI/NotI* fragment of the resulting plasmid was cloned into pEBG cut with *ClaI* and *NotI*, yielding pEBG-B-myb. The *HindIII/BamHI* fragment of pFLAG-WT-B-myb (see below) was used to generate pEBG-WT-B-myb. To generate two deletion constructs, pEBG-B-mybR1 and pEBG-B-mybR2, and a wild-type B-myb construct, pFLAG-WT-B-myb, we performed a PCR using the full-length B-myb cDNA as the template. The forward primers for B-mybR1 (5'-GCGAATTCCTGAACCT CTGGAACAAA-3'), B-mybR2 (5'-GCGAATTCAGGAGG TGCTGCTTCT-3'), and WT-B-myb (5'-GCAAGCTTATGTCTCGGCGGACGCGC-3') contain an *EcoRI* and a *HindIII* site (underlined). The reverse primers for B-mybR1 and B-mybR2 (5'-GCCTCGAGCTGGGC CACTGCTGCCTT-3') and WT-B-myb (5'-GCGAATTCCTCCAAGGGCT-3') contain a *XhoI* and an *EcoRI* site (underlined). The amplified PCR products for deletion mutants were cut with *EcoRI* plus *XhoI* and cloned into pEBG as described above. The pFLAG-WT-B-myb was generated after subcloning of the *HindIII/EcoRI* PCR fragment into the *HindIII/EcoRI* site of pFLAG-CMV-2 containing an *EcoRI/XhoI* fragment of B-myb (nucleotides 930–2627). The identity of all PCR products was confirmed by nucleotide sequencing analysis on both strands with the T7 Sequencing kit (Pharmacia).

2.4. Yeast two-hybrid assay

The 353 amino acids (nucleotides 1010–2068) of the human B-myb were cloned in frame into the LexA coding sequence to generate a bait plasmid, pEG202-B-myb. pEG202-B-myb and a fish plasmid, pJG4-5 harboring B-myb, were introduced into a yeast strain containing a chromosomal copy of the *Leu2* gene (EGY48 (Mat α trp1 ura3-52 leu2::pLeu2-lexAop6(Δ UAS leu2)) and an episomal β -galactosidase gene under the control of a synthetic promoter with LexA binding

Baita	Fish	B-myb
PTP-BAS		+++
B-myb		++
H-PTP-k		–
MPK38		–
KIAA0175		–
pag		–
elastase inhibitor		–

Fig. 1. B-myb gene products interact with each other in the yeast two-hybrid system. Specificity test for determining the specificity of interactors was performed on yeast expressing the B42 transactivation domain fused to B-myb proteins (fish) and indicated heterologous LexA-fused baits. Transformants were assayed for β -galactosidase activity by the colony color assay. The colony color assay showed either white (–), blue (++) or dark blue (+++) colonies. References of sources of the analyzed baits are as follows: PTP-BAS [17]; B-myb [1]; R-PTP-k [15]; MPK38 [21]; KIAA0175, a human counterpart of MPK38 [22]; pag [23]; monocyte/neutrophil elastase inhibitor [24].

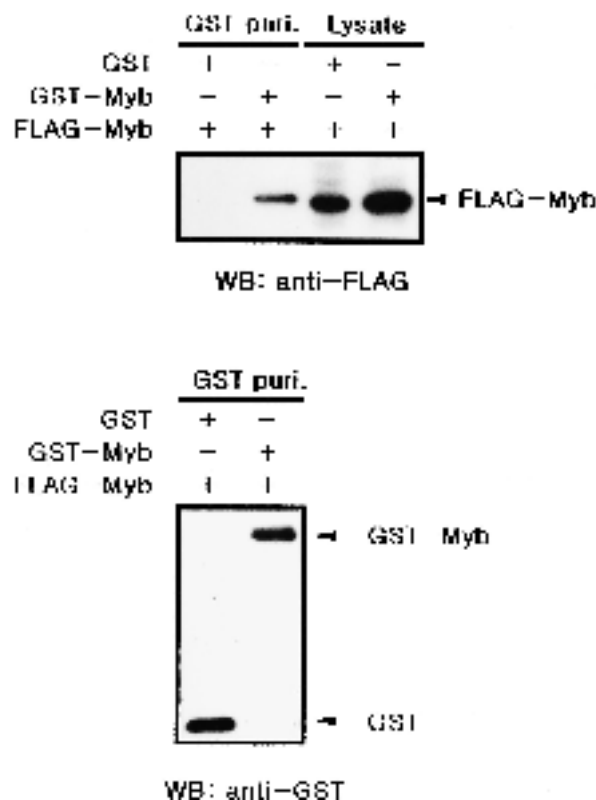


Fig. 2. In vivo self-association of B-myb proteins. GST alone (pEBG), as a control, and pEBG-WT-B-myb were cotransfected with pFLAG-WT-B-myb into 293T cells by Lipofectin. After 48 h, cells were extracted in a lysis buffer containing 0.1% Nonidet P-40. GST fusion proteins were purified on glutathione-Sepharose beads (GST puri.) and analyzed on a SDS-polyacrylamide gel, and the complex formation (upper left) and the FLAG-tagged B-myb (FLAG-Myb) of the amount used for the in vivo binding assay (upper right) were determined by anti-FLAG antibody immunoblot. The same blot was stripped and re-probed with an anti-GST antibody (lower panel) to confirm expression of the GST fusion protein (GST-Myb) and the GST control (GST).

sites [16]. The β -galactosidase activity was used for selection of proteins interacting with the B-myb.

2.5. Cotransfection and in vivo interaction assay

293T cells grown in DMEM supplemented with 10% FBS were plated in 6-well flat-bottomed microplates (Costar, Cambridge, MA, USA) at a concentration of 2×10^5 cells per well the day before transfection. 1 μ g of each plasmid DNA was transfected into 293T cells with Lipofectin (Life Technologies, Inc.). Forty-eight hours after transfection, cells were washed three times with ice-cold phosphate-buffered saline and solubilized with 100 μ l of lysis buffer (20 mM HEPES (pH 7.9), 10 mM EDTA, 0.1 M KCl, and 0.3 M NaCl) containing 0.1% Nonidet P-40, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 10 mM sodium fluoride, 2 μ g/ml α -1-antitrypsin, 2 mM sodium pyrophosphate, 25 mM sodium β -glycerophosphate, 1 mM sodium orthovanadate, and 1 mM PMSF. Detergent-insoluble materials were removed by centrifugation at 13000 rpm for 15 min at 4°C. Approximately 80 μ l of the cleared lysates were mixed with 15 μ l of glutathione-Sepharose beads (Pharmacia, Uppsala, Sweden) and rotated for 2 h at 4°C. Beads were washed three times with the lysis buffer. The bound proteins were eluted by boiling in SDS sample buffer, subjected to SDS-polyacrylamide gel electrophoresis, and then transferred to PVDF membranes. The membranes were probed with an anti-FLAG (M2) antibody and then developed using an enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia Biotech). The relative quantity of the level of B-myb self-association was estimated using a densitometer (Molecular Dynamics) and ImageQuant software.

3. Results

3.1. *B-myb* gene products interact with each other in vivo

B-myb was initially isolated in a two-hybrid screen for a protein interacting with the PDZ domain of PTP-BAS, a protein tyrosine phosphatase [17,18] (data not shown). To verify the interaction specificity of *B-myb*, the *B-myb* library plasmid was rescued from the galactose-dependent $\text{Leu}^+/\text{lacZ}^+$ yeast strain and reintroduced into the original selection PTP-BAS bait strain as well as the other strains containing different baits available in our laboratory, including *B-myb* bait. From transformants, *B-myb* cDNA was found to interact with the *B-myb*

and PTP-BAS but not with the other baits containing unrelated sequences (Fig. 1). To determine whether the self-association of *B-myb* could occur in vivo, we performed cotransfection experiments using GST- and FLAG-tagged eukaryotic expression vectors. In these experiments, the wild-type *B-myb* was coexpressed as a GST fusion protein and a FLAG-tagged protein in 293T cells. The interactions of FLAG-tagged *B-myb* proteins to the GST-*B-myb* fusion proteins were analyzed by immunoblotting with an anti-FLAG antibody. As shown in Fig. 2, the FLAG-tagged *B-myb* was coprecipitated with GST-*B-myb* but not with GST alone used, indicating that these *B-myb* proteins physically interact with each other in

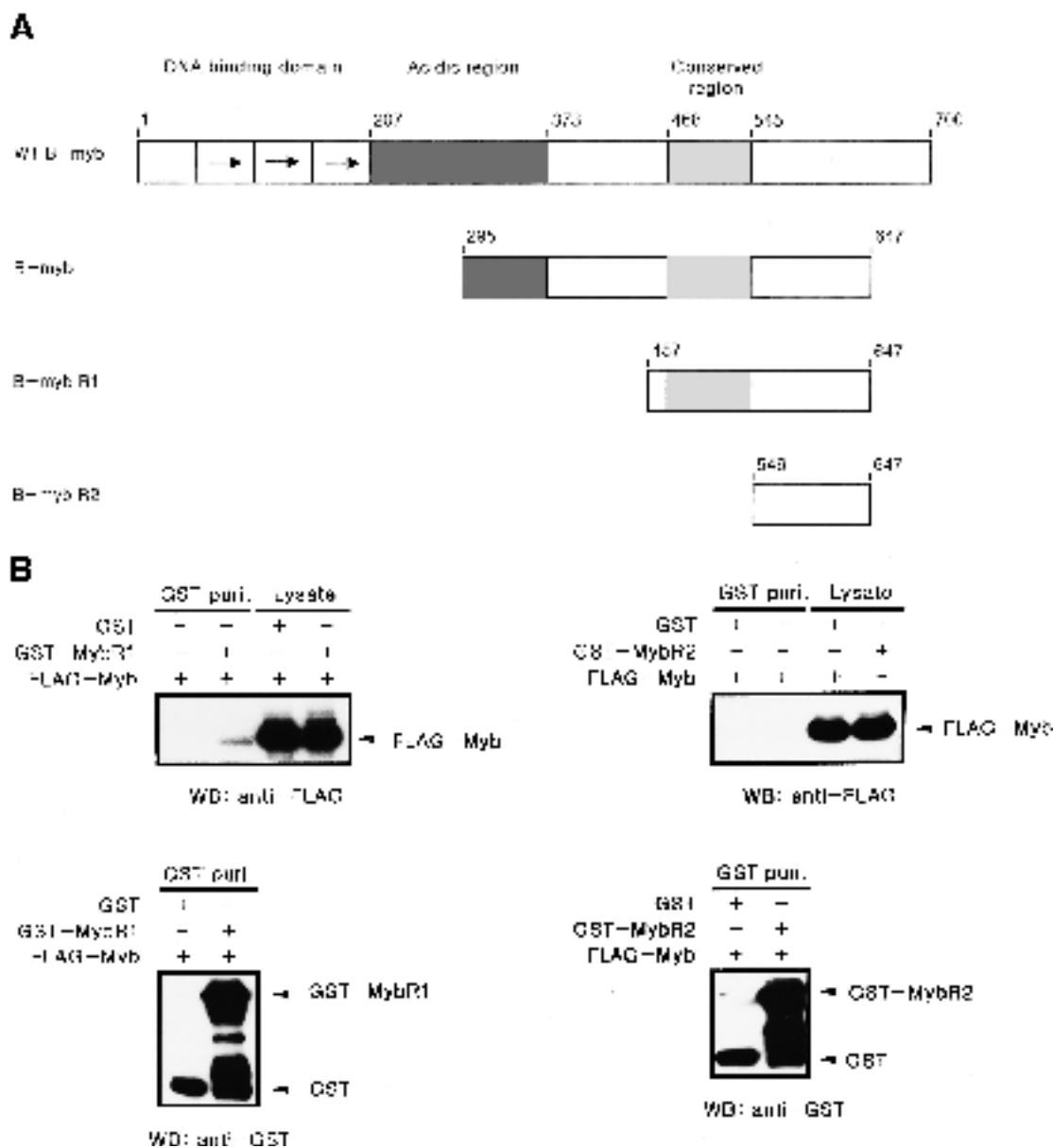
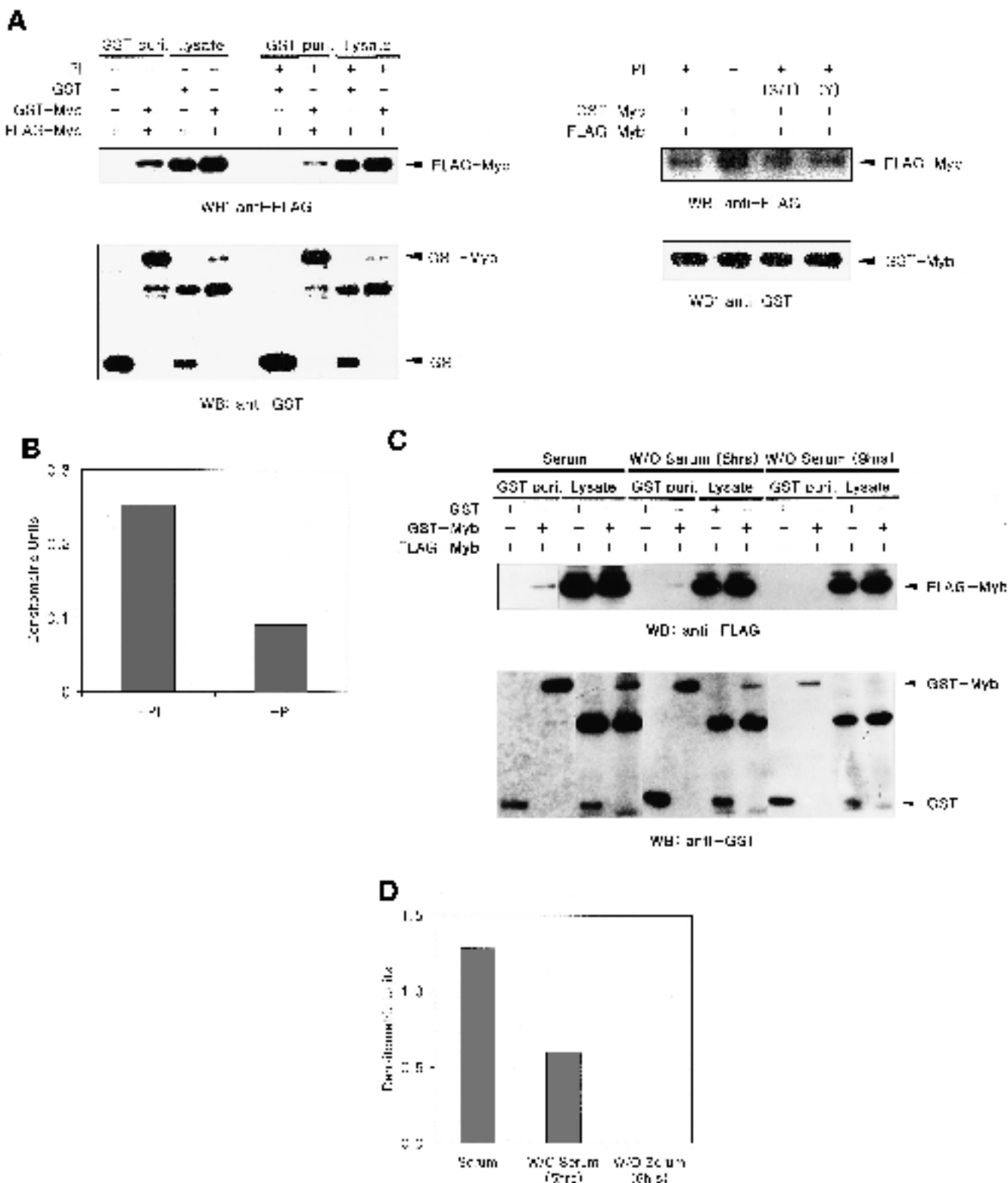


Fig. 3. Immunoblot analysis of the deletion mutants of the *B-myb*. A: Schematic representation of *B-myb* deletion mutants, *B-myb*R1 and *B-myb*R2. The structure of the wild-type human *B-myb* is depicted with the relative locations of its DNA binding domain, acidic region, and conserved region indicated. Amino acid number of domain boundaries is indicated. Arrows represent the three tandem repeats in the DNA binding domain. B: Mapping of the site on *B-myb* involved in the self-association. 293T cells were cotransfected with GST alone (pEBG), pEBG-*B-myb*R1 (GST-MybR1), or pEBG-*B-myb*R2 (GST-MybR2), together with pFLAG-*B-myb* (FLAG-Myb). Transfected cells were extracted and purified with glutathione-Sepharose beads, and immunoblotted with an anti-FLAG antibody as in Fig. 2. The purified GST fusion proteins (upper left, lower) and the total cell lysates (upper right) were analyzed by immunoblotting with anti-GST (lower panel) and anti-FLAG antibodies (upper panel).



mammalian cells. Expression of amounts of GST- and FLAG-tagged *B-myb* proteins (Fig. 2, lower and upper right, respectively) in cells was also confirmed by protein immunoblot analysis with the antibodies to GST and FLAG.

3.2. Mapping of the region of *B-myb* for self-association

To determine which regions of *B-myb* were required for

self-association observed *in vivo*, we generated two deletion constructs (Fig. 3A) and transformed them into yeast with *B-myb*, together with a *B-myb* bait harboring part of the acidic region and a complete conserved region. In the yeast two-hybrid interaction studies, compared with the pEG202:*B-myb*, the pEG202:*B-myb*R1 including a carboxy-terminal conserved region displayed a comparable interaction with

Fig. 4. B-myb self-association is regulated by phosphorylation and serum deprivation. A: Effect of phosphorylation on the self-association of B-myb. 293T cells were cotransfected with GST alone (pEBG) or pEBG-WT-B-myb (GST-Myb), together with pFLAG-WT-B-myb (FLAG-Myb). Cell extracts were prepared in the presence (+PI) and absence (–PI) of phosphatase inhibitors such as sodium pyrophosphate (2 mM), sodium β -glycerophosphate (25 mM), and sodium orthovanadate (1 mM). The purified GST fusion proteins (GST puri.) and the total cell lysates (Lysate) were analyzed by immunoblotting with anti-GST (lower panel) and anti-FLAG antibodies (upper panel). To differentiate phosphatase inhibitors (right panel), the purified GST fusion protein extracts were prepared in the presence of the serine/threonine (S/T) or tyrosine (Y) phosphatase inhibitors. B: Densitometric analysis of B-myb self-association levels (A, upper GST puri.) in the presence and absence of the phosphatase inhibitors. C: Effect of serum deprivation on the self-association of B-myb. 293T cells were transiently cotransfected as described in A. Cells were incubated in the presence (Serum) and absence (W/O Serum) of serum for the indicated times (5, 9 h) prior to transfection. D: Densitometric analysis of B-myb self-association levels (C, upper GST puri.) in the presence and absence of serum. In B and D, the level of B-myb self-association, in arbitrary units, is shown in the graph, after densitometric reading of the autoradiograms and normalization for GST-Myb (lower GST puri.) and FLAG-Myb (upper Lysate) protein levels.

the B-myb, but barely detectable with pEG202:B-mybR2 made by the deletion of a further 89 amino acids (data not shown). These data indicate that the integrity of the carboxy-terminal conserved region is important for B-myb self-association. Using *in vivo* binding assays, we subsequently confirmed that the conserved region is necessary for the self-association of B-myb *in vivo*. As demonstrated in Fig. 3B, we failed to detect association of FLAG-tagged B-myb with the transfected GST-B-mybR2 in 293T cells, but the binding between the FLAG-tagged B-myb and GST-B-mybR1 became readily detectable. These results clearly define the conserved region as essential for B-myb self-association *in vivo* and strongly indicate that the region conserved between *c-myb*, *A-myb* and *B-myb* may act as a potential binding site for some cofactors critical for the transactivation by B-myb.

3.3. Modulations of B-myb self-association

It has been shown that B-myb is specifically phosphorylated by the cyclin A/cdk2 complex during S phase and potentiates its transcriptional transactivation function [10,14,20]. These pieces of evidence led us to investigate the requirement of phosphorylation in the self-association of B-myb. Compared with the control extracts, which were treated with the tyrosine and serine/threonine phosphatase inhibitors, cell extracts prepared without phosphatase inhibitors slightly increased complex formation, indicating a minor role for dephosphorylation in the regulation of B-myb self-association (Fig. 4A,B). Moreover, to distinguish between Ser/Thr and Tyr phosphorylation, different phosphatase inhibitors were employed. As shown in Fig. 4A, we observed that both Ser/Thr and Tyr phosphorylation are important in the regulation of B-myb self-association since the Ser/Thr or Tyr phosphatase inhibitors alone were not sufficient to influence the B-myb self-association. Therefore, it seems reasonable to speculate that the phosphorylation state of the conserved region, although to a somewhat lesser extent, may be involved in the self-association of B-myb compared to the effect of the conserved region itself. It has been demonstrated previously that *B-myb* mRNA expression is strictly regulated in cycling cells [8,19]. Therefore, we assessed whether the cell cycle progression can influence the B-myb self-association in cells following serum withdrawal. As illustrated in Fig. 4C,D, after serum starvation, we observed a slight decrease in the levels of B-myb self-association in a time-dependent manner and did not find a detectable level of B-myb self-association after 9 h starvation prior to transfection, whereas self-association was weakly observed after 5 h starvation. Expression of amounts of the GST- and FLAG-tagged B-myb precipitated by glutathione-Sepharose beads in 293T cells was confirmed by immunoblotting

with either an anti-FLAG antibody (Fig. 4C, upper panel) or an anti-GST antibody (Fig. 4C, lower panel). After 9 h starvation, although there was no detectable B-myb self-association, the expression of the GST- and FLAG-tagged B-myb was easily observed from the coprecipitates with glutathione-Sepharose beads and the cell lysates, respectively (Fig. 4C, lower right and upper right). It is evident from this experiment that, although the kinetics of withdrawal of cells from the cell cycle by serum starvation do not directly correlate with the reduction in the amounts of the expressed *B-myb* proteins, this association is a biologically relevant low affinity interaction and dependent on the *B-myb* protein levels in cells.

4. Discussion

In this study, we demonstrated that *B-myb* proteins interact with each other via the carboxy-terminal conserved region *in vivo*, and that the self-association of B-myb is apparently dependent on its expression levels. Furthermore, B-myb self-association is, at least in part, affected by changes in the phosphorylation state of B-myb.

Comparison of known sequences of the *myb* gene family such as *c-myb*, *A-myb*, and *B-myb* indicates that all three members of the *myb* gene family share a significant degree of homology conserved in evolution, suggesting that the conserved region is likely to be an important determinant for Myb activity in cells. Recently, it was postulated that B-myb might function as a transcriptional activator or repressor depending on the type of cells in various tissues. Currently there is still little information available on the mechanism of regulating the specificity of B-myb transcriptional activity, but analyses of the carboxy-terminal conserved region of B-myb provide evidence that the cellular factors bound to the conserved region are critical for the cell type-specific transcriptional activity of B-myb [13]. These findings, together with the presence of many proline residues in the conserved region, led to the hypothesis that the conserved region may be involved in protein-protein interactions, and eventually modulates the DNA binding and transcriptional activation of B-myb. To test this hypothesis, we prepared a mutant *B-myb* protein lacking the conserved region. *In vivo* binding assays, as demonstrated in Fig. 3B, this protein fails to interact with B-myb. On the other hand, B-myb:B-myb complexes are generated in both the wild-type and the mutant protein containing only the conserved region, indicating that the conserved region may be a major determinant for association with *B-myb* proteins alone.

It has been shown that B-myb is phosphorylated at specific sites in the conserved region during S phase and that the

modification of B-myb, by S phase-specific kinases such as cyclin A/cdk2 complex, may be involved in the B-myb function [14,20]. Thus, we were interested in examining the possibility that the phosphorylation of B-myb might be associated with regulation of B-myb self-association. As illustrated in Fig. 4A,B, the degree of B-myb self-association was slightly increased in the absence of the phosphatase inhibitors compared to the control. These results indicate a possible role for Ser/Thr and/or Tyr phosphorylation in the regulation of B-myb self-association. In our experiments on the serum effect, as shown in Fig. 4C,D, cotransfection of GST- and FLAG-tagged B-myb leads to a dose-dependent increase in the complex formation of B-myb due to the increased expression of exogenous B-myb in the presence of serum. To further investigate whether the expressed B-myb protein levels could influence the self-association, we performed a dose-dependence experiment by decreasing the expression plasmid (data not shown). This study clearly demonstrated that B-myb self-association is dependent on the amounts of the expressed B-myb proteins in cells. One possible explanation for this phenomenon is that a considerable amount of B-myb proteins is required for the self-association since the conserved region thought to mediate this interaction is possibly involved in interactions with other cellular proteins which compete with B-myb proteins for binding.

Based on our observed results, we imagine that rather than directly controlling the transcriptional activity of B-myb, the self-association likely plays a role that is important in fine-tuning the regulation of transactivation by B-myb, probably by competing with the conserved region binding proteins in cells. We also consider that, in addition to the self-association, the conserved region of B-myb may apparently participate in association with other cellular proteins. This would be analogous to the suggested mechanisms by which the cellular co-factors bound to the conserved region of B-myb are critical for the cell type-specific transactivation by B-myb [13]. In this context, the further identification and characterization of the interacting proteins associated with the conserved region will be important to understanding the molecular mechanisms and functions of the *myb* gene family.

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