Increased Affinity of c-Myb for CREB-binding Protein (CBP) after CBP-induced Acetylation

Yuji Sano‡§ and Shunsuke Ishii‡¶

From the %Laboratory of Molecular Genetics, RIKEN Tsukuba Institute and the %CREST (Core Research for Evolutional Science and Technology) Research Project of JST (Japan Science and Technology Corporation), 3-1-1 Koyadai, Tsukuba, Ibaraki 305-0074, Japan.

The c-myb proto-oncogene product (c-Myb) is a sequence-specific DNA-binding protein that functions as a transcriptional activator. The transcriptional coactivator CREB-binding protein (CBP) binds via its KIX domain to the activation domain of c-Myb and mediates c-Myb-dependent transcriptional activation. CBP possesses intrinsic histone acetyltransferase activity, and can acetylate not only histones but also certain transcriptional factors such as GATA1 and p53. Here we demonstrate that the C/H2 domain of CBP, which is critical for the acetyltransferase activity, also directly interacts with the negative regulatory domain (NRD) of c-Myb. Consistent with this observation, CBP acetylated c-Myb in vitro at Lys438 and Lys441 within the NRD. In addition, CBP acetylated c-Myb in vivo not only at the sites found in this study but also at the p300-induced acetylation sites reported recently. Replacement of lysine by arginine at all of these sites dramatically decreased the trans-activating capacity of c-Myb. The results of transcriptional activation assays with c-Myb acetylation site mutants suggested that acetylation of c-Myb at each of these five sites synergistically enhances c-Myb activity. Mutations of these acetylation sites reduced the strength of the interaction between c-Myb and CBP. Thus, acetylation of c-Myb by CBP increases the trans-activating capacity of c-Myb by enhancing its association with CBP. These results demonstrate a novel molecular mechanism of regulation of c-Myb activity.

The c-myb proto-oncogene is the cellular progenitor of the v-myb oncogenes carried by the chicken retroviruses avian myeloblastosis virus and E26, which transform myelomonocytic hematopoietic cells (see Refs. 1 and 2, and for review, see Ref. 3). Analysis of c-myb-deficient mice and transgenic mice expressing dominant negative forms of the c-myb gene product (c-Myb) indicated that c-Myb is essential for the proliferation of immature hematopoietic cells and for the development of T cells (4–6). c-Myb is a transcriptional activator that binds to the specific DNA sequence, 5’-AACNG-3’ (7–10). By inducing transcription of a group of target genes, c-Myb regulates both proliferation and apoptosis of hematopoietic cells (11–13). c-Myb has three functional domains, which are aligned from the N terminus in the order: DNA-binding domain, transcriptional activation domain, and negative regulatory domain (NRD) (10). The DNA-binding domain in the NH2-terminal region of c-Myb consists of three imperfect tandem repeats of 51–52 amino acids, each containing a helix-turn-helix variation motif (14–16). The transcriptional activation domain of c-Myb, which is rich in acidic amino acids, is adjacent to the DNA-binding domain. The transcriptional coactivator CBP (CREB-binding protein) binds to this activation domain to mediate c-Myb-induced transcriptional activation (17, 18). Deletion of the NRD, located in the carboxyl-proximal portion of the molecule, increases both trans-activation and transformation capacity, implying that this domain normally represses c-Myb activity (10, 19–23), although the mechanism of negative regulation by NRD remains unknown.

CBP was originally identified as a coactivator of the transcription factor cAMP response element-binding protein (CREB). CBP acts as a bridging factor between CREB and the general transcription factor TFIIB (24, 25). The CBP gene family contains at least one other member, p300, which was originally identified as a protein that binds to the adenovirus E1A protein (26). Both CBP and p300 bind to the phosphorylated form of CREB and also to E1A (27, 28). CBP binds not only to phosphorylated CREB but also to nonphosphorylated forms of many other transcription factors, including c-Myb (17, 18) and nuclear hormone receptors (29, 30) (for review, see Ref. 31). CBP contributes to the transcriptional activation mediated by each of these factors. CBP possesses intrinsic histone acetyltransferase (HAT) activity (32, 33) and binds to other HAT proteins such as PCAF (34) and ACTR (35), suggesting that it contributes to transcriptional activation by disrupting repressive chromatin structure via acetylation of histones. The finding that certain TAFs (TBP (TATA-binding protein)-associated factors) are integral components of PCAF and yeast SAGA-HAT complexes further suggests a mechanistic connection between the RNA polymerase II machinery and CBP HAT activities (36, 37).

CBP/p300 acetylates not only histones but also transcription factors. p300 directly acetylates p53 and GATA-1, and enhances their sequence-specific DNA binding activity (38, 39). Similar observations were reported for E2F (40, 41) and c-Myb (42). In addition to some sequence-specific DNA-binding proteins, CBP also acetylates other types of transcription factors. Drosophila CBP acetylates T-cell factor, a high mobility group domain protein, and this acetylation blocks the interaction between T-cell factor and its coactivator β-catenin/Armadillo.

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§ Supported by the Special Researcher’s Basic Science Program.

¶ E-mail: sishii@rtc.riken.go.jp.

The abbreviations used are: c-Myb, myb gene product; CREB, cAMP-response element-binding protein; NRD, negative regulatory domain; HAT, histone acetyltransferase; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; RSV, Rous sarcoma virus.
Acetylation of another high mobility group protein, HMG-I, also leads to transcriptional repression of the interferon-β gene promoter (44). Thus, CBP appears to control transcription by acetylating certain transcription factors through multiple mechanisms.

Here, we have identified CBP-induced acetylation sites in the c-Myb protein. Three of the sites correspond to those recently identified as p300-induced acetylation sites, and the two other sites are new. Our results suggest that acetylation of c-Myb at each site synergistically increases the trans-activating capacity of c-Myb. Acetylation of c-Myb enhanced its affinity for CBP, explaining at least partly the mechanism of CBP-activating capacity of c-Myb. 

EXPERIMENTAL PROCEDURES

Plasmid Construction—The plasmids pGEX-KIX, pGEX-Bromo, pGEX-C/H2, and pGEX-C/H3 to express GST fusion proteins containing various regions of CBP were described previously (45). The expression plasmids for GST fusion proteins of ATF-2, Smad3/4, and c-Ski were also described previously (45, 46). To express GST fusion proteins containing various regions of c-Myb, appropriate DNA fragments of c-Myb were introduced into the pGEX vectors (Amersham Pharmacia Biotech). The pSPUTK vector (Stratagene) was used for transcription/translation of the various derivatives of c-Myb. The expression plasmids for GST fusion proteins of ATF-2, Smad3/4, and c-Ski were also described previously (45, 46). The expression plasmid pRc/RSV-CBP-HA was a gift from R. Goodman. The plasmids to express various derivatives of Gal4-c-Myb fusions, in which the DNA-binding domain of Gal4 was fused to c-Myb, were constructed by the polymerase chain reaction-based method using the cytomegalovirus promoter-containing vector pCMX (47).

In Vitro Binding Analysis with GST Fusion Proteins—The GST pull-down assay using GST-CBP and in vitro translated c-Myb was essentially performed as described previously (17). The GST fusion proteins were expressed in Escherichia coli and bacterial lysates containing 20 μg of GST-CBP were rocked for 2–3 h at 4 °C with 100 μl of glutathione-Sephase beads (Amersham Pharmacia Biotech). The beads were washed with 1 ml of 0.6 M NaCl two times, with phosphate-buffered saline containing 0.05% Nonidet P-40, four times, and then with 1 ml of binding buffer (20 mM Hapes, pH 7.7, 150 mM KCl, 0.1 mM EDTA, 2.5 mM MgCl2, 1% skim milk, 1 mM dithiothreitol, 0.05% Nonidet P-40). Various forms of [35S]methionine-labeled c-Myb were synthesized using an in vitro transcription/translation kit according to the procedures described by the supplier (Promega). A sample from the reaction was mixed with GST-CBP affinity resin in 750 μl of binding buffer. After rocking at 4 °C overnight, the resin was washed with 1 ml of binding buffer 5 times, and resuspended in SDS sample buffer, and boiled to release bound proteins. The proteins were analyzed by SDS-PAGE followed by autoradiography.

In Vitro Acetylation Assay using Immunoprecipitated CBP—HepG2 cells were transfected with 6 μg of the CBP expression plasmid pRc/RSV-CBP-HA using LipofectAMINE (Life Technologies, Inc.). Two days
after transfection, cells were disrupted using lysis buffer (50 mM Hepes, pH 7.5, 250 mM NaCl, 0.2 mM EDTA, 0.5% Nonidet P-40) containing a protease inhibitor mixture (Roche Molecular Biochemicals) and centrifuged at 15,000 rpm for 20 min. The anti-CBP CT polyclonal antibodies (Upstate Biotech) were added to the supernatant and incubated on ice overnight. Protein G-Sepharose was added and the mixture was rotated for 1 h. The resulting immunocomplexes were washed with wash buffer 3 times and with acetylation buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40) containing a protease inhibitor mixture and the lysates were centrifuged. The CBP-containing immunocomplexes were precipitated using anti-CBP polyclonal antibodies (New England Biolabs), while a mixture of acetylated and nonacetylated forms of c-Myb were detected by Western blotting using the anti-c-Myb monoclonal antibody 5-1 and a chemiluminescent detection reagent (New England Biolabs).

**Reporter Gene Assay**—The Myb site containing luciferase reporter, in which the thymidine kinase promoter is linked to three tandem repeats of the Gal4-binding site, was transfected into HepG2 cells together with plasmid expressing various forms of c-Myb or the control vector, and the internal control plasmid pRL-CMV using the CaPO4 method. Two days after transfection, the cells were lysed, and luciferase activities were measured as described above. Experiments were repeated 4 times, and the data were averaged. To examine the transactivation capacity of the Gal4-Myb fusion, the Gal4 site containing luciferase reporter, in which the thymidine kinase promoter is linked to three tandem repeats of the Gal4-binding site, was transfected together with plasmid expressing various forms of Gal4-Myb fusions or the control vector and the internal control plasmid pRL-CMV using the CaPO4 method. Two days after transfection, cell lysates were prepared using TNE buffer (10 mM Tris-HCl, pH 7.8, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 10 mM sodium butyrate) and centrifuged at 15,000 rpm. The supernatant was subjected to SDS-PAGE and analyzed by Western blotting. The acetylated forms of c-Myb were detected with anti-acetylated lysine polyclonal antibody (New England Biolabs), while a mixture of acetylated and nonacetylated forms of c-Myb were detected by the anti-c-Myb monoclonal antibody 1-1 or 5-1.

**Coimmunoprecipitation**—To examine the effect of c-Myb acetylation on c-Myb affinity for CBP, 293 cells were transfected together with 3 μg of the CBP expression plasmid pReRSV-CBP-HA together with equal amounts of one of the plasmids expressing various forms of c-Myb. Two days after transfection, the cells were lysed using RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 10 mM sodium butyrate) and centrifuged at 15,000 rpm. The supernatant were subjected to SDS-PAGE and analyzed by Western blotting. The acetylated forms of c-Myb were detected with anti-acetylated lysine polyclonal antibody (New England Biolabs), while a mixture of acetylated and nonacetylated forms of c-Myb were detected by the anti-c-Myb monoclonal antibody 1-1 or 5-1.

**Acetylation**—In vitro acetylation of c-Myb by CBP. The GST-CBP fusion protein was used instead of immunoprecipitated CBP as a source of acetyltransferase enzyme.

**In Vivo Acetylation Assay**—To investigate the in vivo acetylation of c-Myb by CBP, 293 cells were transfected with 3 μg of the CBP expression plasmid pReRSV-CBP-HA together with equal amounts of one of the plasmids expressing various forms of c-Myb. Two days after transfection, cells were lysed using RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 10 mM sodium butyrate) and centrifuged at 15,000 rpm. The supernatant were subjected to SDS-PAGE and analyzed by Western blotting. The acetylated forms of c-Myb were detected with anti-acetylated lysine polyclonal antibody (New England Biolabs), while a mixture of acetylated and nonacetylated forms of c-Myb were detected by the anti-c-Myb monoclonal antibody 1-1 or 5-1.

**Localization of the acetylation site in NRD**—The GST-CBP fusion protein containing the HAT domain (amino acids 1099–1758) was purified using glutathione-Sepharose beads from E. coli extract. The acetylation assay was done as described above except that 1 μg of GST-CBP was used instead of immunoprecipitated CBP as a source of acetyltransferase enzyme.

**Coimmunoprecipitation**—To examine the effect of c-Myb acetylation on c-Myb affinity for CBP, 293 cells were transfected together with 3 μg of the CBP expression plasmid pReRSV-CBP-HA and 3 μg of plasmid expressing various forms of c-Myb using LipofectAMINE (Life Technologies). Two days after transfection, cell lysates were prepared using TNE buffer (10 mM Tris-HCl, pH 7.8, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 10 mM sodium butyrate) containing a protease inhibitor mixture and the lysates were centrifuged. The CBP-containing immunocomplexes were precipitated with anti-CBP CT polyclonal antibody from the supernatant and washed with TNE buffer 3 times. The immunocomplexes were separated on 8% SDS gels and analyzed by Western blotting using the anti-c-Myb monoclonal antibody 5-1 and a chemiluminescent detection reagent (New England Biolabs).
RESULTS

Direct Interaction between the C/H2 Region of CBP and the NRD of c-Myb—We previously reported that CBP directly binds to the transcriptional activation domain of c-Myb via the N-terminal KIX domain (17). Numerous other transcriptional factors such as CREB also bind to the KIX domain. In a more precise analysis of the interaction between c-Myb and CBP, we obtained evidence suggesting that a region other than the KIX domain also directly interacts with c-Myb. To precisely identify this region, we performed the GST pull-down assay using GST-CBP fusion proteins containing various portions of CBP (Fig. 1).

In vitro translated mouse c-Myb directly bound to not only the GST-KIX fusion but also to the GST-CBP fusion containing the C/H2 domain. To determine which region of c-Myb interacts with the C/H2 domain of CBP, we next performed GST pull-down assays using the GST-C/H2 fusion protein bound to glutathione-Sepharose beads and various derivatives of c-Myb translated in vitro (Fig. 2, A and B). The results indicated that the NH₂-terminal two-thirds of the NRD (amino acids 326–448) was responsible for binding to GST-C/H2. To further confirm that the NRD of c-Myb directly binds to the C/H2 domain of CBP, the GST pull-down assays using the GST fusion containing the NRD were performed (Fig. 2C). The in vitro translated CBP fragment containing the C/H2 region (amino acids 1191–1758) bound to the GST fusion containing the NRD of c-Myb (amino acids 326–448). Thus, in addition to the previously reported interaction between the KIX domain of CBP and the transcriptional activation domain of c-Myb, the C/H2 domain of CBP also binds to the NRD of c-Myb.

Acetylation of c-Myb NRD by CBP—Since the C/H2 domain of CBP is critical for the intrinsic HAT activity of CBP, the direct interaction between the C/H2 domain of CBP and c-Myb suggested that CBP might directly acetylate c-Myb via its HAT domain. To test this, various transcription factors expressed in bacteria (Fig. 3A) were incubated with the CBP protein together with [¹⁴C]acetyl-CoA, and the level of acetylation of the transcription factors was investigated (Fig. 3B). CBP protein was prepared by immunoprecipitating CBP with the anti-CBP polyclonal antibody from extracts of HepG2 cells transfected with the mouse CBP expression plasmid. The c-Myb
protein was efficiently acetylated, whereas the other four transcription factors, ATF-2, Smad3, Smad4, and Ski, were not. To identify which region of c-Myb molecule was acetylated by CBP, we used a series of GST-c-Myb fusion proteins containing various portions of c-Myb (Fig. 4B) for in vitro acetylation assays. Among the five GST-c-Myb fusion proteins, only the fusion protein containing the NRD was acetylated by CBP (Fig. 4, A and C). This is consistent with our observation that the C/H2 domain of CBP directly binds to the NRD of c-Myb.

Identification of the Acetylation Sites in the NRD—To determine which lysine residue(s) in the NRD are acetylated by CBP, we first used a series of GST-NRD fusion proteins containing various portions of the NRD truncated from the COOH terminus (Fig. 5A and B). The GST fusion proteins containing the entire NRD (GST-326–563 and GST-326–500) were efficiently acetylated by CBP (Fig. 5C, left panel). Deletion from the COOH terminus to amino acid 449 (GST-326–448) did not abrogate acetylation by CBP, whereas deletion to amino acid 436 (GST-326–435) dramatically reduced the acetylation level. Next, we used one GST fusion protein containing the downstream region from the NRD (GST-501–636) and two GST fusion proteins containing both the downstream region from the NRD and the different C-terminal region of the NRD (GST-473–636 and GST-449–636) (Fig. 5C, right panel). Neither of these three GST fusions were acetylated by CBP. These results indicate that the region between amino acids 436 and 448 contains the CBP-induced acetylation site(s).

This narrow region contains two lysine residues, Lys438 and Lys441, which could serve as potential CBP-mediated acetylation sites (Fig. 6A). We mutated either or both of these lysine residues to arginine, and used them in in vitro acetylation assays containing immunoprecipitated CBP (Fig. 6B). The GST-NRD fusion protein containing amino acids 326–563 was efficiently acetylated by CBP, whereas the construct with arginine at either Lys438 or Lys441 displayed significantly reduced but not total loss of acetylation by CBP (Fig. 6C). Furthermore, the construct possessing the two mutated lysine residues was very weakly acetylated by CBP. To further confirm that CBP really acetylates these two lysine residues, we examined the acetylation of c-Myb by CBP-CBP recombinant proteins expressed in E. coli (Fig. 6D). The GST-c-Myb fusion protein containing amino acids 326–563 was efficiently acetylated by CBP-CBP protein containing the HAT domain. In contrast, GST-CBP acetylated neither the GST-c-Myb mutant protein, in which both Lys438 and Lys441 were mutated to arginine, nor the GST protein alone. Thus, CBP directly acetylates the c-Myb protein in vitro at Lys438 and Lys441.

In Vivo Acetylation of Lys438 and Lys441 by CBP—We next confirmed that the two acetylation sites identified in vitro were also acetylated by CBP in vivo. When the c-Myb expression plasmid was transfected into 293 cells together with increasing amounts of the CBP expression plasmid, the acetylated form of c-Myb was detected by the anti-acetylated lysine antibody (Fig. 7A). The amount of acetylated c-Myb increased in a dose-dependent manner with the amount of the CBP expression plasmid. These results indicated that CBP also acetylates c-Myb in vivo. To investigate which region of c-Myb is acetylated in vivo, plasmids expressing various derivatives of c-Myb were transfected into 293 cells together with the CBP expression plasmid, and the acetylated form of c-Myb was detected by the anti-acetylated lysine antibodies (Fig. 7B). The two mutants lacking the DNA-binding domain or the transcriptional activation domain (ΔDBD or ΔTA) were not acetylated by CBP. The DNA-binding domain contains the nuclear localization signals and the ΔDBD mutant cannot enter the nucleus (10), which means that the ΔDBD mutant would not be able to associate with CBP. The transcriptional activation domain is critical for association with CBP and the ΔTA mutant has probably much lower affinity for CBP compared with wild-type c-Myb (17). Thus, failure to detect acetylated forms of these two mutants may not be due to defects in their acetylation sites, but rather due to inefficient association between c-Myb and CBP. In fact, we observed CBP acetylated in vivo the c-Myb ΔDBD mutant fused to the Gal4 DNA-binding domain which has a nuclear localization signal. CT1 was efficiently acetylated in vivo, whereas CT2 was not. These results indicate that the region between amino acids 404 and 500 in the NRD contains the acetylation sites. These results are consistent with the data from the in vitro acetylation assays described above. When the c-Myb mutant in which Lys438 or Lys441 was replaced by arginine (K438R or K441R) was expressed together with CBP, the density of the band corresponding to the acetylated form of c-Myb was significantly lower than that of wild-type c-Myb (Fig. 7C, left panel). However, the acetylated form of these two c-Myb mutants could still be detected. Furthermore, when the c-Myb mutant in which both Lys438 and Lys441 were replaced by arginine (2K/R) was used, the density of the band corresponding to the acetylated form of c-Myb was about half of that of wild-type c-Myb (Fig. 7C, right panel). Although the results indicate that Lys438 and Lys441 are acetylated by CBP in vivo, other sites of acetylation must exist to explain the residual

\(^2\) Y. Sano, unpublished results.
level of acetylation of the 2K/R mutant. During the course of this study, another group reported the acetylation of three other sites, Lys471, Lys480, and Lys485, in the human c-Myb protein by p300 (42). These three sites in human c-Myb correspond to Lys467, Lys476, and Lys481 in mouse c-Myb, and are located further downstream from the two acetylation sites identified here. Based on this report, we speculated that the acetylation of the 2K/R mutant occurred at these three sites. To confirm this possibility, we constructed a mutant c-Myb that had all five lysine residues (two sites identified in this study and the three sites reported by the other group) mutated to arginines (5K/R), and used it in the in vivo acetylation assay (Fig. 7C, right panel). The mutant displayed almost no acetylation by CBP, suggesting that CBP acetylates c-Myb in vivo at five sites. The use of different substrates for in vitro acetylation used by our group and the other group may explain the discrepancy in the number of detected acetylation sites (see “Discussion”).

Effect of CBP-induced Acetylation on trans-Activating Capacity of c-Myb—To examine whether acetylation of c-Myb affects the trans-activating capacity of c-Myb, we performed co-transfection assays. The luciferase reporter, in which luciferase expression was placed under the control of the SV40 early promoter linked to six tandem repeats of the Myb-binding site, was co-transfected into HepG2 cells together with the c-Myb expression plasmid. Wild-type c-Myb stimulated luciferase expression 8.3-fold, whereas the 2K/R and 5K/R mutants stimulated luciferase expression by 6.0- and 4.3-fold, respectively (Fig. 8A). These results indicate that the CBP-induced acetylation of c-Myb positively regulates the trans-activating capacity of c-Myb. Furthermore, acetylation at Lys438 and Lys441 synergistically enhanced the trans-activating capacity of c-Myb already acetylated at Lys467, Lys476, and Lys481.

In the study of p300-induced acetylation of c-Myb, it was reported that p300-mediated acetylation enhanced the DNA binding activity of c-Myb (42). We also observed that CBP-induced acetylation increased the DNA binding activity of c-Myb. In addition, we observed that the CBP-mediated acetylation of c-Myb stimulated transcriptional activation by the Gal4-c-Myb fusion protein (Fig. 8B). The luciferase reporter containing the Gal4-binding sites was transfected into HepG2...
cells together with the plasmid to express the Gal4 fusion protein containing full-length c-Myb. The luciferase expression from this reporter was enhanced 4.5-fold by Gal4-c-Myb. Replacement of both Lys438 and Lys441 by arginine (2K/R) suppressed activation by Gal4-c-Myb to 3.6-fold. Furthermore, replacement of all five lysine residues by arginines suppressed activation to 2.2-fold. The expression levels of these acetylation site mutants were similar to that of wild-type Gal4-c-Myb (Fig. 8C). In the case of the Gal4-c-Myb fusion, the Gal4 DNA-binding domain, but not the authentic DNA-binding domain of c-Myb, was functional. Therefore, these results indicate that acetylation of c-Myb at five sites specifically stimulates trans-activation activity.

Acetylation of c-Myb Enhances the Association with CBP—During investigation of the mechanism of how CBP-mediated acetylation increases the trans-activating capacity of c-Myb, we found that acetylation of c-Myb enhances its association with CBP. As described above, the two regions of CBP, KIX and C/H2, directly interact with c-Myb. In the GST pull-down assays using the GST-CBP-KIX fusion protein, in vitro translated 2K/R and 5K/R mutants bound to GST-CBP-KIX less efficiently compared with wild-type c-Myb (Fig. 9A). The 2K/R and 5K/R mutants bound to GST-CBP-KIX at levels that were 67 and 39% of that of wild-type c-Myb. In the second GST pull-down assays using GST-CBP-C/H2, similar results were obtained (Fig. 9B). The binding efficiencies of the two mutants, 2K/R and 5K/R, to GST-CBP-C/H2 were 61 and 26% of that of the wild-type, respectively. To confirm that c-Myb acetylation really enhances its affinity for CBP in vivo, coimmunoprecipitation assays were performed (Fig. 9C). The plasmid to express the wild-type or the acetylation site mutants was transfected into 293 cells together with the CBP expression plasmid. Lysates were prepared from transfected cells, and used for immunoprecipitation with the anti-CBP antibody. Under these conditions, apparently less 2K/R and 5K/R mutant proteins were co-immunoprecipitated with CBP compared with wild-type c-Myb. These results indicate that acetylation of c-Myb increases its affinity for CBP.

DISCUSSION

In this study, we have identified Lys438 and Lys441 of c-Myb as the targets of CBP-induced in vivo acetylation. In addition to these two lysine residues, three other lysine residues (Lys467, Lys476, and Lys481 of mouse c-Myb), which were recently reported to be acetylated in vitro by p300, were also found to be acetylated by CBP in vivo. Consistent with our observations, their study suggested that p300 might also acetylate c-Myb at other acetylation sites in addition to the three sites at Lys467, Lys476, and Lys481 on mouse c-Myb. In fact, we observed that immunoprecipitated p300 acetylated c-Myb in vitro at Lys438 and Lys441.2 These results strongly suggest that both CBP and p300 acetylate c-Myb at five lysine residues (Lys438 and Lys441 identified in this study and Lys467, Lys476, and Lys481 of the recent report). We used the GST-c-Myb fusion protein as a substrate for in vitro acetylation, whereas in vitro translated c-Myb was used for acetylation by p300 (42). This suggests that the three lysine residues identified as p300-induced acetylation sites may have been masked in the GST-c-Myb fusion protein by GST-mediated changes in c-Myb protein conformation, leading to no acetylation at these sites. Mutation analysis of the acetylation sites in c-Myb suggested that acetylation at each site synergistically enhanced the trans-activating capacity of c-Myb. Our results indicated that acetylation of c-Myb by CBP enhanced its affinity for CBP. Acetylation of the NRD in c-Myb enhanced its binding not only for the C/H2 domain of CBP but also for the KIX domain of CBP. The C/H2 domain interacts with the NRD containing the acetylated lysines, whereas the KIX domain directly interacts with the activation domain of c-Myb which lacks acetylation sites. Compared with wild-type c-Myb, the c-Myb mutant lacking the activation domain (ΔTA) has a lower affinity for the C/H2 domain of CBP (Fig. 2B). These results suggest that NRD and the activation domain interact with each other in the c-Myb molecule and synergistically stimulate c-Myb association with CBP. Probably, acetylation at NRD affects the protein conformation of the activation domain of c-Myb, leading to enhanced association of c-Myb with the KIX domain. After binding of
Acetylation of c-Myb by CBP

Fig. 9. Decreased affinity between the acetylation site mutants of c-Myb and CBP. A, effect of the acetylation site mutation on the affinity of c-Myb for the KIX domain of CBP. The 35S-labeled wild-type and mutant c-Myb proteins were synthesized in vitro and mixed with the GST-CBP-KIX affinity resin, and bound proteins were analyzed by SDS-PAGE followed by autoradiography. In the input lanes, the amount of protein was 10% of that used for the binding assay. Experiments were repeated three times and the average amount of c-Myb bound to GST-CBP-KIX is indicated in the bar graph along with standard deviations on the right. B, effect of the acetylation site mutations in c-Myb on the affinity of c-Myb for the CH2 domain of CBP. The 35S-labeled wild-type and mutant c-Myb proteins were synthesized in vitro and mixed with the GST-CBP-CH2 affinity resin, and bound proteins were analyzed by SDS-PAGE followed by autoradiography. Experiments were repeated three times and the average amount of c-Myb bound to GST-CBP-CH2 is indicated in the bar graph along with standard deviations on the right. C, effect of the acetylation site mutations in c-Myb on the in vitro affinity of c-Myb for CBP. The plasmid to express wild-type and mutant c-Myb was transfected into 293 cells together with the CBP expression plasmid. Lysates were prepared from transfected cells, and used for immunoprecipitation with anti-CBP antibody. The immunocomplex was analyzed by SDS-PAGE followed by Western blotting with anti-CBP or anti-CBP antibody. In the right panels, an aliquot of lysate was directly used for Western blotting with anti-CBP or anti-CBP antibody.

c-Myb to the enhancer region of c-Myb target genes, CBP may be recruited to c-Myb on the enhancer. However, this association between c-Myb and CBP may be transient or very weak. Once c-Myb is acetylated by CBP, CBP may become tightly associated with c-Myb, and mediate more efficient c-Myb-induced transcriptional activation. In the study of p300-induced acetylation of c-Myb, it was shown that acetylation of c-Myb enhanced the DNA affinity of c-Myb for the Myb recognition sequence (42). We also observed that the DNA-binding capacity of c-Myb was enhanced by CBP. Therefore, acetylation of c-Myb by CBP/p300 has two functional consequences for c-Myb activity: increased DNA binding activity and increased activation potential. Recently, it was also reported that acetylation of E2F stimulates E2F activity via multiple mechanisms including increased DNA binding activity, activation potential, and protein half-life (41). Thus, acetylation may affect multiple characteristics of transcription factors. NRD negatively regulates c-Myb activity by associating with uncharacterized inhibitor(s). Thus, acetylation of the NRD of c-Myb could also serve to inhibit the binding of such inhibitors to c-Myb.

Both A-Myb and B-Myb, other members of the myb gene family, are also acetylated by CBP. Five lysine residues of mouse c-Myb (Lys438, Lys441, Lys467, Lys476, and Lys481), which can be acetylated by CBP, are well conserved in A-Myb. Both c-Myb and A-Myb bind to CBP to elicit strong transcriptional activation. Among these five lysine residues, Lys438 is the only one not conserved in B-Myb, although the other four lysines are present. This difference could be partly related to the observation that B-Myb is a weaker transcriptional activator compared with c-Myb and A-Myb (48, 49). Although B-Myb has a transcriptional activation domain, which is rich in acidic amino acids, the COOH-terminal portion containing the putative acetylation sites appears to be required for transcriptional activation by B-Myb (50, 51). Cyclin A/Cdk2 phosphorylates this region, and stimulates its transcriptional-activating capacity (52–55).

Further study will be required to investigate the interesting possibility that acetylation and phosphorylation of this region synergistically modulate B-Myb activity.

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REFERENCES

1. Roussel, M., Saule, S., Lagrou, C., Beug, H., Graff, T., and Stehelin, D. (1979) Nature 281, 452–455
2. Klempnauer, K.-H., Gonda, T. J., and Bishop, J. M. (1982) Cell 31, 453–463
3. Lipsick, J. S., and Wang, D. M. (1999) Oncogene 18, 3047–3055
4. Muensiri, M. L., McLain, K., Kier, A. E., Swerdlow, S. H., Schereiner, C. M., Miller, T. A., Pietryga, D. W., Scott, W. J., and Potter, S. S. (1993) Cell 65, 677–689
5. Badiani, P., Corbellia, P., Kioussis, D., Maravel, J., and Weston, K. (1994) Genes Dev. 8, 770–782
6. Allen, R. D., III, Bender, T. P., and Siu, G. (1999) Genes Dev. 13, 1073–1078
7. Biedenkapp, H., Borgmeyer, U., Sippel, A. E., and Klempnauer, K.-H. (1988) Nature 335, 835–837
8. Ness, S. A., Marknell, A., and Graff, T. (1989) Cell 59, 1115–1125
9. Weston, K., and Bishop, J. M. (1989) Cell 58, 85–94
10. Sakura, H., Kanei-Ishii, C., Nagase, T., Nakagoshi, H., Gonda, T. J., and Ishii, S. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 5758–5762
11. Kowenz-Leutz, E., Herr, P., Niss, K., and Leutz, A. (1997) Cell 91, 185–195
12. Frampton, J., Ramqvist, T., and Graff, T. (1996) Genes Dev. 10, 2720–2731
13. Ogata, K., Morikawa, S., Nakamura, H., Hojo, H., Yoshimura, S., Zhang, R., Aimo, S., Ametani, Y., Hira, S., Sarai, A., Ishii, S., and Ishiwara, Y. (1994) Cell 79, 639–648
14. Ogata, K., Morikawa, S., Nakamura, H., Sekikawa, A., Inoue, T., Kanai, H., Sarai, A., Ishii, S., and Ishiwara, Y. (1995) Nature Struct. Biol. 2, 309–320
15. Dai, P., Akimaru, H., Tanaka, Y., Hou, D.-X., Yasukawa, T., Kanei-Ishii, C., Takahashi, T., and Ishii, S. (1996) Genes Dev. 10, 528–540
16. Ogle, D., J., M., Brand, R., A., Z., and L., U. (1969) EMBO J. 15, 577–580
17. Gonda, T. J., Buckmaster, C., and Ramsay, R. G. (1989) EMBO J. 8, 1777–1783
18. Hu, L.-Y., Ramsay, R. G., Kanei-Ishii, C., Ishii, S., and Gonda, T. J. (1991) Oncogene 6, 1549–1553
19. Grasser, F. A., Graff, T., and Lipsick, J. S. (1991) Mol. Cell. Biol. 11, 3987–3996
20. Kanei-Ishii, C., Macmillan, E. M., Nomura, T., Sarai, A., Ramsay, R. G., Aimo, S., Ishii, S., and Gonda, T. J. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 3088–3092
21. Dubendorff, J. W., Whittaker, L. J., Emlman, J. T., and Lipck, J. S. (1992) Genes Dev. 6, 2524–2535
22. Christia, J. C., Kwo, K. P. S., Lamb, N., Hagiwara, M., Montminy, M. R., and Goodman, R. H. (1993) Nature 365, 855–859
23. Kwo, K. P. S., Lundblad, J. R., Christia, J. C., Richards, J. P., Banchinger, H. P., Brennan, R. G., Roberts, S. G. R., Green, M. R., and Goodman, R. H. (1994) Nature 370, 223–226
24. Eckner, R., Ewen, M. E., Newsome, D., Gerdes, M., DeCaprio, J. A., Lawrence, J. B., and Livingston, D. M. (1994) Genes Dev. 8, 869–884
25. Lundblad, J. R., Kwo, K. P. S., Laurence, M. E., Harter, M. L., and Goodman, R. H. (1995) Nature 374, 85–88
26. Arany, Z., Newsome, D., Oldred, E., Livingston, D. M., and Eckner, R. (1995) Nature 374, 85–88
27. Kamei, Y., Xu, L., Heinzel, T., Tchoria, J., Kurokawa, R., Goss, B., Lin, S.-C., Heyman, R. A., Rose, D. W., Glass, C. K., and Rosenfeld, M. G. (1996) Cell 85, 403–414
30. Chakravarti, D., LaMorte, V. J., Nelson, M. C., Nakajima, T., Schulman, I. G., Jugulion, H., Montminy, M., and Evans, R. M. (1996) *Nature* **383**, 99–103
31. Giles, R. H., Peters, D. J., and Breuning, M. H. (1998) *Trends Genet.* **14**, 178–183
32. Ogryzko, V. V., Schiltz, R. L., Russanova, V., Howard, B. H., and Nakatani, Y. (1996) *Cell* **87**, 953–959
33. Bannister, A. J., and Kouzarides, T. (1996) *Nature* **384**, 641–643
34. Yang, X.-J., Ogryzko, V. V., Nishikawa, J., Howard, B. H., and Nakatani, Y. (1996) *Cell* **90**, 569–580
35. Grant, P. A., Schieltz, D., Pray-Grant, M. G., Steger, D. J., Reese, J. C., Yates, J. R., III, and Workman, J. L. (1998) *Cell* **94**, 45–53
36. Gu, W., and Roeder, R. G. (1997) *Cell* **90**, 595–606
37. Oh, I. H., and Reddy, E. P. (1998) *Mol. Cell. Biol.* **18**, 499–511
38. Sala, A., Kundu, M., Casella, I., Engelhard, A., Calabretta, B., Grasso, L., Paggi, M. G., Giordano, A., Watson, R. J., Khalili, K., and Peschle, C. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 532–536
39. Ziebold, U., Bartsch, O., Marais, R., Ferrari, S., and Klempnauer, K.-H. (1997) *Curr. Biol.* **7**, 253–260
40. Saville, M. K., and Watson, R. J. (1998) *Oncogene* **17**, 2679–2689
41. Higuchi, R. (1990) in *PCR Technology: Principles and Applications for DNA Amplification* (Erlich, H. A., ed) pp. 61–70, Stockton Press, New York
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Yuji Sano and Shunsuke Ishii

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