CBP as a transcriptional coactivator of c-Myb

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CBP (CREB-binding protein) is a transcriptional coactivator of CREB (cAMP response element-binding) protein, which is directly phosphorylated by PKA (cAMP-dependent protein kinase A). CBP interacts with the activated phosphorylated form of CREB but not with the nonphosphorylated form. We report here that CBP is also a coactivator of the *c-myb* proto-oncogene product (c-Myb), which is a sequence-specific transcriptional activator. CBP directly binds to the region containing the transcriptional activation domain of c-Myb in a phosphorylation-independent manner *in vitro*. The domain of CBP that touches c-Myb is also required for binding to CREB. A c-Myb/CBP complex *in vivo* was demonstrated by a yeast two-hybrid assay. CBP stimulates the c-Myb-dependent transcriptional activation. Conversely, the expression of antisense RNA of CBP represses c-Myb-induced transcriptional activation. In addition, adenovirus E1A, which binds to CBP, inhibits c-Myb-induced transcriptional activation. Our data thus identify CBP as a coactivator of c-Myb. These results suggest that CBP functions as a coactivator for more transcriptional activators than were thought previously.

[Key Words: CBP; Myb; coactivator; CREB; adenovirus E1A]

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The *c-myb* proto-oncogene is the cellular progenitor of the *v-myb* oncogenes carried by the chicken retroviruses AMV and E26, which transform myelomonocytic hematopoietic cells (for review, see Graf 1992). The level of expression of *c-myb* is high in immature cells of various hematopoietic lineages and is down-regulated during terminal differentiation (Westin et al. 1982; Gonda and Metcalf 1984; Duprey and Boettiger 1985). Artificial expression of *c-myb* blocks the induced differentiation of immature erythroid cells (Clarke et al. 1988; Todokoro et al. 1988), and treatment with an antisense oligonucleotide directed to *c-myb* blocks the proliferation of precursor cells (Gewirtz and Calabretta 1988). Furthermore, homozygous *c-myb* mutant mice are severely anemic and die in utero because of defective fetal hematopoiesis (Müclenski et al. 1991). Therefore, c-Myb is important in maintaining the proliferative state of immature hematopoietic cells.

c-Myb is a transcriptional activator that binds to a specific DNA sequence, AACNG (Biedenkapp et al. 1988; Nakagoshi et al. 1990; Tanikawa et al. 1993). Both artificial promoter constructs containing c-Myb-binding sites (Nishina et al. 1989; Weston and Bishop 1989; Nakagoshi et al. 1990) and natural promoters such as the *mim-1* and *c-myc* genes (Ness et al. 1989; Evans et al.

1990) are activated by c-Myb. Deletion analyses of c-Myb indicates that it has three functional domains responsible for DNA binding, transcriptional activation, and negative regulation (Sakura et al. 1989). The DNA-binding domain in the amino-terminal region of c-Myb consists of three imperfect tandem repeats of 51–52 amino acids, each containing a helix–turn–helix variation motif (Sakura et al. 1989; Ogata et al. 1992, 1995). The second and third repeats are closely packed in the major groove of the DNA, and the two recognition helices in these repeats bind to the specific base sequence cooperatively (Ogata et al. 1994). The transcriptional activation domain of c-Myb, which is rich in acidic amino acids, is adjacent to the DNA-binding domain. Two *c-myb*-related genes, *A-myb* and *B-myb*, were identified by cross-hybridization (Nomura et al. 1988). *A-myb* has a very similar structure of functional domains to c-Myb and is the strongest transcriptional activator among the three members of the *myb* gene family (Golay et al. 1994; Trauth et al. 1994; Takahashi et al. 1995). On the other hand, *B-myb* is a cell-type-specific transcriptional activator, and the specific cofactor that binds to the carboxy-proximal portion appears to be required for transcriptional activation by *B-myb* (Tashiro et al. 1995).

The RNA polymerase II transcriptional machinery is composed of the enzyme and a complement of accessory factors (TFIIA, TFIIB, TFIID, TFIIIE, TFIIF, and TFIIH) (for

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review, see Buratowski 1994). Among these, only TFIID is a sequence-specific DNA-binding protein composed of the TATA-binding protein (TBP) and multiple TBP-associated factors (TAFs) (Goodrich and Tjian 1994). Several TAFs are essential as coactivators of transcription through their interaction with specific enhancer-binding activators (Tjian and Maniatis 1994). Different transcriptional activators target distinct TAFs in the TFIID complex for their functional activation of transcription. However, there is no information about the coactivator for c-Myb that binds to its acidic activation domain.

CBP (CREB-binding protein) is a transcriptional coactivator of CREB (cAMP response element-binding protein) that binds to the cAMP response element (CRE), TGACGTCA (Chrivia et al. 1993; Kwok et al. 1994). An increase in intracellular cAMP concentration leads to the activation of the cAMP-dependent protein kinase A (PKA) that directly phosphorylates CREB at Ser-133 (Gonzalez and Montminy 1989). This Ser-133 is in the transcriptional activation domain, and CBP binds to CREB only when Ser-133 is phosphorylated. As CBP interacts with TFIIB, which contacts TBP and also functions in the recruitment to the promoter of RNA polymerase II, CBP mediates CREB-dependent transcriptional activation as a bridge between phosphorylated CREB and the RNA polymerase II complex. Thus, the phosphorylation-dependent binding of CBP to CREB is a critical step for transcriptional activation through the cAMP pathway. Microinjection of anti-CBP antiserum into fibroblasts inhibited transcription from the promoter containing not only CRE but also a TPA response element (TRE) and serum response element (SRE) (Arias et al. 1994). In addition, CBP was suggested to bind to c-Jun phosphorylated by c-Jun amino-terminal kinase [JNK; also known as stress-activated protein kinases (SAPK)]. These facts suggest that CBP functions as a coactivator for certain phosphorylated activators. In mammals, the CBP gene family has two members, CBP and p300 (Arany et al. 1994). p300 was originally identified as a protein that binds to the adenovirus E1A protein (Eckner et al. 1994). Both CBP and p300 are large proteins composed of ~2400 amino acids and have high homology overall. Recently, CBP was also shown to bind to E1A, and in addition, p300 was found to function as a coactivator of CREB, indicating that the proteins have very similar functions (Arany et al. 1995; Lundblad et al. 1995). The CREB-binding domain is localized in the amino-proximal region of CBP and p300, whereas the E1A-binding domain is in the carboxy-proximal region. Binding of E1A to CBP inhibits CREB-induced transcriptional activation. The recent finding that Rubinstein-Taybi syndrome is associated with mutations in CBP indicates the important physiological role of CBP (Petrij et al. 1995).

We report here that CBP binds to the activation domain of c-Myb and functions as a coactivator. Our results indicate that CBP not only interacts with phospho-CREB but also other transcription factors in a phosphorylation-independent manner. Thus, CBP may be a more general coactivator than was thought previously.

Results

Specific binding of CBP to the nonphosphorylated form of c-Myb

Recently we have identified a *Drosophila* mutant of the CBP gene (dCBP), and found that the tissue and developmental stage specificity of its expression is very similar to that of the *Drosophila myb* gene (H. Akimaru and S. Ishii, unpubl.). This allowed us to speculate that CBP may function as a coactivator of c-Myb. To test this, we first examined whether CBP interacts directly with c-Myb (Fig. 1). Protein affinity resins, in which the glutathione S-transferase (GST) or GST-CBP fusion protein containing the 201-amino-acid region of CBP was used as a ligand, were prepared (Fig. 1A). This 201-amino-acid region of CBP contains the domain responsible for binding to the phosphorylated CREB (Chrivia et al. 1993). The full-length form of mouse c-Myb was synthesized using an in vitro transcription/translation system and mixed with this affinity resin. Approximately 15% of input c-Myb bound to the resin containing the GST-CBP fusion protein but not to GST resin at all (Fig. 1B, lanes 1–3). We confirmed that the in vitro-translated CREB bound to this GST-CBP resin (~12% of input) only when the in vitro-synthesized CREB was treated with the catalytic subunit of PKA (Fig. 1B, lanes 5–8). However, c-Myb bound to the GST-CBP resin without treatment with PKA, and the PKA treatment did not increase the efficiency of binding of c-Myb to CBP (Fig. 1B, cf. lanes 3 and 4). To examine the possibility that the in vitro-translated c-Myb is already phosphorylated by some kinases and that this is why PKA treatment did not affect the binding to CBP, we treated the in vitro-translated c-Myb with potato acid phosphatase and then used it for a binding assay. Previously, we showed that 0.08 unit of phosphatase was sufficient to dephosphorylate the transcription factor HIV-TF1 and decrease its DNA-binding activity (Maekawa et al. 1991b). In this study we used 120-fold more phosphatase (10 units) to treat the in vitro-translated c-Myb, but the phosphatase treatment did not affect the binding of c-Myb to CBP (Fig. 1C, lanes 1–6). In addition, the full-length form of c-Myb that was produced as a soluble form in *Escherichia coli* also bound to the GST-CBP resin efficiently (~10% of input) (Fig. 1C, lanes 7–9). These results indicate that CBP binds to nonphosphorylated c-Myb.

In vitro binding of CBP to the region containing the activation domain of c-Myb

To examine further whether CBP interacts with the activation domain of c-Myb, which is rich in acidic amino acids, we used deletion mutants of c-Myb for a binding assay (Fig. 2A,B). We had identified the region between amino acids 238 and 325 as a transcriptional activation domain (Sakura et al. 1989). The Δ TA mutant lacking this activation domain still partly retained the capacity to bind to CBP. About 12% of the input wild-type c-Myb protein bound to the GST-CBP resin, and 6% of the input Δ TA bound. The weaker binding activity of Δ TA was

confirmed by performing the binding assays using increasing amounts of wild-type and Δ TA proteins (Fig. 2C). Consistent with this, the small protein TA, containing only the activation domain, has a CBP-binding activity, but its activity is also lower than that of wild type (~6% of the input) (Fig. 2A,B). Again, the weaker binding activity of TA was confirmed by using increasing amounts of TA (Fig. 2C). These results suggest that CBP binds to the region containing the activation domain and its adjacent region. Three carboxy-terminally truncated mutants CT1, CTV, and CT2 bound to CBP as efficiently as wild type (~10–12% of the input), but CT5 did not interact with CBP at all. These results indicate that CBP binds to the region between the carboxy-terminal ends of CT5 and CT2 (amino acids 194–401).

Domain of CBP that interacts with c-Myb

We examined whether the domain of CBP required for interaction with c-Myb is the same as the CREB-binding domain. In addition to the GST-fusion protein containing the 201-amino-acid region of CBP (amino acids 461–661) that was used in Figure 1, three fusion proteins containing the broader 265-amino-acid region [GST-CBP265], and the amino-terminal 129-amino-acid region

(GST-CBP129) or carboxy-terminal 80-amino-acid region (GST-CBP80) of the original 201 amino acids, were prepared and used for binding assays (Fig. 3A,B). In the carboxy-terminal 80-amino-acid region (amino acids 590–669 of CBP), the amino acid sequence is highly conserved between CBP, p300, and the two putative homologs of *Caenorhabditis elegans* R10E11.1 and K03H1.10 (Arany et al. 1994; Lundblad et al. 1995). As expected from this highly conserved sequence, phosphorylated CREB bound to this 80-amino-acid region (10% of input) as efficiently as to the 201- and broader 265-amino-acid regions (11% of input in both cases) and did not bind to its adjacent 129-amino-acid region (<1% of input) (Fig. 3C). Although 15% of the input c-Myb bound to the 201- or 265-amino-acid regions, only 6% of input c-Myb bound to this 80-amino-acid region. To confirm these results, binding assays using increasing amounts of c-Myb and CREB were done (Fig. 3D). The binding affinity of c-Myb to GST-CBP80 was significantly lower than that to GST-CBP265, but phosphorylated CREB bound to these two fusion proteins with almost the same affinity. Thus, the 80-amino-acid region of CBP (amino acids 590–669) is required for binding with either phosphorylated CREB or c-Myb, and its adjacent region (amino acids 461–589) increases the binding with c-Myb but not with CREB.

Figure 2. Binding of CBP to the region containing the activation domain of c-Myb. (A) Structure of the c-Myb proteins used for binding assay. (Top) The functional domains of c-Myb are shown schematically. The structures of various forms of c-Myb are shown below. Mutants that bind to CBP are indicated by stippled bars, and a mutant that does not bind, by an open bar. The results of binding assays shown in B are indicated at right. The relative binding activities of the mutants are designated ++, +, and -, which indicate the binding of 10–12%, 5–7%, and <1% of the input protein, respectively. (B) Binding of c-Myb mutants to CBP. In lanes 1–7, various forms of c-Myb shown above each lane were synthesized in vitro and analyzed by 15% SDS-PAGE. In lanes 8–14, the 35 S-labeled proteins shown above each lane were mixed with the GST–CBP affinity resin, which contains amino acids 461–661 of CBP, and the bound

proteins were analyzed on 15% SDS-PAGE followed by autoradiography. In lanes 1–7, the amount of proteins was 10% of that used for binding assay. Less than 0.5% of the input c-Myb proteins bound to the control GST resin (data not shown). (C) Weaker binding of Δ TA and TA to CBP. To compare the binding affinities of wild-type c-Myb, Δ TA, and TA mutants to CBP, increasing amounts of in vitro-translated c-Myb proteins were mixed with affinity resin containing 200 μ g of GST–CBP, and bound proteins were analyzed as described above. (Right) The amounts of the c-Myb proteins bound to GST–CBP are shown.

In vivo binding of c-Myb to CBP

To examine the in vivo interaction between c-Myb and CBP, we used the yeast two-hybrid system. The *Saccharomyces cerevisiae* strain L40 used here contains two integrated reporter constructs, the yeast *HIS3* gene and the bacterial *lacZ* gene, which contain binding sites for the LexA protein (Vojtek et al. 1993). Four plasmids that encode the fusion proteins containing the VP16 activation domain and various portions of c-Myb were constructed (211/360, CT2, CTY, and CT1) (Fig. 4A). All of these fusion proteins contained the domain interacting with CBP, which was identified by the in vitro-binding assay shown in Figure 2. Expression in yeast L40 cells of any of these four fusion proteins together with the fusion protein consisting of the DNA-binding domain of the yeast transcription activator LexA (amino acids 1–211) and the CREB-binding domain of CBP (amino acids 461–682) led to the expression of the reporter gene *HIS3* and

the growth of colonies on the plates lacking histidine (Fig. 4B). In the control experiments with the control plasmid encoding only the LexA DNA-binding domain, none of these four fusion proteins induced the expression of *HIS3* and growth of colonies on the plates lacking histidine. In addition, the VP16 activation domain itself did not activate the expression of *HIS3* when it was expressed with the LexA–CBP fusion protein. The interaction of c-Myb with CBP was also assessed by the ability to trans-activate the *lacZ* reporter construct (Fig. 4C). The four VP16–c-Myb constructs (211/360, CT2, CTY, and CT1) produced 250–380 units of β -galactosidase activity when they were used with the LexA–CBP expression plasmid for transformation of yeast L40 cells. In contrast, the VP16 control vector lacking the c-Myb-coding region generated only the background level of activity (2–3 units) when it was used with the LexA–CBP expression plasmid or with the control plasmid pBTM for transformation. These results indicate that the region

Figure 3. Domain of CBP required for binding to c-Myb. (A) Structure of the GST-CBP fusion proteins used for binding assay. (Top) The structure of murine CBP is shown schematically. The structures of four types of GST-CBP fusion proteins are shown below. The regions of CBP used for binding to c-Myb in Fig. 1 are shown by hatched bars. The results of the binding assays shown in B are indicated at right. The relative binding activities are designated ++, +, and -, which indicate the binding of 10%–14%, 5%–7%, and <1% of the input protein, respectively. (B) Analysis of the GST-CBP fusion proteins. The bacterial lysates containing 3–5 μ g of various GST-CBP fusion proteins or control GST were mixed with the glutathione-Sepharose resin and washed. The bound proteins were analyzed on 10% SDS-PAGE followed by Coomassie brilliant blue staining. In lanes 2 and 3, the GST protein that were generated by degradation of the fusion proteins is visible below. (C) Binding of c-Myb and CREB to four types of GST-CBP fusion proteins. The binding assays were performed using the affinity resin containing 200 μ g of each of the fusion proteins or control GST and shown as described in Fig. 1. The amount of c-Myb and CREB in lane 1 is 10% that used for binding assay. (D) Comparison of the binding affinities using increasing amounts of in vitro-translated protein. To compare the binding affinities of c-Myb or CREB to the 80- and 201-amino-acid regions of CBP, increasing amounts of in vitro-translated c-Myb or phosphorylated CREB proteins were mixed with the affinity resin containing 200 μ g of GST-CBP, and bound proteins were analyzed as described above. (Right) The amounts of c-Myb and CREB bound to GST-CBP are shown.

between amino acids 211 and 360 of c-Myb interacts with CBP in vivo.

Coactivation of c-Myb-dependent gene expression by CBP

To examine whether CBP functions as a coactivator of c-Myb, we then did some chloramphenicol acetyltransferase (CAT) cotransfection experiments using CV-1 cells (Fig. 5). The plasmid pA10CAT6MBS-I, which contains six tandem repeats of the Myb-binding site MBS-I linked to the SV40 early promoter, was used as a reporter. The CAT expression from this reporter plasmid is activated by c-Myb, and cotransfection of this reporter with 3 μ g of the c-Myb expression plasmid increased the level of CAT activity about four-fold in our standard as-

say system (Nakagoshi et al. 1990; Nomura et al. 1993; Fig. 5, cf. lanes 1 with or without c-Myb). As we reported previously (Nomura et al. 1993), 0.5–1 μ g of the c-Myb expression plasmid DNA gives maximum *trans*-activation, and the degree of *trans*-activation decreases with additional increases in the amount of the c-Myb expression plasmid. Here we used 3 μ g of the c-Myb expression plasmid because we expected that an excess of c-Myb would activate transcription by using CBP expressed from the CBP expression plasmid. Increasing amounts of the mouse CBP expression plasmid were cotransfected with this reporter in the presence or absence of 3 μ g of the c-Myb expression plasmid. Cotransfection of increasing amounts of the CBP expression plasmid increased the degree of *trans*-activation up to a maximum of 18-fold with 6 μ g of CBP expression plasmid in the presence of the c-Myb expression plasmid. In the control experi-

Figure 4. In vivo interactions of c-Myb and CBP. (A) Structure of the VP16-c-Myb fusion proteins used for yeast two-hybrid assay. (Top) The functional domains of c-Myb are shown schematically. The structures of various forms of VP16-c-Myb fusion proteins are shown below. 211/360, CT2, CTY, and CT1 contain the amino acids 211–360, 211–400, 211–460, and 211–500 of c-Myb, respectively. (B) Assay of *HIS3* reporter construct. The *S. cerevisiae* reporter strain L40 was transformed with the pBTM-CBD plasmid encoding the fusion protein, which consists of the LexA DNA-binding domain (amino acids 1–211) and the CREB-binding domain of CBP (amino acids 461–682), and the plasmid to express the indicated VP16-c-Myb fusion protein (1–5). In the control experiments (6–10), L40 was transformed with the pBTM vector encoding only the LexA DNA-binding domain and the plasmid to express the indicated VP16-c-Myb fusion protein. Individual *Leu⁺ Trp⁺* transformants were streaked on synthetic medium plates lacking tryptophan, histidine, leucine, uracil, and lysine (– His) but including 15 mM 3-aminotriazole and on synthetic medium plates lacking leucine and tryptophan (+ His) but containing 15 mM 3-aminotriazole. The plates were incubated at 30°C for 3 days. (C) β-Galactosidase activity from the *lacZ* reporter construct. Three independent transformants harboring the plasmids indicated at left were isolated and grown, and their β-galactosidase activities were measured. Values shown are the averages of duplicate assays of three independent transformants with the standard deviation indicated.

ment without any c-Myb expression plasmid, addition of the CBP expression plasmid did not affect the level of CAT activity at all. Thus, CBP potentiates c-Myb-induced *trans*-activation.

To confirm that CBP does not affect the level of c-Myb expression from the transfected DNA, the levels of c-Myb in the transfected cells were examined in the presence or absence of the CBP expression plasmid (Fig. 5B). Cell lysates were prepared from cells transfected with a mixture of the c-Myb expression plasmid and the CBP expression plasmid or the control plasmid and used for Western blotting with the c-Myb-specific antibody. The level of c-Myb in the presence of the CBP expression plasmid was almost the same as that observed without the CBP expression plasmid.

We next asked whether CBP is required for the c-Myb-dependent transcriptional activation by using the plas-

mid to express the antisense RNA of mouse CBP (Fig. 6A). Expression of the antisense RNA in mouse NIH-3T3 cells can be expected to block the synthesis of endogenous CBP. Increasing amounts of the antisense CBP RNA expression plasmid were cotransfected with the reporter plasmid pA10CAT6MBS-I in the presence or absence of the c-Myb expression plasmid. The antisense CBP RNA inhibited the c-Myb-dependent transcriptional activation in a dose-dependent manner but did not affect the basal promoter activity of the reporter plasmid in the absence of c-Myb. Western blot analysis of the level of c-Myb in the transfected cells indicated that the antisense CBP RNA expression plasmid did not affect the c-Myb expression from the transfected c-Myb expression plasmid (Fig. 6B). These results suggest that endogenous CBP is required for c-Myb-induced transcriptional activation.

Figure 5. Effects of CBP on c-Myb-dependent transcriptional activation. (A) Potentiation of c-Myb-activated gene expression by CBP. A mixture of 0, 1, 2, 3, 4, 5, or 6 μ g (lanes 1–7) of the CBP expression plasmid pRc/RSV-CBP, 4 μ g of a CAT reporter plasmid pA10CAT6MBS-I, 3 μ g of the c-Myb expression plasmid pact-c-myb (*top*) or the control plasmid pact-1 (*bottom*) lacking the c-Myb-coding region, and 1 μ g of the internal control plasmid pact- β -gal was transfected into CV-1 cells. The total amount of DNA was adjusted to 14 μ g by adding the control plasmid pRSV0 lacking the CBP-coding region. CAT assays were done as described in Materials and methods. A typical result is shown at *left*. The degree of transactivation (compared to samples without the c-Myb expression plasmid) shown at *right* is the average of three independent experiments with standard deviations indicated. (B) Effects of CBP on the expression of c-Myb in transfected cells. CV-1 cells were transfected with a mixture of 6 μ g of the CBP expression plasmid pRc/RSV-CBP (+) or the control plasmid pRc/RSV0 (–), 4 μ g of the CAT reporter plasmid pA10CAT6MBS-I, 3 μ g of the c-Myb expression plasmid pact-c-myb, and 1 μ g of the internal control plasmid pact- β -gal. Proteins were prepared from the transfected cells and separated by SDS-PAGE on a 10% gel. The c-Myb proteins were detected by Western blot analysis. The precise amounts of lysates used for Western blotting were normalized with respect to β -galactosidase activity. Lanes 1 and 2 contain the proteins from $\sim 2 \times 10^6$ cells. The amount of proteins in lanes 3 and 4, and lanes 5 and 6 is $\frac{1}{2}$ and $\frac{1}{4}$, respectively, of the amount in lanes 1 and 2.

Figure 6. Inhibition of c-Myb-induced transcriptional activation by expression of the antisense CBP RNA. (A) Repression of c-Myb-dependent gene expression by antisense CBP RNA. A mixture of 0, 2, 4, 6, 8, or 10 μ g (lanes 1–6) of the antisense CBP RNA expression plasmid pSR α -anti-CBP, 4 μ g of a CAT reporter plasmid pA10CAT6MBS-I, 3 μ g of the c-Myb expression plasmid pact-c-myb (*top*) or the control plasmid pact-1 (*bottom*) lacking the c-Myb-coding region, and 1 μ g of the internal control plasmid pact- β -gal was transfected into NIH-3T3 cells. The total amount of DNA was adjusted to 14 μ g by adding the control plasmid pRSV0 lacking the CBP-coding region. CAT assays were done as described in Materials and methods. The results are as shown in Fig. 5. (B) Effects of antisense CBP RNA on the expression of c-Myb in transfected cells. NIH-3T3 cells were transfected with a mixture of 6 μ g of the antisense CBP RNA expression plasmid pSR α -anti-CBP (+) or the control plasmid pRc/RSV0 (–), 4 μ g of the CAT reporter plasmid pA10CAT6MBS-I, 3 μ g of the c-Myb expression plasmid pact-c-myb, and 1 μ g of the internal control plasmid pact- β -gal. Western blotting was performed, and the results are as shown in Fig. 5.

Effects of E1A on c-Myb-induced transcriptional activation

E1A binds to CBP and inhibits CREB-dependent transcriptional activation (Arany et al. 1995; Lundblad et al. 1995). We therefore examined the effects of E1A on c-Myb-induced transcriptional activation (Fig. 7). Here we used 1 μ g of the c-Myb expression plasmid to observe the maximum *trans*-activation by c-Myb. Cotransfection of the reporter plasmid pA10CAT6MBS-I with the E1A 12S expression plasmid did not affect the basal level of CAT activity (lanes 1–4). However, in the presence of the c-Myb expression plasmid, E1A 12S decreased the level of CAT activity in a dose-dependent manner (lanes 5–8). As a control, two E1A mutants, Δ 30–85 and Δ 121–

150, were also used. It had been demonstrated that CBP and E1A interact *in vivo* with the same specificity as do p300 and E1A (Arany et al. 1995; Lundblad et al. 1995). Δ 30–85 is known neither to interact efficiently with p300 (Whyte et al. 1989) nor to block p300-dependent *trans*-activation, but Δ 121–150, which is unable to interact with any retinoblastoma family protein (Stein et al. 1990), interacts with p300 and inhibits p300-dependent *trans*-activation. As shown in Figure 7, c-Myb-induced transcriptional activation was blocked by Δ 121–150 (lanes 17–24) but not by Δ 30–85 (lanes 9–16). Thus, the repression by E1A 12S is specific and depends on the ability of E1A to interact with CBP/p300. When we also added the CBP expression plasmid, the inhibition of c-Myb-induced transcriptional activation by E1A 12S

Figure 7. Repression of c-Myb-dependent transcriptional activation by E1A. (A) Typical result of CAT assay. In the experiments (lanes 1–24) examining the effects of three forms of E1A on c-Myb-dependent gene expression, a mixture of 4 μ g of the reporter plasmid pA10CAT6MBS-I, 1 μ g of the c-Myb expression plasmid *pact-c-myb* (+) or the control plasmid *pact-1* (–), 0 (lanes 1, 5, 9, 13, 17, 21), 2 (lanes 2, 6, 10, 14, 18, 22), 4 (lanes 3, 7, 11, 15, 19, 23), or 6 (lanes 4, 8, 12, 16, 20, 24) μ g of the E1A 12S (lanes 1–8), Δ 30–85 (lanes 9–16), or Δ 121–150 (lanes 17–24) expression plasmid, and 1 μ g of the internal control plasmid *pact- β -gal* was transfected into CV-1 cells. The total amount of DNA was adjusted to 12 μ g with the control plasmid *pE1A*[–] (lanes 1–8) or *pCMV0* lacking the E1A-coding region (lanes 9–24). In the experiments examining the effects of CBP on the repression of the c-Myb-induced transcription by E1A (lanes 25–30), a mixture of 4 μ g of the reporter plasmid pA10CAT6MBS-I, 1 μ g of the c-Myb expression plasmid *pact-c-myb* (+) or the control plasmid *pact-1* (–), 2 μ g of E1A 12S expression plasmid (+) or the control plasmid *pE1A*[–] (–), 0 (lanes 25–27), 6 (lane 28), 10 (lane 29), or 14 (lane 30) μ g of the CBP expression plasmid *pSR α -CBP* DNA, and 1 μ g of the internal control plasmid *pact- β -gal* was transfected into CV-1 cells. The total amount of DNA was adjusted to 22 μ g with the control plasmid *pE1A*[–] lacking the E1A-coding region and *pSR α 0* lacking the CBP-coding region (lanes 9–24). (B) The results of three independent experiments. The average of three experiments are shown as relative CAT activity with standard deviations indicated.

was abolished (lanes 25–30). These results indicate that E1A suppresses c-Myb-dependent transcriptional activation and repression by E1A is mediated by CBP.

Discussion

Our results indicate that CBP binds to the region containing the activation domain of c-Myb and functions as a coactivator of c-Myb. CBP binds to phosphorylated CREB, but not to the nonphosphorylated form. In contrast, in vitro-translated c-Myb bound to GST–CBP without phosphorylation by PKA. This result was confirmed by phosphatase treatment of in vitro-translated c-Myb and the experiment with the bacterially expressed c-Myb: Recombinant c-Myb also bound efficiently to CBP without any phosphorylation. These results indicate that CBP binds to nonphosphorylated c-Myb, although we cannot exclude the possibility that phosphorylation of c-Myb by some kinase increases the efficiency of binding to CBP. Recently, CBP has been suggested to be a coactivator specific to a group of phosphorylated activators, including CREB, Jun, and SRF (Arias et al. 1994). Our results indicate that CBP is a coactivator not only for phosphorylated CREB but also for nonphosphorylated transcription activators such as c-Myb. During preparation of this paper Bannister and Kouzarides (1995) reported that CBP also binds to c-Fos in a phosphorylation-independent manner and functions as a coactivator for c-Fos. However, c-Fos binds to the carboxy-terminal domain of CBP, which is also used to contact E1A, and this Fos-binding domain is different from the c-Myb- or CREB-binding domain. In addition, Lee et al. (1995) reported that the CBP-related protein p300 binds to the transcriptional repressor YY1, a human glioblastoma–Krüppel-related protein (Shi et al. 1991), and that binding of E1A to p300 relieves transcriptional repression by YY1. In this case, CBP also binds to nonphosphorylated YY1. Therefore, CBP binds to the transcriptional activators CREB, c-Fos, c-Myb, and the repressor YY1. Thus, CBP may function as a coactivator for more enhancer-binding factors than expected previously.

Our results indicate that the 80-amino-acid region of CBP (amino acids 590–669) is sufficient for binding to phosphorylated CREB and necessary for binding to c-Myb. Thus, CREB and c-Myb appear to bind to the same region of CBP. Interestingly, mutations in the human CBP gene cause Rubinstein–Taybi syndrome through haploinsufficiency (Petrij et al. 1995), suggesting that the amount of CBP in cells is not excessive and a 50% decrease in the amount affects normal development. Transfection of only 2 μ g of the plasmid to express antisense CBP RNA resulted in a 50% decrease in *trans*-activation by c-Myb (Fig. 6A). These results may also be consistent with this speculation. Thus, cross talk between the c-Myb and cAMP pathway through competition with phosphorylated CREB for binding to CBP is possible. Therefore, it is important to examine whether c-Myb activity is inhibited by activation of the cAMP

pathway. Although YY1 and c-Fos bind to different domains of CBP or p300 from c-Myb and CREB, we cannot exclude the possibility that the YY1- and/or c-Fos-interacting domain is also necessary to mediate c-Myb- and CREB-induced transcriptional activation. In this context, it is also of interest whether c-Myb affects not only the CREB- but also the AP-1-, or YY1-dependent transcriptional regulation by competing for binding to CBP.

To bind to CBP efficiently, the region between amino acids 194 and 401 of c-Myb is necessary, and the transcriptional activation domain identified previously (amino acids 238–325) containing the acidic amino acid-rich region binds to CBP with lower efficiency. These results suggest that CBP binds to multiple regions, including the acidic amino acid cluster in the activation domain of c-Myb. Thus far, the presence of multiple subdomains in the carboxy-terminal half of c-Myb that negatively regulate the c-Myb activity was reported (Sakura et al. 1989; Kalkbrenner et al. 1990; Dubendorff et al. 1992; Kanei-Ishii et al. 1992). Although the mechanism of negative regulation of c-Myb activity by these subdomains is unclear, some of them were suggested to modulate transcriptional activation, not DNA binding. Analyses of the effects of these subdomains on binding to CBP may lead to understanding the role of these subdomains.

Among the three members of the mammalian *c-myb* gene family, A-Myb and c-Myb function as a transcriptional activator in all cells examined, but the transcriptional activating capacity of B-Myb depends on the cell type (Takahashi et al. 1995; Tashiro et al. 1995). B-Myb activates transcription through binding to the Myb-binding site, MBS-I, in CV-1 cells but not in NIH-3T3 cells. It was suggested that the putative cofactor that binds to the carboxy-terminal region in B-Myb is required for cell type-specific *trans*-activation by B-Myb. Although it is not known whether CBP binds to B-Myb, the interesting possibility is that this putative cofactor supports the interaction between CBP and B-Myb.

E1A can down-regulate transcriptional activation mediated by c-Myb. This result is similar to the recent report that E1A can repress CREB- and c-Fos-induced transcriptional activation (Arany et al. 1995; Bannister and Kouzarides 1995; Lundblad et al. 1995). Because the growth-inhibitory and differentiation-inducing effects of cAMP are well documented, the ability of E1A to interfere with the cAMP pathway through CBP could be important for cell immortalization by E1A. However, c-Myb and c-Fos are positive regulators of proliferation, so that the block of the c-Myb- or c-Fos-induced transcriptional activation by E1A appears not to be consistent with the view that E1A-induced cell immortalization may depend on interactions between E1A and CBP. One idea to resolve this discrepancy is that c-Myb and c-Fos are implicated in both the proliferative and differentiation pathways and that E1A preferentially blocks the differentiation-specific transcription induced by c-Myb and c-Fos. Interestingly, it was postulated that the promoters of proliferation genes regulated by c-Myb possess Myb-binding sites with higher affinity or greater

number than do differentiation genes (Dini and Lipsick 1993). It is of interest whether E1A leads to preferential inhibition of the activity of promoters to which c-Myb binds weakly by partly repressing the function of CBP. Analyses of the effect of CBP or antisense CBP RNA expression plasmid on the c-Myb-induced *trans*-activation of various promoters may contribute to examination of this possibility.

Materials and methods

Plasmids

To express various forms of GST–CBP fusion proteins in *E. coli*, the plasmids pGST–CBP265, pGST–CBP201, pGST–CBP129, and pGST–CBP80 were made by inserting the *Bam*HI–*Eco*RI fragment encoding the regions of 265 (amino acids 454–718), 201 (amino acids 461–661), 129 (amino acids 461–589), and 80-amino-acids (amino acids 590–669) of mouse CBP, respectively, which was prepared by PCR, into the *Bam*HI–*Eco*RI site of the pGEX–4T-3 vector (Smith and Johnson 1988; Pharmacia). The modified pSP65 vector pSPUTK (Stratagene) was used for *in vitro* transcription/translation of various forms of c-Myb, except for the Δ TA mutant. This vector contains the *Xenopus* β -globin 5'-untranslated region and the consensus Kozak translation initiation site. To generate the plasmids to express various forms of c-Myb, the *Nco*I–*Xba*I fragment containing the c-Myb-coding region was prepared from the actin promoter-based expression plasmids and inserted into the *Nco*I–*Xba*I site of pSPUTK. To construct the plasmid pSPUTKmybTA, which was used to synthesize *in vitro* the TA protein containing only the activation domain of c-Myb, the *Nco*I–*Bam*HI fragment containing the region between amino acids 243 and 325 of c-Myb was prepared by a PCR-based method (Higuchi 1990). This fragment was inserted into the *Nco*I–*Bam*HI site of pSPUTK. The plasmid pSP65NcoImyb Δ TA, expressing the c-Myb Δ TA mutant in the *in vitro* transcription/translation system, was constructed by inserting the *Nco*I–*Xba*I fragment containing the c-Myb-coding region into the *Nco*I–*Xba*I site of the modified pSP65 vector pSP65NcoI, which has an *Nco*I site at the original *Sma*I site.

The plasmids expressing c-Myb in cultured cells have been described (Sakura et al. 1989). The pA10CAT6MBS-I reporter plasmid containing tandem repeats of the Myb-binding site, the control effector plasmid pact-1, and the internal control plasmid pact- β -gal were also described previously (Nakagoshi et al. 1990; Maekawa et al. 1991a). The CBP expression plasmid pRc/RSV–CBP (Chrivia et al. 1993) and the CREB expression plasmid pRSV–CREB (Gonzalez and Montminy 1989) were kindly provided by Drs. R.H. Goodman (Vollum Institute, Oregon Health Sciences University, Portland) and M.R. Montminy (Salk Institute, La Jolla, CA), respectively. The plasmids expressing CBP or antisense CBP RNA, pSR α –CBP, or pSR α –anti-CBP were constructed by transfer of the *Bam*HI CBP cDNA insert of pRc/RSV–CBP into the *Eco*RI site of the SR α promoter construct in either direction after converting both sites into flush ends (Takebe et al. 1988). The E1A expression plasmid pE1A12S and the control plasmid lacking the E1A-coding region (Leff et al. 1984) were kindly donated by Dr. C. Keding. The plasmids to express E1A mutants Δ 30–85 and Δ 121–150 are gifts from Dr. R. Eckner (The Dana Farber Cancer Institute and Harvard Medical School, Boston, MA).

The plasmid pBTM–CBD, expressing in yeast cells a fusion protein consisting of the LexA DNA-binding domain (amino acids 1–211) and the CREB-binding domain of CBP (amino acids

461–682), was a gift from Dr. R.H. Goodman. The parental vector pBTM116 for pBTM–CBP was described by Vojtek et al. (1993). The four plasmids expressing the fusion proteins consisting of the VP16 activation domain and various portions of c-Myb were constructed as follows using the plasmid pVP16, which was also described by Vojtek et al. (1993). The *Bam*HI–*Not*I fragment encoding the amino acids 211–360 of c-Myb was prepared by PCR and inserted into the *Bam*HI–*Not*I cloning site of pVP16 to generate the plasmid pVP16–211/360. To construct the three other plasmids, pVP16–CT2, pVP16–CTY, and pVP16–CT1, the *Sph*I–*Bam*HI fragments encoding the carboxy-terminal half of the c-Myb-coding region were prepared from the β -actin promoter-based expression plasmids for CT2, CTY, and CT1 (Sakura et al. 1989; Hu et al. 1991), and the *Sph*I–*Not*I fragment of pVP16–211/360 was replaced by either of these fragments after converting the *Bam*HI and *Not*I sites into flush ends. These three plasmids encode amino acids 211–400, 211–460, and 211–500 of c-Myb, respectively.

In vitro-binding analysis with GST fusion proteins

Expression of the GST–CBP fusion protein or GST in *E. coli* and preparation of the bacterial lysates containing these proteins were done as described (Nomura et al. 1993). Samples of bacterial lysate containing 200 μ g of GST or GST–CBP were rocked for 2 hr at 4°C with 80 μ l of glutathione–Sepharose beads (Pharmacia). The beads were washed five times with 1 ml of PBS and then with 1 ml of binding buffer [20 mM HEPES (pH 7.7), 75 mM KCl, 0.1 mM EDTA, 2.5 mM MgCl₂, 1% skim milk, 1 mM DTT, 0.05% NP-40]. Various forms of c-Myb were synthesized with [³⁵S]methionine using an *in vitro* transcription/translation kit according to the procedures described by the supplier (Promega). Then, 20 μ l of reaction was mixed with 1.5 ml of binding buffer and GST or GST–CBP affinity resin. After rocking at 4°C for 2 hr, the resin was washed five times with 1 ml of PBS and mixed with SDS–sample buffer, and the bound proteins were released by boiling. The proteins were analyzed by SDS–PAGE followed by autoradiography. In experiments examining the effects of phosphorylation by PKA, 50 μ l of lysate containing *in vitro*-translated c-Myb or CREB was mixed with 150 μ l of the kinase buffer to adjust to a final concentration of 20 mM Tris–HCl (pH 7.5), 100 mM NaCl, 12 mM MgCl₂, and 2.5 mM ATP and incubated with or without 100 units of the catalytic subunit of bovine protein kinase A (Sigma). In experiments examining the effects of phosphatase, 20 μ l of lysates containing ³⁵S-labeled c-Myb was incubated with or without 10 units of potato acid phosphatase (Sigma) in 500 μ l of the phosphatase buffer [10 mM Tris–HCl (pH 7.5), 50 mM KCl, 1 mM DTT, 0.05% NP-40, 100 μ g/ml of bovine serum albumin (BSA), and 5% glycerol]. To examine the interaction of bacterially expressed c-Myb and CBP, full-length c-Myb was expressed as a soluble form using our newly developed *E. coli* expression system, in which many mammalian proteins were expressed as soluble forms by coexpression of the bacterial thioredoxin (Yasukawa et al. 1995). The bacterial lysates containing the soluble form of c-Myb was prepared using buffer M [50 mM Tris–HCl (pH 8.0), 0.5 mM EDTA, 10 mM DTT, 125 mM PMSF], similarly to the case of GST–CBP fusion proteins, and then passed through DEAE–cellulose. The flowthrough fraction containing c-Myb was used for binding analysis. The binding assays with the bacterially made c-Myb were done similarly.

In vivo interaction analysis using the yeast two-hybrid system

Yeast two-hybrid assays were performed using a modified version (Vojtek et al. 1993) of the system developed by Fields and

Song (1989). The genotype of the *S. cerevisiae* reporter strain L40 was described by Vojtek et al. (1993). The *S. cerevisiae* L40 reporter strain was transformed with two kinds of plasmids encoding the LexA-CBP fusion protein or the c-Myb-VP16 fusion protein. Transformants were plated on a synthetic medium lacking leucine, tryptophan, and uracil. The plates were incubated at 30°C for 3 days. Individual Leu⁺ Trp⁺ transformants were picked up and streaked on synthetic medium plates lacking tryptophan, histidine, leucine, uracil, and lysine but including 15 mM 3-aminotriazole, a chemical inhibitor of imidazole glycerol phosphate dehydratase which restores histidine auxotrophy [Durfee et al. 1993], and on control synthetic medium plates lacking leucine, tryptophan, and uracil but containing 15 mM 3-aminotriazole. The plates were incubated at 30°C for 3 days. To measure β -galactosidase activity, three independent transformants grown in synthetic control medium lacking leucine, tryptophan, and uracil were isolated and grown at 30°C until A_{600} reached 0.8–1.0. β -Galactosidase activity was measured as described by Guarente (1983).

CAT cotransfection experiments

The CAT cotransfection assay was done essentially as described [Nakagoshi et al. 1990]. A mixture of DNAs described in the legends to Figures 5–7 was transfected into African green monkey kidney cells (CV-1) or mouse fibroblast NIH-3T3 cells. The total amount of DNA was adjusted by adding the control effector plasmid DNA. The amounts of lysates used for the CAT assay were normalized with β -galactosidase activity expressed from the internal control plasmid pcat- β -gal. All CAT cotransfection experiments were repeated at least three times. Typical results and the average of three independent experiments with standard deviations are shown in Figures 5–7.

Western blot analysis of c-Myb proteins

A mixture of the plasmid DNAs described in the legends to Figs 5 and 6 was transfected into CV-1 or NIH-3T3 cells as described above for CAT cotransfection experiments. Forty-eight hours after transfection, the cell lysates were prepared and β -galactosidase activity was measured using a sample of cell lysates as was performed for CAT assays. Appropriate amounts of extracts (the precise amounts were normalized with respect to β -galactosidase activity) from each transfection sample were separated by 10% SDS-PAGE followed by blotting to nitrocellulose membranes. Blots were blocked in phosphate-buffered saline (PBS: 130 mM NaCl, 2.7 mM KCl, 10 mM potassium phosphate buffer at pH 7.2) containing 1.5% BSA and 1.5% skim milk. c-Myb was detected by using the anti-c-Myb monoclonal antibody 1-1 [Ramsay et al. 1989], alkaline phosphatase-conjugated anti-mouse IgG, and a color development reagent (Promega).

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