# Enzymic characterization of a novel member of the regulatory B-like carboxypeptidase with transcriptional repression function: stimulation of enzymic activity by its target DNA

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The adipocyte-enhancer binding protein (AEBP) 1 is a novel transcriptional repressor with carboxypeptidase (CP) activity. AEBP1 binds to a regulatory sequence (termed adipocyte enhancer 1, AE-1) located in the proximal promoter region of the adipose P2 (*aP2*) gene, which encodes the adipocyte fatty-acid binding protein. Sequence comparisons and kinetic studies using known carboxypeptidase substrates, activators and inhibitors have characterized AEBP1 as a member of the regulatory B-like CP family. Significantly, the inherent CP activity of AEBP1 is stimulated by the AE-1 sequence. Our results indicate that AEBP1 is activated by a novel mechanism, wherby the direct binding of DNA enhances its protease activity. These results represent the first demonstration of DNA-mediated regulation of CP activity.

Key words: adipocyte differentiation, gene expression, pre-adipocyte factor.

## INTRODUCTION

The use of preadipocyte cell lines, such as 3T3-L1 and 3T3-F442A, as models of adipocyte differentiation is common, since the process in these models closely resembles that of precursor cells from adipose tissue [1]. The stimulation of differentiation in 3T3-L1 cells is characterized by an increased lipogenic capacity, a change from a fibroblast morphology to the unilocular appearance of mature adipocytes and an alteration in the expression levels of hundreds of proteins. In particular, studies have been focused on the regulation of adipose P2 (*aP2* or *422*) gene expression and its significance in adipocyte differentiation [2,3].

We have defined previously a *cis*-controlling element, adipocyte enhancer 1 (AE-1) and its *trans*-acting factors, which include the positive murine (3T3) adipocyte factor, CCAAT/enhancer binding protein $\alpha$ [4], human preadipocyte factors [5] and the negative 3T3 preadipocyte factor, adipocyte-enhancer binding protein (AEBP) 1 [6], which are involved in either positive or negative regulation of *aP2* gene expression. Past work has focused on the transcriptional repressor factor AEBP1. The remarkable findings were that AEBP1 is a novel carboxypeptidase (CP) and that the CP activity was involved in *aP2* repression. AEBP1, by binding to the regulatory AE-1 site in the proximal promoter region of *aP2* gene, acts as a negative regulator to this important adipocyte gene.

CPs are proteases that cleave C-terminal amino acids from polypeptides. There are two broad classes or families of CPs, the digestive type and the regulatory CP-B-like type. The digestive CPs play an important role in general protein and peptide degradation. CP-A cleaves C-terminal aromatic and aliphatic amino acids, whereas CP-B cleaves basic amino acids and glycine. The lysosomal CP-B removes all C-terminal amino acids except proline [7]. The second family of CPs is the B-like or regulatory CPs. These enzymes remove C-terminal arginine and lysine residues, and play an important role in the processing of certain polypeptides and hormones. Members of this family of enzymes participate in the release of active peptides, alter membrane receptor specificity for polypeptides and terminate polypeptide activity ([8] and references therein). The physiological importance of these B-like CPs is exemplified by CP-E, which is involved in insulin processing. Mature insulin is produced by a series of proteolytic cleavages. Pro-insulin is cleaved by an endoprotease at dibasic arginine–arginine and lysine–arginine sequences. CP-E then removes the remaining arginine–arginine sequences found at the C terminus, resulting in the mature insulin molecule.

CPs have been shown to be involved in the onset of obesity. Naggert et al. [9] reported that mice homozygous for the *fat* mutation are obese and hyperglycemic. The mutation responsible for this phenotype was found in the *CP-E* gene. A single amino acid substitution, $Ser^{202} \rightarrow Pro$, in a highly conserved region of CP-E destabilized and abolished enzyme activity. Another interesting example of the diversity and importance of CPs is seen in the function of tubulin CP. This enzyme cleaves C-terminal tyrosine residues from subunits of tubulin, a modification that occurs during neural and muscle differentiation. The modification of tubulin by this CP may be required to modulate tubulin during these differentiation processes [10]. CP-M is a membrane-bound enzyme that removes C-terminal arginine or lysine residues from various polypeptides [8]. McGuire and Skidgel [11] showed that CP-M cleaves epidermal growth factor at the C-terminal arginine to form des-$Arg^{53}$-epidermal growth factor. This modified form of epidermal growth factor was found to bind to its receptor better, although the physiological function of this cleavage is unknown.

The above examples demonstrate the diverse functions of the regulatory B-like CPs in several cell functions, including differentiation and cell signalling.The main body of AEBP1 contains a domain with a high degree of similarity to the family of B-like CPs [6]. Sequence comparisons suggested that AEBP1 may function similarly to the CP-B-like proteases. In this study we

Abbreviations used: aP2, adipose P2; AE-1, adipocyte enhancer 1; AEBP, adipocyte-enhancer binding protein; CP, carboxypeptidase.

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present a detailed characterization of the enzymic activity of AEBP1. These studies indicated that AEBP1 functions as a CP-B-like enzyme in terms of its response to substrates, activators and inhibitors. In addition, our results indicated that the CP activity of AEBP1 is stimulated by a novel mechanism, whereby the direct binding of its target DNA enhances its CP activity.

## MATERIALS AND METHODS

### Materials

The expression plasmid pET-16b was from Novagene. Restriction enzymes were purchased from either New England Biolabs or Gibco/BRL. Talon metal-affinity resin was purchased from Clontech. Hippuryl-L-arginine, hippuryl-L-lysine and hippuryl-L-phenylalanine were purchased from Sigma.

### Induction of recombinant protein

The AEBP1 cDNA insert [6], cloned into the vector pET-16b, was transformed into DH5$\alpha$ cells, and transformants were screened by isolation of plasmid DNA followed by endonuclease restriction digests to identify positive clones. Once clones were identified, they were transformed into DE3 cells. Single colonies were selected and grown overnight in 2 ml Luria-Bertani medium containing ampicillin (100 $\mu$g/ml) at 37 °C. From these overnight cultures, 1 ml was transferred to 500 ml of Luria-Bertani medium containing ampicillin (100 $\mu$g/ml). The cells were grown at 37 °C for approx. 4 h until the $D_{600}$ was between 0.5 and 0.6. To induce protein expression, isopropyl thio-$\beta$-D-galactoside (2 mM) was added and the cells were grown at 37 °C for a further 3.5 h. Cells were collected from a 500 ml culture by centrifugation at 4000 ***g*** for 5 min. The pellet was resuspended and lysed by incubation in 2.5 ml of lysis buffer (50 mM $NaH_2PO_4$, 10 mM Tris/HCl, 6 M guanidinium HCl, 100 mM NaCl, pH 8.0) per 50 ml of culture, with gentle mixing for 1 h at room temperature. The resuspended material was sonicated for 30–60 s and then centrifuged at 27000 ***g*** for 10 min to remove cell debris. The soluble cell lysate was stored at −20 °C until needed for further purification.

### Purification of recombinant protein

Purification of His-tagged recombinant protein was carried out using Talon metal-affinity resin according to the manufacturer's specifications but with a number of minor modifications. The resin was equilibrated by incubation with lysis buffer. Lysate was added to the equilibrated resin and incubated for 1 h at room temperature with constant agitation. After the incubation, the resin was collected by centrifugation at 500 ***g*** for 5 min and the eluent was discarded. The resin was washed with 10 ml of lysis buffer, mixed by gentle shaking for 10 min, collected by centrifugation for 5 min at 500 ***g*** and the supernatant was discarded. This washing step was repeated twice. The washed Talon metal-affinity resin was resuspended in 2 ml of lysis buffer and transferred to a column (BioRad), washed again with 4–6 ml of lysis buffer followed by further washing with wash buffer (50 mM $NaH_2PO_4$, 8 M urea, 100 mM NaCl) adjusted to pH 8.0 for the first wash and to pH 7.0 for the second wash, until the $A_{280}$ of the eluate was less than 0.01. The recombinant protein was eluted from the resin with 6 ml of elution buffer (50 mM $NaH_2PO_4$, 8 M urea, 20 mM Pipes, 100 mM NaCl, pH 6.3) and 1 ml fractions were collected; protein was monitored at $A_{280}$. A second elution was performed using elution buffer adjusted to pH 5.0, the fractions were similarly collected and monitored. Samples of each fraction were analysed by SDS/PAGE [12] to determine which fractions contained purified recombinant protein. The most concentrated recombinant protein fractions, as determined by SDS/PAGE, were renatured using a step-wise dialysis procedure at 4 °C. The following series of dialysis solutions were used: (1) wash buffer containing 4 M urea, pH 7.5 for 2 h; (2) wash buffer containing 2 M urea, pH 7.5 for 2 h; (3) wash buffer containing 1 M urea, pH 7.5 for 2 h; (4) wash buffer containing 0.5 M urea, pH 7.5 for 2 h; (5) wash buffer without urea but containing 10% (v/v) glycerol, pH 7.5, overnight. After renaturation, protein concentrations were determined using a Coomassie Blue dye-binding method (Bio-Rad) [13] with bovine gammaglobulin as a standard. The renatured recombinant protein samples were divided into portions and stored at −20 °C.

### CP assay

CP activity was measured by a method developed by Folk et al. [14] and modified by Fricker and Devi [15]. In this method, the conversion of hippuryl-L-arginine, hippuryl-L-lysine or hippuryl-L-phenylalanine to hippuric acid was monitored spectrophotometrically at $A_{254}$. Purified recombinant AEBP1 protein (2 $\mu$g) was mixed with each of the hippuryl substrates (1 mM) in 0.1 M sodium acetate, pH 5.5, incubated at 37 °C and the enzyme activity was measured at $A_{254}$ at 4 min intervals. All assays were repeated four times and values given are means ± S.D. For all assays with activators or inhibitors, the compounds were pre-incubated with the enzyme, on ice, for 1 h before the enzyme assay.

## RESULTS AND DISCUSSION

### CP activity of AEBP1

Comparisons between AEBP1 and regulatory B-like CPs showed that these proteins vary greatly in the C- and N-terminus [16]. As these differences are thought to determine the location of the CP in the cell, and there are also variations in the C- and N-terminus of the protease region of AEBP1, which has an extra 165 amino acids in the N-terminus and 168 amino acids in the C-terminus, these additional amino acids may contribute to a multifunctional role for AEBP1 within the cell (S.-W. Kim, A. Muise and H.-S. Ro, unpublished work, [6,17]). Although the mechanism by which CP-B-like proteases remove C-terminal basic amino acids is unknown, comparison of the $Zn^{2+}$-binding residues, substrate-binding residues and the catalytic residues with similar residues in CP-A [18] may give an insight into possible mechanisms of the CP-B-like protease. AEBP1 has conserved $Zn^{2+}$-binding residues, $His^{236}$, $Glu^{239}$, and a similar residue $Asp^{374}$ ($His^{69}$, $Glu^{72}$ and $His^{196}$ are found in CP-A [16,19]), and the conserved catalytic residue $Glu^{481}$ ($Glu^{270}$ in CP-A [16]) is found in all CPs. AEBP1 also has conserved catalytic residues $Gly^{375}$ and $Gly^{376}$, which are also found in CP-N and CP-E (residues 197 and 198 and 207 and 208 respectively [16]). Other residues ($Arg^{127}$, $Ser^{197}$ and $Tyr^{198}$ [16]) found to be important in CP-A activity are not conserved in any of the CP-B-like proteins, and conserved residues $Arg^{145}$ and $Tyr^{248}$ of CP-A are not conserved in AEBP1. Although these sequence comparisons suggest that the CP-B-like proteases function using a mechanism similar to that defined for CP-A, the number of non-conserved catalytic residues between the CP-B-like and CP-A enzymes indicates that a slightly different mechanism may be involved in CP-B-like protease activity. Residues $His^{236}$ and $Glu^{481}$ ($His^{69}$ and $Glu^{270}$ in CP-A) have been shown to be involved in a mechanism important in a wide variety of metallopeptidases, including bovine angiotensin I-converting enzyme (peptidyl-dipeptidase A), rabbit neutral endopeptidase, enkephalinase, human fibroblast collagenase and bacterial ther-

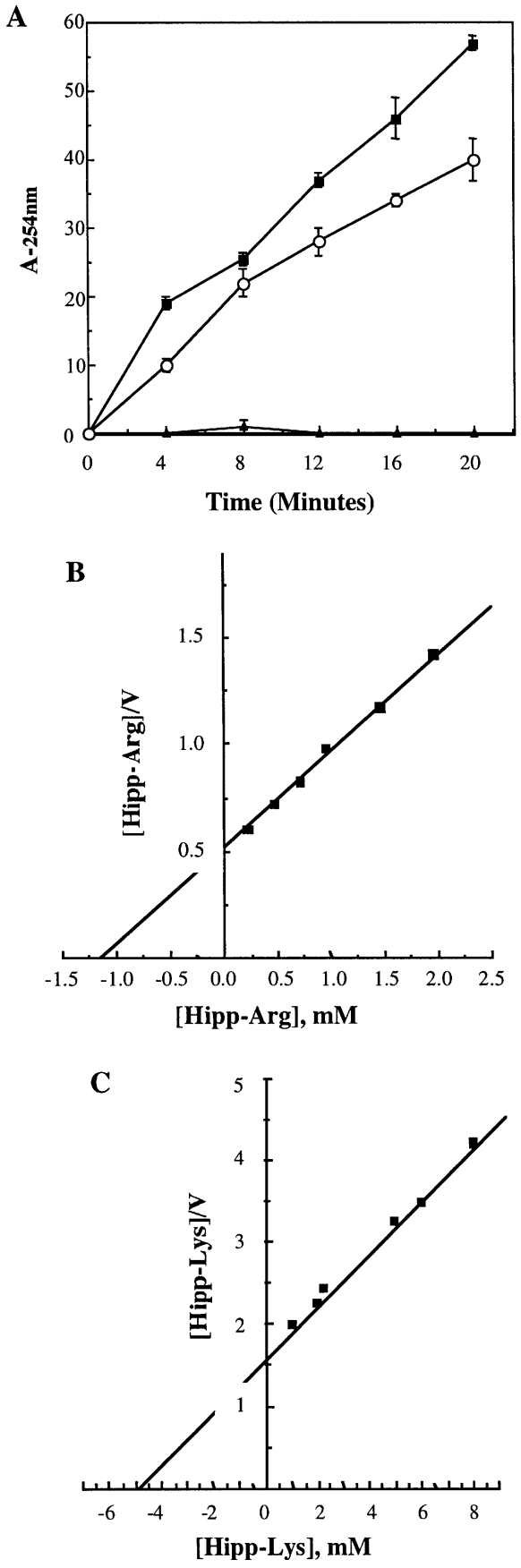


**Figure 1 CP activity of AEBP1**

(**A**) CP assays were carried out using the recombinant, His-tagged AEBP1 protein. The conversion of 1 mM hippuryl-arginine (■), 1 mM hippuryl-lysine (○) or 1 mM hippuryl-phenylalanine (▲) to hippuric acid was monitored at $A_{254}$. (**B**, **C**) Kinetic properties of AEBP1 CP. These are representative plots which illustrate the effects of various substrate concentrations on the CP activity of AEBP1. The $K_m$ values for hippuryl-arginine (**B**) and hippuryl-lysine (**C**) were determined by plotting substrate concentration/velocity versus substrate concentration in a Hanes–Woolf plot. The range of substrate concentrations was 0.25–2.0 mM hippuryl-arginine and 1.0–8.0 mM hippuryl-lysine. The intercept on the *x*-axis gives the $K_m$.

**Table 1 Kinetic parameters of AEBP1 CP**

For $K_m$ determination, various substrate concentrations were used, as shown in the legend to Figure 1, and the conversion of hippuryl-arginine or hippuryl-lysine to hippuric acid was measured. Concentrations of activators and inhibitors used to determine $K_a$ and $IC_{50}$ values are shown in the legends to Figures 2, 3 and 5. $K_a$ values were determined from the curves in Figures 3(A) and 3(B) or 5(B) using non-linear regression analysis to fit the data to the equation $V_{max} - 1 = C[Activator]/(K_a + [Activator])$, where C is the maximal value of $V_{max}$ at saturating metal-ion or AE−1 DNA concentrations. $IC_{50}$ is the inhibitor concentration when $V_{max}/V_{inh} = 2$.

| Compound | Parameter | Value (±S.D.) |
|---|---|---|
| Hippuryl-lysine | $K_m$ | 5.0±0.4 mM |
| Hippuryl-arginine | $K_m$ | 1.2±0.3 mM |
| $ZnCl_2$ | $K_a$ | 0.29±0.09 mM |
| $CoCl_2$ | $K_a$ | 0.55±0.09 mM |
| $NiCl_2$ | $K_a$ | 0.71±0.11 mM |
| AE-1 DNA | $K_a$ | 110±23 ng/ml |
| Captopril | $IC_{50}$ | 4.5±0.8 μM |
| *o*-Phenanthroline | $IC_{50}$ | 7.2±0.4 mM |

molysin [16]. These comparisons suggest that a wide variety of metallopeptidases employ common mechanisms in their protease functions, and it is possible that a similar protease mechanism exists in AEBP1.

To determine if the AEBP1 CP domain was functional, enzyme assays were performed using the synthetic substrates hippuryl-arginine, hippuryl-lysine and hippuryl-phenylalanine. Upon cleavage of the amino acid, the product, hippuric acid, can be measured spectrophotometrically at $A_{254}$. Figure 1(A) shows that recombinant His-tagged AEBP1 was capable of cleaving the synthetic substrates hippuryl-arginine and hippuryl-lysine, indicating that the protein has functional CP activity. AEBP1 did not cleave the synthetic substrate hippuryl-phenylalanine, a substrate for CP-A. These results further indicate that AEBP1 functions similarly to CP-B-like enzymes.

To ensure that the enzyme activity observed was due specifically to AEBP1, a number of controls were carried out. As the His-tagged AEBP1 used in the enzyme assay was a recombinant protein expressed in and purified from bacteria, controls containing other His-tagged recombinant proteins (AEBP2 [20] and a ribosomal protein S 24 [21]), and controls containing bacterial extracts purified using similar procedures, were tested in the CP assay. These purified His-tagged recombinant proteins and purified bacterial extracts showed no CP activity, indicating that the CP activity observed in the AEBP1 assays was due to AEBP1 and not to other proteins co-purified in the purification procedure.

Kinetic constants were determined using recombinant, purified His-tagged AEBP1 and various concentrations of the substrates (Figure 1B, Figure 1C and Table 1). The results showed that AEBP1 had a greater affinity for hippuryl-arginine than it did for hippuryl-lysine. The > 4-fold difference in $K_m$ values for the substrates suggests that arginine may be the preferred C-terminal amino acid of an unknown substrate which AEBP1 cleaves.

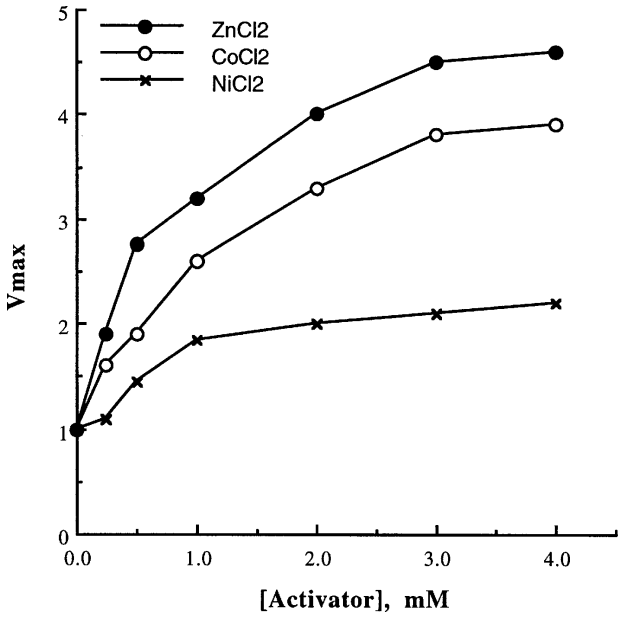


**Figure 2 Activation of AEBP1 CP activity by metal salts**

CP assays were carried out using the recombinant, His-tagged AEBP1 protein in the presence of metal activators. The salts were incubated with AEBP1 for 1 h, after which conversion of hippuryl-arginine (1 mM) to hippuric acid was measured spectrophotometrically at $A_{254}$. Representative plots are shown which illustrate the effects of various activator concentrations on the CP activity of AEBP1. The $K_a$ values for the activators for $ZnCl_2$ (●), $CoCl_2$ (○) and $NiCl_2$ (×) were determined by plotting $V_{max}$ versus activator concentration. The concentration ranges for activators were from 0–4.0 mM.

### Modulation of AEBP1 CP activity by activators and inhibitors

To further explore the enzymic activity of AEBP1, known CP activators and inhibitors were tested. The CP activators $NiCl_2$, $CoCl_2$ and $ZnCl_2$ (CP-N [22], CP-E and CP-D [23], and CP-M [11]) were all found to enhance AEBP1 CP activity. At an activator concentration of 1 mM, a 1.4-fold activation with $NiCl_2$, a 1.6-fold activation with $CoCl_2$ and a 1.9-fold activation with $ZnCl_2$ was observed after a 20-min assay period (results not shown). Kinetic constants were determined using recombinant, purified, His-tagged AEBP1 and various concentrations of activators (Figure 2 and Table 1). AEBP1 had a greater affinity for $ZnCl_2$ ($K_a = 0.29$ mM) than the other metals, $CoCl_2$ ($K_a = 0.55$ mM) and $NiCl_2$ ($K_a = 0.71$ mM).

The general CP inhibitor and zinc chelator, *o*-phenanthroline (CP-M [24], CP-N [25], CP-E and CP-D [23], and CP-M [11]), and the CP-specific competitive inhibitor, captopril (CP-N [25]), inhibited AEBP1 CP activity. Captopril (1 $\mu$M) reduced AEBP1 CP activity to 54% after a 20-min assay period, and a concentration of 10 $\mu$M completely abolished enzyme activity (results not shown). *o*-Phenanthroline (5 mM) reduced CP activity to 40% after a 20-min assay; 20 mM *o*-phenanthroline completely abolished AEBP1 CP activity (results not shown). The $IC_{50}$ was determined, using recombinant, purified, His-tagged AEBP1 and various concentrations of inhibitors (Figure 3 and Table 1). AEBP1 was more sensitive to the CP-specific competitive inhibitor captopril ($IC_{50} = 4.5$ $\mu$M) than to the general chelator *o*-phenanthroline ($IC_{50} = 7.2$ mM). These results indicate that both the substrate-binding and $Zn^{2+}$-binding residues are important for AEBP1 CP activity, as a known inhibitor, which chelates zinc, inhibits CP activity and a known CP competitive inhibitor also inhibits enzyme activity. These results show that AEBP1 behaves like other members of the CP-B-like CPs in terms of its response to activators and inhibitors.

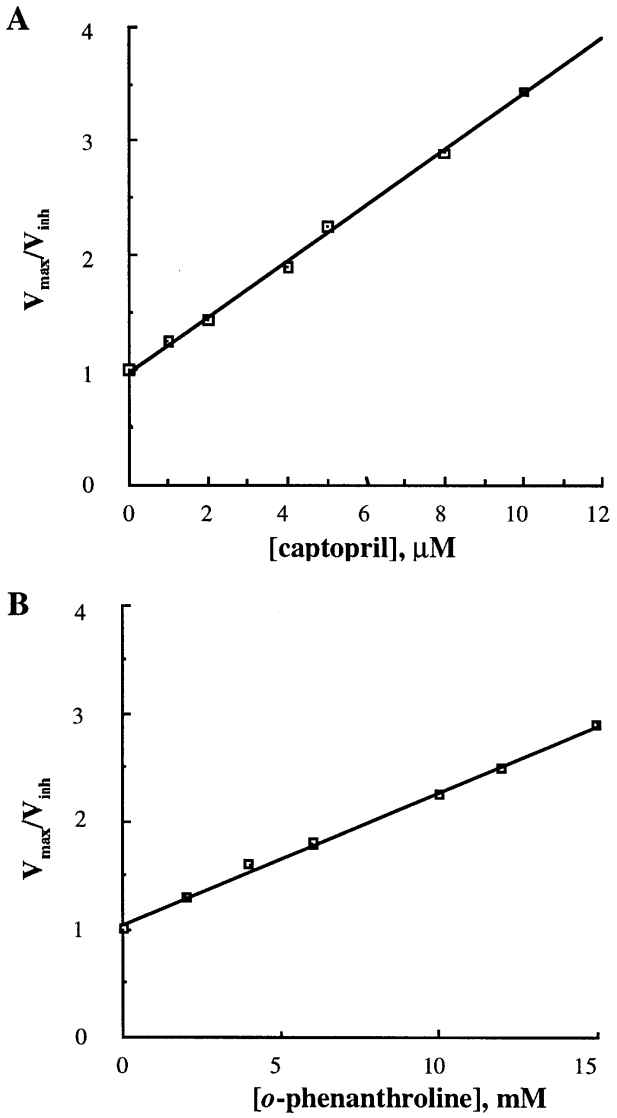


**Figure 3 Inhibition of AEBP1 CP activity by CP inhibitors**

CP assays were carried out using the recombinant His-tagged AEBP1 protein and CP inhibitors. The inhibitors were incubated with AEBP1 for 1 h, after which the conversion of hippuryl-arginine (1.5 mM) to hippuric acid was measured spectrophotometrically at $A_{254}$. The $IC_{50}$ values for captopril (**A**) and *o*-phenanthroline (**B**) were determined by plotting $V_{max}/V_{inhibitor}$ versus inhibitor concentration. Captopril concentrations ranged from 0–10 $\mu$M and *o*-phenanthroline concentrations from 0–15 mM.

### Stimulation of AEBP1 CP activity by its target DNA

The ability of AEBP1 to bind to a specific DNA sequence derived from the AE-1 promoter region of the *aP2* gene [6] raised the possibility that the AEBP1–DNA complex may affect AEBP1 enzymic activity. To test this idea, AEBP1 was incubated with the AE-1 DNA sequence and then used in the CP assay. As Figure 4(A) illustrates, AEBP1 CP activity was enhanced 1.9-fold in the presence of the specific DNA sequence after a 20-min assay. To ensure that the activation by the AE-1 oligonucleotide was not due to non-specific activation by DNA, a control assay containing the SP1 oligonucleotide (used as a control in a gel

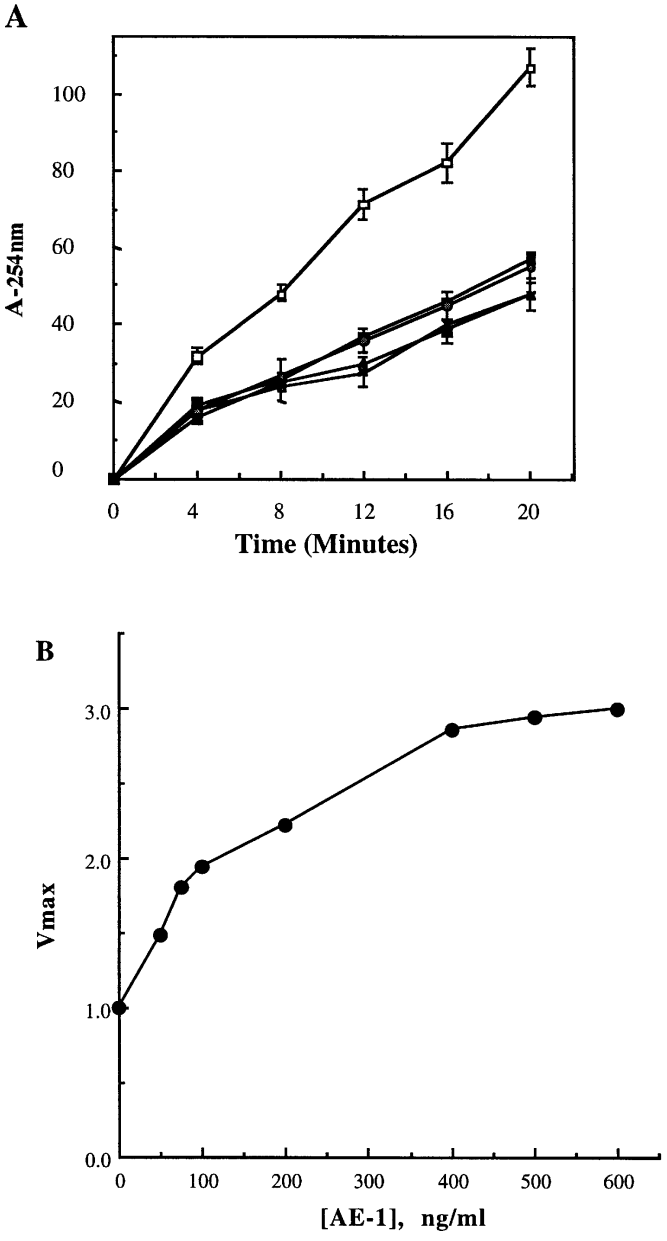


**Figure 4 Stimulation of AEBP1 CP activity by the AE-1 DNA sequence**

(**A**) The recombinant AEBP1 proteins were incubated with the AE-1 or control oligonucleotides for 1 h, after which conversion of hippuryl-arginine to hippuric acid was measured spectrophotometrically at $A_{254}$. The reactions contained AEBP1 (■) alone, 100 ng of AE-1 DNA (□) or 100 ng SP1 DNA (●). Control reactions contained AEBP1ΔSty (▲) alone or 100 ng of AE-1 DNA (×). (**B**) Representative plot illustrating the effects of various concentrations of AE-1 DNA on the CP activity of AEBP1. The $K_a$ values for the activator AE-1 DNA was determined by plotting $V_{max}$ against AE-1 concentration. The concentration range for AE-1 DNA was 0–600 ng/ml.

mobility-shift assay [6]) was performed. This control DNA sequence had no effect on AEBP1 CP activity (Figure 4A). To confirm that this was specific activation of AEBP1 CP activity due to AE-1 DNA, the assay was carried out using the C-terminal-deletion mutant of AEBP1, AEBP1ΔSty, which is unable to bind the AE-1 sequence (A. Muise and H.-S. Ro, unpublished work). AEBP1ΔSty displayed CP activity [6] but was not activated by the AE-1 oligonucleotide (Figure 4A). These results indicate that AEBP1 is activated by a novel mechanism whereby the direct binding of DNA enhances its protease activity. Kinetic constants were determined using recombinant, purified, His-tagged AEBP1 and various concentrations of AE-1; the results are shown in Figure 4(B) and summarized in Table 1.

In summary, the enzyme kinetic studies of the recombinant AEBP1 protein revealed that AEBP1 is a novel member of the regulatory B-like CP family. The results indicate that AEBP1 functions as a CP-B-like enzyme, as it responds in a similar fashion as other CP-B-like proteases to known substrates, activators and inhibitors. Our studies further suggest that, by specifically binding to the AE-1 region of the *aP2* promoter, the inherent CP activity of AEBP1 is stimulated. Other DNA regions did not activate AEBP1 CP activity and the enzymically active mutant form of AEBP1, which was unable to bind DNA, was not influenced by the AE-1 DNA sequence. These results suggest that the ability of AEBP1 to bind DNA, its protease activity and its transcriptional repression function are all correlated. The enhanced protease activity, which was mediated by DNA binding, may be required for AEBP1 to repress transcription of the *aP2* gene by an active repression mechanism [6].

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## REFERENCES

1 Cornelius, N., MacDougald, O. and Lane, M. D. (1994) Annu. Rev. Nutr. **14**, 99–129
2 Hunt, C. R., Ro, H.-S., Min, H.-Y., Dobson, D. E. and Spiegelman, B. M. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 3786–3790
3 Distel, R. J., Ro, H.-S., Rosen, B. S., Groves, D. L. and Spiegelman, B. M. (1987) Cell **49**, 835–844
4 Herrera, R., Ro, H.-S., Robinson, G. S., Xanthopoulos, K. G. and Spiegelman, B. M. (1989) Mol. Cell. Biol. **9**, 5331–5339
5 Ro, H.-S. and Roncari, D. A. K. (1991) Mol. Cell. Biol. **11**, 2303–2306
6 He, G. P., Muise, A., Li, A. W. and Ro, H.-S. (1995) Nature (London) **378**, 92–96
7 Fricker, L. D. (1988) Ann. Rev. Physiol. **50**, 309–321
8 Skidgel, R. A. (1988) Trends Pharmacol. Sci. **9**, 299–304
9 Naggert, J. K., Fricker, L. D., Varlamov, O., Nishina, P. M., Rouille, Y., Steiner, D. F., Carroll, R. J., Paigen, B. J. and Leiter, E. H. (1995) Nat. Genet. **10**, 1355–1342
10 Webster, D. R., Modesti, N. M. and Bulinski, J. C. (1992) Biochemistry **31**, 5849–5856
11 McGuire, G. B. and Skidgel, R. A. (1995) J. Biol. Chem. **270**, 17153–17158
12 Aushbel, F. M., Brent, R. E., Kingston, M., Moore, D. D., Seidman, J. A. and Struhl, K. (1989) Current Protocols in Molecular Biology, Wiley Interscience and Green Publishing Associates, New York
13 Bradford, M. M. (1976) Anal. Biochem. **12**, 248–253
14 Folk, J. E., Piez, K. A., Carrol, W. R. and Gladner, J. A. (1960) J. Biol. Chem. **235**, 2272–2277
15 Fricker, L. D. and Devi, L. (1990) Anal. Biochem. **184**, 21–27
16 Gebhard, W., Schube, M. and Eulitz, M. (1989) Eur. J. Biochem. **178**, 603–607
17 Park, J.-G., Muise, A., He, G.-P., Kim, S.-W. and Ro, H.-S. (1999) EMBO J. **18**, 4004–4012
18 Christianson, D. W., David, P. R. and Lipscomb, W. N. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 1512–1515
19 Roth, W. W., Mackin, R. B., Spiess, J., Goodman, R. H. and Noe, B. D. (1991) Mol. Cell. Endocrinol. **78**, 171–178
20 He, G.-P., Kim, S.-W. and Ro, H.-S. (1999) J. Biol. Chem. **274**, 14678–14684
21 Xu, L., He, G.-P., Li, A. and Ro, H.-S. (1994) Nucleic Acids Res. **22**, 646–655
22 Skidgel, R. A. (1984) Biochem. Pharmacol. **33**, 3471–3478
23 Song, L. and Fricker, L. D. (1995) J. Biol. Chem. **270**, 25007–25013
24 Deddish, P. A., Skidgel, R. A., Kriho, V. B., Li, X.-Y., Becker, R. P. and Erdos, E. G. (1990) J. Biol. Chem. **265**, 15083–15089
25 Barabe, J. and Huberdeau, D. (1991) Biochem. Pharmacol. **41**, 821–827

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