The Transactivation Domain within Cysteine/Histidine-rich Region 1 of CBP Comprises Two Novel Zinc-binding Modules*

Anthea L. Newton‡, Belinda K. Sharpe‡, Ann Kwan, Joel P. Mackay, and Merlin Crossley§

From the Department of Biochemistry, G08, University of Sydney, Sydney, New South Wales 2006, Australia

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The regulation of transcription is coordinated primarily by sequence-specific DNA-binding proteins that recognize elements within the promoters and enhancers of their target genes. Although the mechanisms by which DNA-binding proteins activate or repress transcription are not completely understood, these proteins often work by recruiting coactivators such as cAMP-response element-binding protein (CBP)1 and p300 (1–3).

CBP was first isolated as a coactivator that is recruited by the phosphorylated form of cAMP-response element-binding protein (CBP)1 and p300 (1–3). Many classes of zinc finger domains have been described and ascribed roles in nucleic acid binding, protein recognition, or both (19, 20). However, neither CH1 nor CH3 resemble any previously reported zinc-binding modules. In this study, we show that the CH1/CH3 regions of CBP proteins contain a highly conserved arrangement of cysteine and histidine residues. These regions can be broken down into discrete modules that each contain four putative metal-ligating residues and share the consensus sequence Cys-X2–4-Cys-X5–9-His-X5–9-Cys. We have termed these modules “zinc bundles.” We show that chemically synthesized peptides corresponding to individual zinc bundles from both CH1 and CH3 are capable of forming stable secondary structures in the presence of Zn(II) and that this structure appears to contain a substantial degree of helical character. These results are consistent with 1H NMR studies of the first zinc bundle of CH1 (CH11); 2H second hydrogen chemical shifts suggest that the domain is largely helical in nature. Finally, we demonstrate that the integrity of both zinc bundles in the CH1 domain of CBP is essential for its interaction with of these proteins (4–9). The molecular mode of action of CBP is complex. It has been shown that it possesses intrinsic histone acetylase activity and that it is able to recruit P/CAF, another acetyl transferase (10, 11). These results suggest that CBP is involved in the remodeling of chromatin, since it has been shown that the degree of histone acetylation significantly alters chromatin structure (12). Furthermore, CBP can interact directly with the basal transcriptional machinery (13, 14), underlining its central role as a transcriptional adaptor. The intracellular concentration of CBP is thought to be limiting, and it has consequently been proposed that CBP may be instrumental in coordinating different signaling pathways. Thus, for example, the inhibitory effects of nuclear hormone receptors on AP-1 activity have been attributed to the competition between hormone receptors and Jun/Fos for CBP (15). CBP has consequently been termed a transcriptional coactivator (15).

One of the most remarkable properties of CBP is its ability to physically associate with and modulate the activity of so many different and apparently unrelated DNA-bound transcription factors. A diagram of CBP and some of its partner proteins is shown in Fig. 1. A substantial number of these partner proteins associate with either CH1 or CH3 or both. Almost nothing is known about the molecular nature of these CH1/CH3-mediated interactions, although other interactions made by CBP, such as that made with cAMP-response element-binding protein (which does not involve the Cys/His-rich domains), have been studied in some detail (1, 16, 17). In this case the solution structure of the complex formed between the KIX domain of CBP and the cAMP-response element-binding protein KID domain has been determined (18).

The fact that CH1 and CH3 contain a large number of residues that are capable of chelating zinc ions has led to the suggestion that these domains may constitute zinc fingers (1, 2). Many classes of zinc finger domains have been described and ascribed roles in nucleic acid binding, protein recognition, or both (19, 20). However, neither CH1 nor CH3 resemble any previously reported zinc-binding modules. In this study, we show that the CH1/CH3 regions of CBP proteins contain a highly conserved arrangement of cysteine and histidine residues. These regions can be broken down into discrete modules that each contain four putative metal-ligating residues and share the consensus sequence Cys-X2–4-Cys-X5–9-His-X5–9-Cys. We have termed these modules “zinc bundles.” We show that chemically synthesized peptides corresponding to individual zinc bundles from both CH1 and CH3 are capable of forming stable secondary structure in the presence of Zn(II) and that this structure appears to contain a substantial degree of helical character. These results are consistent with 1H NMR studies of the first zinc bundle of CH1 (CH11); 2H second hydrogen chemical shifts suggest that the domain is largely helical in nature. Finally, we demonstrate that the integrity of both zinc bundles in the CH1 domain of CBP is essential for its interaction with

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§ To whom correspondence should be addressed. Tel.: 61 2 9351 2233; Fax: 61 2 9351 4726; E-mail: crossley@biochem.usyd.edu.au.
1 The abbreviations used are: CBP, cAMP-response element-binding protein-binding protein; d2-TSP, d2-(trimethylsilyl)propionic acid; bis-Tris propane, 1,3-bis[tris(hydroxymethyl)methylamine]propane.
some partner proteins and for the transactivation activity of CH1 in cellular assays. Taken together, these results indicate that the zinc bundles of CBP constitute novel protein domains that mediate protein-protein interactions.

**EXPERIMENTAL PROCEDURES**

**Materials**—Peptides were synthesized by Auspep Pty. Ltd. (Victoria, Australia). All other reagents were of analytical grade.

**Circular Dichroism Spectroelctrometry**—Samples of CH1 and CH3 were prepared by dissolving each of the lyophilized peptides (as provided) in a solution containing 1 mM ZnSO4 and 0.5 mM tris-(carboxyethyl)phosphine, to a final concentration of 30–40 μM peptide. The pH was then adjusted slowly to either 5.0 (CH3) or 6.5 (CH1) using 0.01 M NaOH. Spectra were collected on a Jasco J-720 spectropolarimeter, using a 1-mm path length cell, and the temperature was controlled with a water-jacketed cell holder. Spectra were collected at 20 °C over the wavelength range 184–260 nm, using a resolution of 0.5 nm and a bandwidth of 1 nm. Final spectra were the sum of five scans accumulated at a speed of 20 nm min⁻¹ with a response time of 1 s. Spectra were also base line-corrected. For the thermal denaturation of CH1, the ellipticity at 195 nm was monitored over a temperature range of 5–95 °C, using a resolution of 0.2 °C, a bandwidth of 1 nm, and a temperature gradient of 1 °C min⁻¹.

**Sedimentation Equilibrium Experiments**—Sedimentation equilibrium experiments were carried out at 20 °C using a Beckman Optima XL-A ultracentrifuge equipped with an Anti-60 rotor, and at rotor speeds of 48,000 and 56,000 rpm. CH1 was dissolved in a solution containing 10 mM bis-Tris propane, 0.5 mM tris-(carboxyethyl)phosphine, 1 mM ZnSO4, and 150 mM NaCl, so that the absorbance of CH1 was 0.8 at 230 nm. A far-UV CD spectrum of the sample was recorded prior to the sedimentation equilibrium experiments in order to confirm that the peptide was folded. Two dilutions of this sample (1:2 and 1:4) were also prepared. Data were recorded in three double-sector cells as absorbance versus radius scans (0.001-cm increments, 10 scans). Scans were collected at 3-h intervals and compared with ensure that the sample remained unchanged. Simultaneous analysis of the six resulting data sets was carried out using the NONLIN (21) software, by carrying out nonlinear least squares fits of the data to a number of different models. The final parameters were determined by a nonlinear least squares fit to a model incorporating a single nonassociating species. The goodness of fit was determined by examination of the residuals derived from the fit. The partial specific volume of CH1 was calculated from tabulated values for each major solution component (22). The solvent density was approximated by that containing 95:5 H2O:D2O, 600 μM tris-(carboxyethyl)phosphine, 20 μM d4-(trimethylsilyl)propionic acid (d4-TSP), and 600 μM ZnSO4, pH 3.2. The pH was then increased slowly by 1-M additions of 0.1 M NaOH, until it reached ~6.5.

**NMR Spectroscopy**—CH1 was dissolved in a solution (0.5 ml) containing 95.5 H2O:D2O, 600 μM tris-(carboxyethyl)phosphine, 20 μM d4-(trimethylsilyl)propionic acid (d4-TSP), and 600 μM ZnSO4, pH 3.2. Circular dichroism spectra were recorded at 2 °C on a Bruker DRX600 equipped with a triple resonance (HCN) probe and three-axis pulsed-field gradients. Spectra were referenced to d4-TSP at 0.00 ppm. Both one-dimensional 1H NMR spectra and two-dimensional total correlation spectroscopy (23) were recorded. Two-dimensional nuclear Overhauser effect spectroscopy (24) and DQF-COSY (25) experiments were also prepared. Data were recorded in three double-sector cells as were also base line-corrected. For the thermal denaturation of CH1, the absorbance at 195 nm was monitored over a temperature range of 5–95 °C, using a resolution of 0.2 °C, a bandwidth of 1 nm, and a temperature gradient of 1 °C min⁻¹.
recorded as described previously (26). Data were processed by zero-filling once in each dimension and applying either Lorentzian-Gaussian or squared shifted sine bell transformations prior to Fourier transformation.

**Plasmid Construction and Site-directed Mutagenesis—**Site-directed mutagenesis was performed using standard methods, either single primer mismatch or overlap polymerase chain reaction, as described previously (27). *Pfu* polymerase (Stratagene) and mutant oligonucleotide primers (Life Technologies, Inc.) were used in the reactions. Details of oligonucleotides and plasmids are available on request.

**Cell Culture and Transient Transfection Assays—**All cell culture manipulations were carried out using standard techniques (28). Briefly, NIH3T3 cells were co-transfected with 2 μg of the Gal4-dependent human growth hormone reporter, p(Gal4)-hGH, and 20 ng of pcDNA3-Gal4-DBD-CH11-CH12 (CBP residues 347–450) wild-type and mutant constructs using the calcium phosphate method (28). Growth hormone assays were carried out using Nichols' Institute Allegro GH assay kits according to the manufacturer's instructions. The data are the result of three independent experiments and have been normalized to LacZ levels derived from co-transfection with the β-galactosidase-encoding plasmid pEF-β-LacZ.

**RESULTS**

**CH1 Contains Two Conserved Zinc Bundle Motifs—**We are interested in the molecular mechanisms through which the CH1 and CH3 domains of CBP recognize such an extensive array of transcription factors (Fig. 1). As a first step toward characterizing these regions of CBP, we searched for related domains in sequence data bases. Using a section of CH1 from the murine CBP protein, we were able to detect homology with human CBP and p300 as well as with related proteins in *Caenorhabditis, Drosophila,* and plants (Fig. 2A). Like murine CH1, these related domains are also rich in the zinc-chelating residues cysteine and histidine, but interestingly, only a subset of these residues is conserved in all of the domains shown. The conserved residues conform to the consensus Cys-X-Cys-X-His-X-Cys; this sequence occurs twice in the CH1 region. The CH3 domain also contains a Cys-X-Cys-X-His-X-Cys motif as well as the related sequence Cys-X-Cys-X-His-X-Cys (Fig. 2B). These motifs do not correspond to any zinc-binding domains of known structure, suggesting that they constitute a novel protein domain. We termed these motifs "zinc bundles."

**The Folding of Cys-X-Cys-X-His-X-Cys Zinc Bins Is Metal Ion-dependent—**In order to determine whether the zinc bundles of CBP are indeed independently folding protein domains, peptides corresponding to one bundle from CH1 (CH11) and one from CH3 (the deviant Cys-X-Cys-X-His-X-Cys bundle; CH32) were synthesized using standard solid phase peptide chemistry (Auspep). Far-UV CD spectra of each of these peptides were recorded under a range of solution conditions. At low pH (Fig. 3, A and B; filled circles), these spectra both show features typical of largely unstructured polypeptides, i.e. minima at ~195 nm and no positive signal at shorter wavelengths. The addition of Zn(II) and an increase in the pH resulted in substantial increases in secondary structure content for both peptides, as demonstrated by a shift of the main minimum to 208 nm, the appearance of a second minimum at ~222 nm, and the appearance of a positive maximum at ~195 nm (Fig. 3, A and B; open squares). Note that neither the increase in pH nor the addition of Zn(II) alone is sufficient to induce the formation of secondary structure (data not shown). The features of these spectra are consistent with the presence of a substantial amount of α-helical structure. Further, the addition of 5 mM EDTA (Fig. 3, A and B; open triangles) resulted in a loss of the induced secondary structure, consistent with a metal-dependent folding process. The thermal stability of CH1 was also investigated by monitoring the far-UV CD signal (at 195 nm) with increasing temperature. Over the temperature range 5–95 °C, there was no indication that significant levels of unfolding had occurred (data not shown). These results suggest that the zinc bundle is a stable protein domain capable of folding independently of the remainder of the protein.

**CH1 Is Monomeric in Solution—**Given that there have been a number of recent reports suggesting that zinc-binding domains are capable of acting as protein dimerization motifs (for a review, see Ref. 20), we sought to determine the aggregation state of CH1. Fig. 4 shows data from sedimentation equilibrium experiments conducted on CH1 (bottom panel), together with fits of these data to a model described by a single, ideal species and the residuals from that fit (upper three panels). This model provided the best fit to the data, yielding a solution molecular mass of 3,590 Da, with 95% confidence limits of 3,530 and 3,640 Da. This is in good agreement with the predicted molecular mass of CH1 (3,131 Da), given that there is some uncertainty regarding both the buffer density and the effect of the zinc atom on the partial specific volume of the
peptide. These data therefore indicate that the CH1 domain does not undergo significant self-association in solution.

CH1 Is Predominantly α-Helical in Solution—Having established that CH1 does not self-associate in solution, we next used NMR spectroscopy to investigate its conformation in more detail. Fig. 5A shows a one-dimensional 1H NMR spectrum of CH1. The good chemical shift dispersion and narrow line widths are consistent with the peptide existing in a stable, folded conformation, and we were able to use the well-established sequential assignment strategy (29) to assign resonances to specific protons in CH1. A plot of chemical shift index (CSI; Ref. 30) for the Hα protons of CH1, versus residue number is presented in Fig. 5B. The CSI is essentially a digital filter, in which Hα protons that are shifted upfield from their random coil value by >0.1 ppm are assigned a value of −1, while Hα protons that are shifted downfield by >0.1 ppm are assigned a value of 1. Other Hα protons are assigned a value of 0. Sequences of more than three indices with values of −1 are indicative of the presence of an α-helix, while sequences of three or more indices with values of 1 are consistent with a β-strand conformation. Values of 0 may be present within these sequences, so long as the “density” of nonzero values does not fall below 70% over any four-residue window. Using these criteria, we can see that there are clearly two helical regions in CH1, namely residues 382–388 and residues 390–395. In addition, it is possible that residues 375–377 exist in a helical conformation. The few values of 1 located between the helical regions most probably represent reverse turns connecting the helices.

The Integrity of the Zinc Bundles Is Required for the Transactivation Function of CH1—CBP is a large protein that appears to activate transcription by means of a number of distinct mechanisms. In particular, it contains a histone acetyltransferase domain (Fig. 1, HAT), and it is thought that CBP may reduce the repressive effects of chromatin by acetylating histones (10, 11). CBP also contains a number of additional domains that appear to contribute to its ability to activate transcription. In particular, CH1 has been shown to act as a potent activation domain when fused to a heterologous DNA-binding domain such as the Gal4 DNA-binding domain. We next sought to investigate whether the integrity of zinc bundles CH11 and CH12 was required for the transcriptional activation function of CH1.

We first cloned a region encompassing CH11 and CH12 downstream and in frame with the Gal4 DNA-binding domain in a mammalian expression vector and then tested this construct for its ability to activate a Gal4-dependent reporter gene in transient transfection experiments in NIH3T3 cells. As shown in Fig. 6A, and consistent with previous reports (31–33), the Gal4-CH1 fusion protein was a potent activator of transcription. We then prepared a panel of mutants, each containing a single amino acid substitution of a putative zinc-coordinating residue. Initially focusing on CH11, we individually mutated each of the four key residues in the Cys-Xα-Cys-Xβ-His-Xγ-Cys motif to alanine. We also prepared proteins containing mutations in other potential zinc-coordinating residues within the domain (i.e. H383A and H396A, residues that have the potential to chelate zinc but do not fall within the zinc bundle consensus identified here). The results are shown in Fig. 6A. Mutants containing substitutions of putative zinc-coordinating residues in CH1 could not activate transcription. Importantly, mutations in histidine residues outside the bundle consensus (which are not expected to necessarily be as important for the structure) did not significantly interfere with the activity of this domain. CH1 was analyzed in a similar manner (Fig. 6B), and again mutation of residues in the bundle consensus significantly impaired the ability of the domain to activate transcription, while mutations of other potential zinc-coordinating residues had little effect. These results are significant in that they suggest that the zinc-binding residues are important in vivo.

Fig. 4. CH11 is monomeric in solution. The bottom panel shows sedimentation equilibrium data recorded on CH1 as plots of A_{280} versus radial position (r0^2 cm^2) for each of the three protein loading concentrations. The fits of these data sets to a model incorporating a single ideal species are also shown. The three upper panels illustrate the residuals for the fit to the three data sets. Data were recorded at 20 °C and 48,000 rpm.

Fig. 5. CH11 is largely α-helical. A, one-dimensional 1H NMR spectrum of CH11 recorded at 2 °C, illustrating the good chemical shift dispersion and line widths displayed by this domain. B, plot of the Hα chemical shift index for CH11. Predicted α-helices are indicated above the graph.
and are consistent with the conclusion that each bundle coordinates zinc through the motif Cys-X4-Cys-X8-His-X3-Cys.

Moreover, these data indicate that both bundles are required for the transcriptional activity of CH1.

The Integrity of the Zinc Bundles Is Required for Interaction with Some but Not All CH1 Protein Partners—Many zinc-binding modules function either in DNA or protein recognition. We carried out a two-hybrid screen against CH1 and identified three previously reported partner proteins, Hif-1α (34), NF-κB p65 (35), and Mrg1/Srj35 (36). Both Hif-1α and NF-κB p65 are well characterized DNA-binding proteins involved in transcriptional activation (37, 38). Mrg1/Srj35 has been implicated as a transcriptional coactivator but has also been shown to inhibit the activity of CBP in certain assays (36, 39). We tested our panel of mutant constructs for binding to these proteins to determine whether the zinc bundle domains were required for physical interaction with these partners. The results are shown in Fig. 7. In the case of Hif-1α and NF-κB p65, mutation of any of the putative zinc-coordinating residues in CH11 or CH12 abrogated binding, whereas mutations in residues not included in the consensus had no effect. This result suggests that the integrity of both bundles is required for interaction with these proteins. In contrast, Mrg1 showed a different profile, retaining its ability to interact with several of the constructs with mutations that disrupted either CH11 (i.e. H393A) or CH12 (i.e. C408A, H417A, and C421A). These results indicate that the zinc bundles are directly involved in the interactions made by some but not all proteins that bind to the general region defined as CH1.

**DISCUSSION**

CBP Is a Zinc-binding Protein—It is clear that the mechanisms by which CBP modulates eukaryotic gene expression are complex. In this study, we have investigated the functions of individual domains within CBP, namely the cysteine/histidine-rich regions CH1 and CH3. A sequence alignment of CBP-related proteins from a range of species has revealed a conserved motif that appears twice in CH1 (CH11 and CH12); this motif conforms to the consensus Cys-X4-Cys-X8-His-X3-Cys. CH3 contains one motif (CH31) that shares this spacing of cysteine and histidine residues and has additional homology to CH11, and a related motif (CH32) that has the form Cys-X2-Cys-X9-His-X3-Cys. The conservation and spacing of residues that are known to be capable of binding metal ions suggested that these domains might comprise zinc-binding modules.

A number of zinc-binding modules with different consensus sequences and structural features have previously been described (19), but the domains identified within CBP CH1 and CH3 appear to be unrelated to previously recognized zinc-binding domains. Evidence from both far-UV CD and 1H NMR spectroscopy, however, confirms that the secondary structure

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**Fig. 7. The integrity of the zinc bundles in CH1 is required for interaction with protein partners.** Wild-type and mutant forms of CH1 were tested for their ability to interact with Hif-1α, NF-κB p65, and Mrg1 using the yeast two-hybrid assay. **Uppercase letters** indicate zinc bundle residues conserved between species and implicated in coordinating zinc. **Boldface and boxed letters** show the residue mutated in each experiment. +, growth on selective media; −, no growth.
content of peptides corresponding to CH1, and the variant motif CH3, is dependent on the presence of Zn(II). Secondary chemical shifts for the H-protons of CH1, (represented as the chemical shift index) and CD data suggest that the secondary structure of this domain is predominantly helical. The fold probably comprises two or three helical regions connected by reverse turns and held together largely through ligation of a single Zn(II) ion. These results, in combination with the novel consensus sequence, lead us to propose that these domains represent a new protein fold, which we have termed a zinc bundle. While previously described zinc-binding modules, such as zinc fingers, are generally associated with nucleic acid binding, it is now clear that several more recently recognized classes of zinc-binding domains are able to contact other proteins. These include LIM domains (40), RING fingers (41), and the CCHC fingers of FOG family proteins (42), and our results indicate that zinc bundles also mediate protein–protein interactions.

The Zinc Bundle Sequences Are Required for Transactivation by CH1—Both CH1 and CH3 have been implicated in mediating interactions with a large array of different gene regulatory proteins. It has also been noted that CH1 can function as a potent transactivation domain. The transactivation ability of this domain is abrogated by mutations to the putative zinc-coordinating residues that comprise the zinc bundle consensus sequences. The precise mechanism by which CH1 functions to increase transactivation from minimal promoters is not known. It is possible that CH1 acts in part by recruiting additional transcriptional proteins (such as those shown in Fig. 1) or recruits coactivators or makes direct contacts with the basal transcriptional machinery at the promoter. It has recently been reported that one known partner protein of CH1, Mrg1/Srj35, can bind TATA-binding protein and act as a coactivator for the LIM domain protein Lhx2 (39). It has also been demonstrated that the Mrg1-related protein, Msg, functions as a coactivator for the Smad family of transcription factors (43). In the light of the Mrg1-related protein, Msg, functions as a coactivator for these proteins recognize related elements within CH1/CH3. Although the zinc bundles of CH1 and CH3 are not identical, the individual bundles CH1 and CH3 share considerable homology (Fig. 2) in addition to the minimal zinc bundle motif, Cys-X5-Cys-X5-His-X5-Cys. CH1 also contains the bundle consensus but shows little additional homology, while CH3 is the most diverged unit in that it contains the variant zinc-binding motif Cys-X2-Cys-X2-His-X2-Cys. It will be interesting to compare the features of different proteins that specifically recognize different bundles within CBP.

Finally, it should be remembered that CBP contains additional regions, such as CH2 (residues 1180–1476), that also contain a number of cysteine and histidine residues. The sequence of CH2 conforms to the accepted but somewhat loose consensus of a leukemia-associated protein domain (44) or plant homeodomain finger (45). These domains are often found in gene-regulatory proteins, but their structural and functional characteristics have yet to be defined. It is possible that the CH2 domain of CBP is also a zinc-binding module that is instrumental in mediating protein–protein interactions. There is an additional cysteine- and histidine-rich motif (residues 1708–1724) upstream of CH3, and its properties also await characterization. The definition of the structural features of CH1, CH3, and the other potential zinc-binding modules within CBP and further investigations of their protein binding properties should provide insights into how these domains associate with such a diverse array of partner proteins.

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