

Functional interaction of chicken p46 polypeptide with histones, histone deacetylase-1 and histone acethyl transferase-1

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Abstract

To better understanding of DNA replicating-coupled chromatin assembly and transcription regulation, we cloned and sequenced cDNA encoding the chicken p46 polypeptide, chp46, homologous to the p48 subunit of chicken chromatin assembly factor-1, chCAF-1p48. The cDNA encoding a protein consists of 424 amino acids including a putative initiation Met, is a member of the WD protein family, with seven WD repeat motifs, and exhibits 90.3% identity to chCAF-1p48, and 94.3% identity to the human and mouse p46 polypeptides. The p46 polypeptide fusion protein were synthesized by *in vitro* translation system and expressed in *Escherichia coli* under induction by 50 μ M IPTG and single step purified with glutathione-agarose beads, showed that GST-tagged protein of approximately 72 kDa, were dramatically synthesis in *Escherichia coli* BL-21 cells. The *in vitro* experiment established that chp46 interacts with chicken histones, chHDAC-1, and chHAT-1. The *in vitro* immunoprecipitation experiment, involving truncated mutants of chp46, revealed not only that two regions comprising amino acids 33-179 and 375-404 are necessary for its binding to H2B, but also that two regions comprising amino acids 1-32 and 405-424 are necessary for its binding to H4. Furthermore, the GST pulldown affinity assay, involving truncated mutants of chp46, revealed that a region comprising amino acids 359-404 binds to chHAT-1 *in vitro*. Taken together, these results indicate not only that chp46 should participate differentially in a number of DNA-utilizing processes through interactions of its distinct regions with histones and chHAT-1, but also that the proper propeller structure of chp46 is not necessary for its interaction with chHAT-1.

Keywords: Chromatin, immunoprecipitation experiment, histones, propeller structure

Introduction

Alterations in chromatin structure are preferentially involved in the regulation of cell functions, including gene expression in eukariotes. In recent years, knowledge concerning the characteristics of the p48 subunit of CAF-1 (CAF-1p48), which has seven WD repeat motifs and is a member of the WD protein family, in numerous DNA-utilizing processes has been accumulated (1). CAF-1p48 was identified as a polypeptide that is tightly associated with the catalytic subunit of human histone deacetylase-1 and 2 (HDAC-1 and -2) (2). On the other hand, the p46 polypeptide (p46), with seven WD (Trp-Asp) repeat motifs, is a member of the WD protein family and is originally characterized as an Rb-binding protein (RbAp46). We have found that chicken CAF-1p48, chCAF-1p48, interacts with chHDAC-1 and 2 *in vivo*, in an immunoprecipitation experiment followed by Western blotting (3). Furthermore, the GST pulldown affinity assay, involving deletion mutants of both chCAF-1p48 and chHDAC-2, revealed not only that chCAF-1p48 binds to two regions of chHDAC-2 comprising amino acids 82-180 and 245-314, respectively, but also that two N-terminal, two C-terminal, or one N-terminal and one C-terminal WD repeat motif of chCAF-1p48 are required for this *in*

vitro interaction. On the other hand, the p46 polypeptide (p46), which is a CAF-1p48 homolog, together with the latter, is contained in repressor complexes with HDAC-1 and -2, and mSin3 (the mammalian homolog of Sin3), Rb (the retinoblastoma protein), or Mi2 plus MeCP2 to repress transcription (4).

In this study we cloned cDNA encoding the chicken p46, chp46, comprising 424 amino acids including a putative initiation Met, exhibiting 90.3% homology to chCAF-1p48, and carrying seven WD repeat motifs. The p46 polypeptide fusion protein were synthesized in *Escherichia coli* with induction by 50 μ M IPTG and single step purified with glutathione-agarose, showed that protein of approximately 72 kDa, were dramatically synthesis in *Escherichia coli* BL-21 cells. The *in vitro* experiment established that chp46 interacts with chicken histones, chHDAC-1, and chHAT-1.

The *in vitro* immunoprecipitation experiment, involving truncated mutants of chp46, revealed not only that two regions comprising amino acids 33-179 and 375-404 are necessary for its binding to H2B, but also that two regions comprising amino acids 1-32 and 405-424 are necessary for its binding to H4. The GST pulldown affinity assay revealed not only that chp46, as well as chCAF-1p48, associates with chHAT-1, but

also that a C-terminal region, comprising amino acids 359-404 is essential for this interaction. These results suggest not only that the proper propeller structure of chp46, probably due to its WD repeat motifs, should not be necessary for its *in vitro* interaction with chHAT-1, but also that chp46 should be involved in a number of DNA-utilizing processes in distinct manners.

Materials and Methods

Materials

pBluescript II SK(-) and pCite4a(-) were purchased from Stratagene and Novagen, respectively. pGEX-2TK plasmid and glutathione-agarose beads were a product of Amersham Pharmacia Biotech. A single Tube Protein™ System 3 were purchased from Novagen. The anti-HA and anti-FLAG M2 beads were purchased from Santa Cruz, Biotech. Inc. and Eastman Kodak Co., respectively.

Cloning and sequencing of cDNA encoding chp46

A PCR product of 435 bp, corresponding to a part of the coding region of hup46, was first prepared from chicken DT40 cDNA, using sense and antisense oligonucleotide primers containing sequences 5'-CTGATGATCAGAACTTATGATATGGG-3' and 5'-AGCAAATGACCCAAG GTTCATTGGG-3', respectively, which were constructed based on the amino acid sequences (DDQKLMiWD and PNEPWWiCS) in hup46 deduced from its cDNA (5). To obtain full-length chp46 cDNA, using the resultant PCR product as a probe, we screened the DT40 lambda ZAP II cDNA library (3) as described (6). The entire nucleotide sequences of both strands of the largest cDNA insert were sequenced by the dye terminator method (Applied Biosystems Division, Perkin Elmer).

Plasmid construction

We constructed the pCitep46 and/or pCiteFLAGp46 plasmid carrying the gene encoding chp46 as described (3,7). First a sense primer containing NcoI (5') with the sequence 5'-CCATGGCGAGTA AGGAAGTGCTGGAGG-3' and an antisense primer containing SalI (3') with the sequence 5'-CTCGAGTTACTGTCTTGACCTTCCAG-3' were constructed. Next we prepared the DNA fragment encoding the full-length coding region of chp46 by PCR using the parental plasmid (pB(II)SKp46) carrying the full-length chp46 cDNA as a template with these primers, followed by digestion with NcoI plus SalI. We replaced the resultant NcoI/SalI fragment with the NcoI/SalI fragment of the pCiteFLAGp48 plasmid carrying the gene encoding

chCAF-1p48 (8). The deletion mutants of chp46 were obtained as described (8). We also use pCiteHAH2A, pCiteHAH2B, pCiteHAH3, pCiteHAH4, pCiteHAHDAC-1 and pCiteHAp60 were obtained as described (3, 8). Expression of the chp46 in *E. coli* and by *in vitro* translation system *E. coli* BL-21 cells were transformed with pGEX-2TKchp46 and pGEX-2TKchHAT-1, respectively, harboring the full-length chp46 and chHAT-1 cDNAs, and grown to $A_{600nm} = \sim 0.2$ in 400 ml of LB medium supplemented with 200 µg/ml ampicillin. After induction by the addition of 50 µM isopropyl β-D-thiogalactopyranoside (IPTG) overnight at 20°C, the cells were collected by centrifugation and purification by glutathione-agarose beads, essentially as described (3).

Production of [³⁵S]Met-labeled proteins

To produce [³⁵S]Met-labeled FLAG-chp46 fusion protein, chp46 polypeptide, CAF-1p48, CAF-1p60, HDAC-1, HAT-1, and histone H4 a Single Tube Protein™ System 3 (Novagen) was used with a following modification. The reaction mixture was set up at room temperature: 1 µl DNA template (free from RNase, Mg²⁺ and salt) plus 1 µl nuclease-free water and 8 µl STP3 transcription kit. It was mixed by stirring with the pipet tip and incubated at 30°C for 30 minute. Next, 5 µl (15 mCi/ml) ³⁵S-Met (Amersham Pharmacia Biotech.) and 30 µl *in vitro* STP3 translation kit were added to the transcription reaction products as mentioned above. Reactions were typically performed in 50 µl volumes by adding 5 µl nuclease-free water and translation reactions were incubate at 30°C about 1 hr. After the reaction was completed, the final products were saved as [³⁵S]Met-labeled protein and stored at -20°C until used.

Immunoprecipitation experiment and GST pulldown affinity assay

The *in vitro* immunoprecipitation experiment was performed with 5 µl of [³⁵S]Met-labeled chp46 polypeptide, CAF-1p48, CAF-1p60, HDAC-1, HAT-1, and histone H4, in 200 µl of bead-binding buffer (50 mM potassium phosphate buffer, pH 7.5, 450 mM KCl, 10 mM MgCl₂, 10% glycerol, 1% Triton X-100, 1% BSA). After standing for 60 min, 20 µl of the reaction mixture was removed as an input sample, and the remaining mixture was added to 20 µl of anti-HA beads (Santa Cruz, Biotech. Inc.) or anti-FLAG M2 beads (Eastman Kodak Co.), followed by gentle rotation for 60 min at 4°C. The affinity beads were collected by centrifugation at 9,500 X g for 10 sec, and then washed with 1 ml of the bead-binding buffer containing 0.1% 4-(2-aminoethyl)-benzoesulfonyl fluoride (AEBSF) three times. The beads were suspended in 30 µl of 2X SDS sample buffer and then

boiled for 5 min. Aliquots (15 μ l) of the resultant eluates were analyzed by 10 or 12% SDS-PAGE, and then the gels were washed with a fluorographic reagent (Amersham Pharmacia Biotech), dried, and subjected to fluorography. The GST pulldown affinity assay essentially as described (3), in 200 μ l of the bead-binding buffer.

Results and Discussion

Cloning of cDNA encoding chp46

To study the characteristics of chp46, we cloned and sequenced cDNA encoding it. Based on conserved amino acid sequences in hup46 and mouse p46 (mop46) (5), we prepared the 435 bp PCR fragment, corresponding to a part of cDNAs encoding hup46 and mop46, by PCR using cDNAs from DT40 cells. Sequence analysis of the largest cDNA insert of 1799 bp among them revealed that the clone contained both an initiation codon and a 3' poly(A) tail, and also appeared to contain the full-length chp46 cDNA sequence. The amino acid sequence deduced from the nucleotide sequence, comprising 424 residues including a putative initiation Met. This chicken polypeptide exhibits 94.3% identity in amino acid sequences to hup46 and mop46, and 90.4% identity to chCAF-1p48.

Expression of recombinant chp46 by in vitro translation system and in E. coli

First, to construct a chimeric plasmid, pGEX-2TKchp46, expressing the GST-chp46 fusion protein, chp46 cDNA was subcloned into the pGEX-2TK plasmid in frame. GST fusion proteins were synthesized in *E. coli*, extracted, and purified essentially as described previously (3). As shown in Figure 1A, the electrophoretic patterns on SDS-PAGE of whole cell lysates before and after induction with IPTG revealed that GST-chp46 fusion proteins of approximately 72 kD were dramatically accumulated in *E. coli* BL-21 cells containing the pGEX-2TKchp46 plasmid. In lane 1 and 2, whole cell lysate of BL-21 cells containing the pGEX-2TKchp46 and pGEX-2TKchHAT-1 plasmid without induction by IPTG, lane 3 and 4, lysate of BL-21 cells containing the pGEX-2TKchp46 and pGEX-2TKchHAT-1 plasmid with induction by 50 μ M IPTG, lane 5 and 6, complex beads containing chp46 and HAT-1, lane 7 and 8, chp46 and chHAT-1 fraction purified with glutathione-agarose beads. In this data revealed, the GST-chp46 fusion proteins were purified to more than 95% homogeneity, using glutathione-agarose beads (see lane 7 in Figure 1A). Furthermore, chp46 also have high and stable expression level by in vitro transcription and translation system (Figure 1B) using a single Tube ProteinTM kit (Novagen).

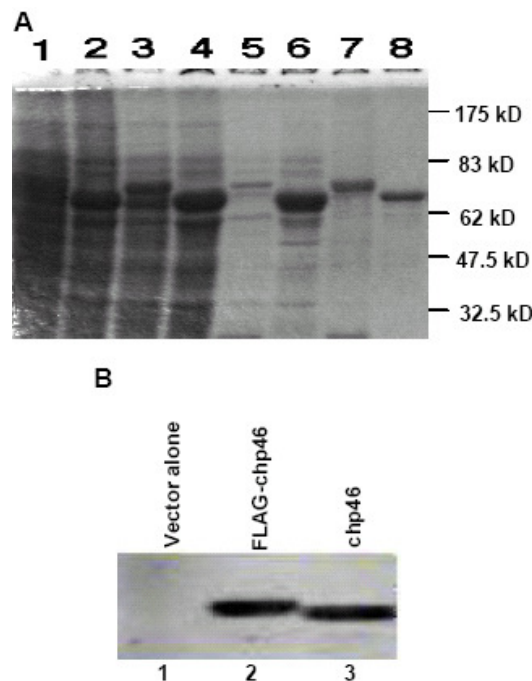


Figure 1 Expression followed SDS gel electrophoretic patterns of GST-chp46 (A) and FLAG-chp46 fusion protein (B).

Interaction of chp46 with histones, HDAC-1, CAF-1p60 and HAT-1

We have revealed that chCAF-1p48 binds to chHDACs (3) and chHAT-1 (10) in vivo and in vitro. On the other hand, it has been reported that a heterodimer of hup46 and HAT-1 is involved in the chemical modification of core histones with acetyl groups, especially in the acetylation of Lys-5 and Lys-12 of histone H4 (11). Therefore, to determine whether or not chp46 bound to these chromatin-related proteins, such as histone H4, HDAC-1 and CAF-1p60, we carried out an in vitro immunoprecipitation experiment, using [³⁵S]Met-labeled FLAG-tagged chp46 and [³⁵S]Met-labeled HA-tagged histones, HDAC-1 and CAF-1p60. As shown in Figure 3A and 3C, chp46 bound to histone H4 and HDAC-1 but not to CAF-1p60. To determine whether or not the chp46 polypeptide, together with chCAF-1p48 as a positive control, bound also to HAT-1, the GST pulldown affinity assay was carried out. The chp46 was translated in vitro in the presence of [³⁵S]Met, and then assayed its ability to interact with HAT-1. As shown in Figure 3B and 3C, the chp46 bound to GST-HAT-1 fusion protein, as well as the binding activity of CAF-1p48 to HDAC-2 and HAT-1 as a positive control, whereas under the same conditions β -galactosidase as a negative control, did not bind to it.

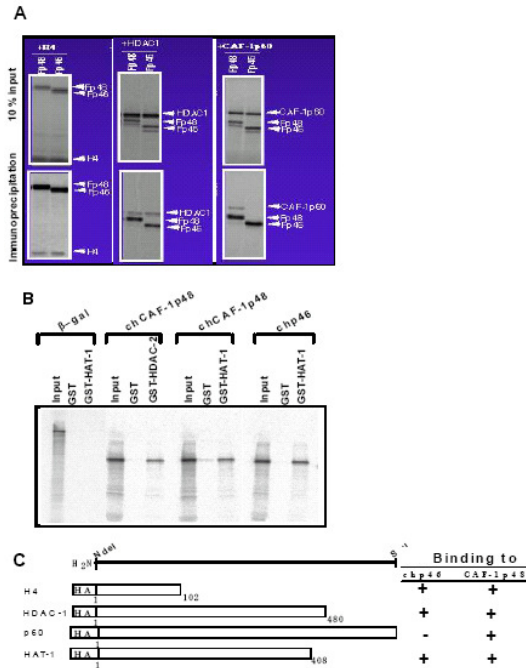


Figure 2 In vitro interaction of chp46 with histone H4, HDAC-1, CAF-1p60 and HAT-1.

Two regions, two other regions and one distinct region of chp46 are necessary for the interaction with H2B, H4 and HAT-1. Next, we determined the region(s) of chp46 necessary for its binding ability as to H2B, H4 and HAT-1, because the interaction of p46 with one or more of these proteins could be thought to play a key role in the DNA-utilizing processes. We constructed C-terminal and N-terminal truncated mutants of FLAG-tagged chp46, and studied their in vitro interaction with the H2B, H4 and HAT-1 protein. An in vitro immunoprecipitation experiment, involving truncated mutants of chp46, revealed not only that two regions comprising amino acids 33-179 and 375-404 are necessary for its binding to H2B, but also that two regions comprising amino acids 1-32 and 405-424 are necessary for its binding to H4 (Figure 3). Furthermore, a GST pull down affinity assay, involving truncated mutants of chp46, revealed that a region comprising amino acids 375-404 bind to HAT-1 in vitro (Figure 3).

These results indicate not only that chp46 should participate differentially in a number of DNA-utilizing processes through the interaction of its distinct regions with H2B, H4 and HAT-1, but also that the propeller structure of chp46, based on the WD repeat motifs, is not necessary for its interaction with HAT-1. Very recently, that sucrose gradient fractionation of semi-purified tagged Asf1-complexes showed the presence of HAT-1, RbAp48 is homolog of chp46 and histones H3/H4 at 5-6S fractions in the complexes (12). These findings suggest the possible involvement of HAT-1

in regulating cytosolic H3/H4 pool mediated by Asf1-containing cytosolic H3/H4 pre-deposition complex.

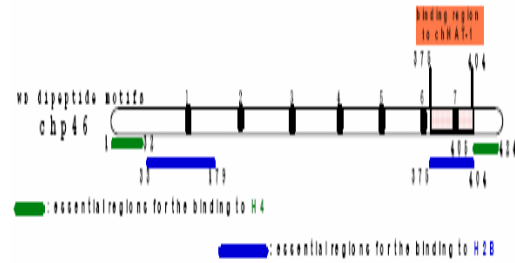


Figure 3 Essential regions of the chp46 polypeptide for interaction with histone H2B and H4, and HAT-1.

Conclusions

In summary, chp46 should be involved in numerous DNA-utilizing processes in different manners for its binding to H2B, H4 and HAT-1, and the proper propeller structure of chp46 is not necessary for the interaction with HAT-1. The overall picture concerning the in vitro and in vivo interaction of chp46 with chHDACs and chHAT-1 will be clarified, using gene targeting techniques, with the DT40 chicken B cell line, which incorporates foreign DNA by targeted integration at frequencies similar to those for random integration for a number of different genomic loci, including genes encoding core histones.

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