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WD Repeats of the p48 Subunit of Chicken Chromatin Assembly Factor-1 Required for *in Vitro* Interaction with Chicken Histone Deacetylase-2*

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Chromatin assembly factor-1 (CAF-1) is essential for chromatin assembly in eukaryotes, and comprises three subunits of 150 kDa (p150), 60 kDa (p60), and 48 kDa (p48). We cloned and sequenced cDNA encoding the small subunit of the chicken CAF-1, chCAF-1p48. It consists of 425 amino acid residues including a putative initiation Met, possesses seven WD repeat motifs, and contains only one amino acid change relative to the human and mouse CAF-1p48s. The immunoprecipitation experiment followed by Western blotting revealed that chCAF-1p48 interacts with chicken histone deacetylases (chHDAC-1 and -2) *in vivo*. The glutathione *S*-transferase pulldown affinity assay revealed the *in vitro* interaction of chCAF-1p48 with chHDAC-1, -2, and -3. We showed that the p48 subunit tightly binds to two regions of chHDAC-2, located between amino acid residues 82–180 and 245–314, respectively. We also established 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 interaction, using deletion mutants of the respective regions. These results suggest that chCAF-1p48 is involved in many aspects of DNA-utilizing processes, through alterations in the chromatin structure based on both the acetylation and deacetylation of core histones.

Understanding the process of chromatin assembly in eukaryotes is a fundamental goal, because alterations in the chromatin structure have been thought to be predominantly involved in DNA-utilizing processes, such as replication, recombination, repair, and gene expression (1–9). Chromatin assembly factor-1 (CAF-1)[1] was originally purified from human cells and promotes *de novo* chromatin assembly on replicating SV40 DNA in the presence of a cytosol replication system (10, 11). CAF-1 is a complex of three polypeptides of 150 kDa (p150), 60 kDa (p60), and 48 kDa (p48) (12). During DNA replication, CAF-1 assembles new nucleosomes through a two-step reaction (10, 13). Coupled to DNA replication, as the first step, histones H3 and H4 are deposited through a reaction that is preferentially dependent upon CAF-1, but histones H2A and H2B are added later to this immature nucleosome precursor, even in the absence of CAF-1 (12, 14, 15). These results indicate that CAF-1 interacts preferentially with H3 and H4, whereas NAP-1 binds to H2A and H2B (2, 16).

The WD protein family members, which are made up of highly conserved WD repeating units, found in eukaryotes, but not in prokaryotes, are involved in numerous biological processes such as signal transduction, RNA processing, gene expression, vesicular trafficking, and cell division (17–19). Thus, most of them seem to be regulatory, and none is an enzyme. The consensus core of the repeating unit, the WD repeat, usually ends with the characteristic sequence, Trp-Asp (WD), and such a conserved unit occurs four to ten times within each polypeptide (19). Each repeat comprises a region of variable length preceding a conserved core of about 30 amino acids (maximum range, 23–41 amino acids), ending with Gly-His (GH) and WD dipeptide residues. In addition, the number of amino acids from WD to the next downstream GH is very variable (6–94 amino acids), although shorter sequences are more common (112 residues long). All of these WD repeat proteins have been proposed to fold into propellers in which the internal β-strands form a rigid skeleton that is fleshed out on the surface by specialized loops to which other proteins bind (19–22). The amino acid residues in the WD repeats of Tup1, the yeast repressor, that are required for the interaction of Tup1 with homeodomain protein α2 have been genetically identified (23). Point mutations in the WD40 domains of the Eed (embryonic ectoderm development) protein block its interaction with Ezh2, a mammalian homolog of the *Drosophila enhancer of zeste* [E(z)] (24).

CAF-1p48, with seven WD repeat motifs, is a member of this WD repeat protein family. In recent years, knowledge concerning the characteristics of CAF-1s in the DNA-utilizing processes has been rapidly accumulated (9). For instance, CAF-1p48 was identified as a polypeptide that is tightly associated with the catalytic subunit of human histone deacetylase-1 (HDAC-1) (25). In addition, the smallest subunit of *Drosophila* CAF-1, p55, is homologous to a mammalian factor, RbAp48, associated with HDAC (26). Interestingly, *Drosophila* p55 was reported to be an integral subunit of the nucleosome remodeling factor (NURF) (27). However, there was little information concerning the detailed mechanisms for the protein-protein interaction of CAF-1p48 in higher eukaryotes.

In this study we first cloned the cDNA encoding chicken CAF-1p48, chCAF-1p48, and demonstrated that it tightly binds to chHDACs *in vivo* and *in vitro*. We describe the *in vitro* interaction of chCAF-1p48 with two regions of chHDAC-2, comprising amino acid residues 82–180 and 245–314, respectively. We also describe that this interaction requires two N-terminal, two C-terminal, or one N-terminal plus one C-terminal WD repeat of chCAF-1p48, as deletion of the respective regions results in a loss of the binding activity.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF097750.

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[1] The abbreviations used are: CAF-1, chromatin assembly factor-1; ch, chicken; GST, glutathione *S*-transferase; HDAC, histone deacetylase; PCR, polymerase chain reaction; HA, hemagglutinin; PAGE, polyacrylamide gel electrophoresis; IPTG, isopropyl β-D-thiogalactopyranoside; BSA, bovine serum albumin.

EXPERIMENTAL PROCEDURES

Materials—In this study the XL1-Blue MRF′ *Escherichia coli* strain, *E. coli* SORL strain (Stratagene), and *E. coli* BL-21 strain (Amersham Pharmacia Biotech) were used. pBluescript II SK(−) and pCite4a(−) were purchased from Promega. The pGEX-2TK gene fusion vector and glutathione-Sepharose beads were products of Amersham Pharmacia Biotech.

Cloning and Sequencing of cDNA Encoding chCAF-1p48—Based on conserved amino acid sequences (LVMTHALEWP and PNEPWVICSV) in the mouse and human CAF-1p48s deduced from their cDNAs (15, 25), sense and antisense degenerate oligonucleotide primers containing sequences 5′-YYTGGTBATGACCCATGCYCTKSAGTGGCCC-3′ and 5′-AYACDGARCAAATSACCCAAGGYTCATTGGG-3′, respectively, were constructed. A PCR product of 1082 base pairs, corresponding to a part of the coding regions of mammalian CAF-1p48s, was prepared from the chicken DT40 cDNAs using the two degenerate primers. To obtain full-length chCAF-1p48 cDNAs, using the resultant PCR product as a probe, we screened the DT40 λZAP II cDNA library constructed by us,[2] using poly(A) mRNAs prepared from the chicken DT40 cell line, essentially as described (28). The entire nucleotide sequences of both strands of the largest cDNA insert were sequenced by the dye terminator method (Applied Biosystems Division, Perkin-Elmer).

Immunoprecipitation and Western Blotting—To construct a vector, designated ptetHAchCAF-1p48, expressing chCAF-1p48 under the control of the tetracycline operator (tetO) and cytomegalovirus minimal promoter, cDNA encoding influenza HA epitope-tagged chCAF-1p48 was inserted into pUHD13-3 plasmid (29), concurrently replacing its constituent luciferase gene. To construct ptTA-bleo, a cassette of the bleomycin-resistant gene driven by β-actin promoter was inserted into the pUHD15-1 plasmid that contains tet-transactivator gene controlled by cytomegalovirus promoter (29). DT40 cells were co-transfected with ptet-HAchCAF-1p48 and ptTA-bleo, and the DT40 cell lines that express the HA-tagged chCAF-1p48 were selected by incubation in medium containing 0.3 mg/ml phleomycin (Sigma). Of these subclones, the clone (designated tetHAp48) that overexpresses the HA-tagged chCAF-1p48 in the absence of tetracycline was further selected.

Cells (1.5×10^7) were lysed in 1 ml of RIPA buffer (25 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride) with occasional inversion. After standing for 30 min at 4 °C, the lysate was centrifuged at 15,000 rpm for 10 min, and the supernatant obtained was divided into two equal portions. To each portion, 5 μg of anti-HA antibody (12CA5, Roche Molecular Biochemicals) or anti-FLAG antibody (Eastman Kodak Co.) were added. After standing for 60 min on ice, protein G-Sepharose beads (40 μl) were added to the incubation mixture. Following gentle rotation for 5 h at 4 °C, the protein G-Sepharose beads were pelleted by centrifugation, washed with 1 ml of RIPA buffer 4 times, and boiled in SDS sample buffer for 5 min. The resultant immunoprecipitated proteins were resolved by 12% SDS-PAGE, essentially as described (28). Upon transfer to a nitrocellulose membrane, proteins were probed with anti-HA antiserum or rabbit anti-chHDAC-1 and -2 antisera (against, respectively, recombinant chHDAC-1 and -2 C-terminal peptide-GST fusion proteins),[2] using an ECL kit according to the manufacturer's protocol (Amersham Pharmacia Biotech). Horseradish peroxidase-conjugated anti-mouse IgG for HA antibody (Dako) and horseradish peroxidase-conjugated anti-rabbit IgG for chHDACs antibody were used as secondary antibodies.

Plasmid Construction—To construct the pGEX-2TKchCAF-1p48 plasmid, the *Nco*I/*Sca*I fragment containing the full-length chCAF-1p48 cDNA was excised from the parental chimeric plasmid (pB(II)SKp48), blunt-ended with T4 polymerase, and then subcloned into the *Sma*I site of the pGEX-2TK plasmid in frame. We also constructed the pGEX-2TKchHDAC-2 plasmid as follows. The parental chimeric plasmid (pB(II)SKchHDAC-2) carrying the full-length chHDAC-2 cDNA[2] was digested with *Kpn*I, followed by treatment with T4 polymerase. From the resultant blunt-ended material, the *Kpn*I blunt-end/*Xba*I fragment containing the full-length chHDAC-2 cDNA was excised and subcloned between the *Bam*HI (blunt-ended by T4 polymerase) and *Xba*I sites of the pGEX-2TK plasmid.

We constructed the pCiteHDAC-1, -2, and -3 plasmids as follows. The three parental plasmids, pB(II)SKchHDAC-1, -2, and -3,[2] were digested with *Cla*I, blunt-ended with T4 polymerase, and then digested with *Not*I. The resultant blunt-ended *Cla*I/*Not*I fragments, carrying the full-length chHDAC-1, -2, and -3 cDNAs, respectively, were subcloned between the *Msc*I and *Not*I sites of the pCite4a(−) plasmid.

Deletion mutants of chHDAC-2 were constructed as follows. The *Stu*I/*Pst*I fragment encoding amino acids 82–488, the *Hin*cII/*Pst*I fragment encoding amino acids 115–488, the *Xcm*I/*Pst*I fragment encoding amino acids 181–488, the *Nde*I/*Pst*I fragment encoding amino acids 315–488, and the *Fsp*I/*Pst*I fragment encoding amino acids 371–488 of chHDAC-2, respectively, were deleted by digestion of pCiteHDAC-2 with the corresponding enzymes and religated after being blunt-ended with T4 polymerase to generate pCiteHDAC-2-(1–81), pCiteHDAC-2-(1–114), pCiteHDAC-2-(1–180), pCiteHDAC-2-(1–314), and pCiteHDAC-2-(1–370). pCiteHDAC-2-(82–370), pCiteHDAC-2-(162–370), and pCiteHDAC-2-(245–370), respectively, were generated by digestion of pCiteHDAC-2-(1–370) with *Nco*I plus *Stu*I, *Nco*I plus XcmI, and *Nco*I plus *Hin*cII, followed by religation. pCiteHDAC-2-(315–488) was generated by digestion of pCiteHDAC-2 with *Nco*I plus *Nde*I to delete the *Nco*I/*Nde*I fragment encoding amino acids 1–314, followed by religation. pCiteHDAC-2-(82–180) and pCiteHDAC-2-(245–314), respectively, were generated from pCiteHDAC-2-(82–370) and pCiteHDAC-2-(245–370) by digestion with *Xcm*I plus *Bam*HI and *Nde*I plus *Bam*HI, followed by religation after being blunt-ended with T4 polymerase.

We constructed the pCiteHAp48 plasmid, carrying both the full-length chCAF-1p48 cDNA and the HA fragment, as follows. The *Nco*I/*Sca*I fragment carrying the full-length chCAF-1p48 cDNA was first excised from the parental pB(II)SKp48 plasmid, blunt-ended with T4 polymerase, and then ligated into the *Sma*I site of pGBT9 to yield pGBT9p48. The *Nco*I/*Sal*I fragment carrying the full-length chCAF-1p48 cDNA was excised from the resultant plasmid and introduced between the *Nco*I and *Sal*I sites of the pCiteHA plasmid, carrying the HA sequence derived from pAS.1 (kindly provided by Dr. C. M. Tiree), which we constructed.

We generated deletion mutants of chCAF-1p48 as follows. pCiteHAp48-(1–267) and pCiteHAp48-(1–375), respectively, were generated by excision of the *Xcm*I/*Sal*I fragment encoding amino acids 268–425 and the *Eco*RV/*Bam*HI fragment encoding amino acids 376–425 from pCiteHAp48 by digestion with *Xcm*I plus *Sal*I and *Eco*RV plus *Bam*HI, followed by religation after blunt-ending with Klenow polymerase. To generate pCiteHAp48-(1–328), the *Nco*I/*Bsa*I fragment of chCAF-1p48 cDNA was excised from pCiteHAp48 and introduced between the *Nco*I and *Sal*I sites of the same plasmid. pCiteHAp48-(55–425), pCiteHAp48-(139–425), and pCiteHAp48-(268–425), respectively, were generated by digestion of pCiteHAp48 with *Nco*I plus *Stu*I, *Nco*I plus *Nsi*I, and *Nco*I plus *Xcm*I, and blunt-ended with T4 polymerase before religation. To generate pCiteHAp48-(181–425), we first constructed sense and antisense primers, containing sequences 5′-TATGGGTTGTCATGGAACCCAAACC-3′ and 5′-CATGGCCATATGACCACCCAAGCTA-3′, respectively, corresponding to amino acids 181–188 of chCAF-1p48 and amino acids 12–19 of HA. Next we prepared the DNA fragment without that encoding amino acids 1–180, by PCR using pCiteHAp48 as a template with these two primers and then ligated the resultant PCR product to yield pCiteHAp48-(181–425). pCiteHAp48-(55–375) and pCiteHAp48-(139–375) were generated by excision of the *Eco*RV/*Bam*HI fragment encoding amino acids 376–425 from pCiteHAp48-(55–425) and pCiteHAp48-(139–425), respectively, and religated after blunt-ending with T4 polymerase. Each end point of the deletions was determined by sequence analysis involving the dye terminator method.

Expression and Purification of GST Fusion Proteins—*E. coli* BL-21 cells were transformed with pGEX-2TKchCAF-1p48 and pGEX-2TKchHDAC-2, respectively, harboring the full-length chCAF-1p48 and chHDAC-2 cDNAs and grown to $A_{600\ nm}$ = ~0.2 in 400 ml of LB medium supplemented with 200 μg/ml ampicillin. Upon induction with 50 μM isopropyl β-D-thiogalactopyranoside (IPTG) overnight at 20 °C, the cells were collected by centrifugation and suspended in 10 ml of lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA) containing 0.1% (v/v) phenylbenzosulfonyl fluoride and 1 mg/ml lysozyme, in liquid N_2 for 2 min, followed by ultrasonication for 3 min. Continuously, the cell lysate was added to Sarkosyl to a final concentration of 1%. After standing for 60 min, Triton X-100 was added to a final concentration of 1%, and then the cell lysate was allowed to stand for an additional 30 min. The cell homogenate thus prepared was subjected to centrifugation at 15,000 rpm for 30 min. The supernatant obtained was mixed with 1 ml of a 50% slurry of glutathione-agarose beads for 4 h with gentle rotation. The GST fusion protein-bound beads were collected by centrifugation at 3,500 rpm for 2 min and washed with the lysis buffer containing 1% Triton X-100 and 0.1% phenylbenzosulfonyl fluoride four times, then with phosphate-buffered saline buffer once. The GST fusion proteins were eluted with 4 ml of 20 mM glutathione in 50 mM Tris-HCl,

[2] Y. Takami, H. Kikuchi, and T. Nakayama, manuscript in preparation.

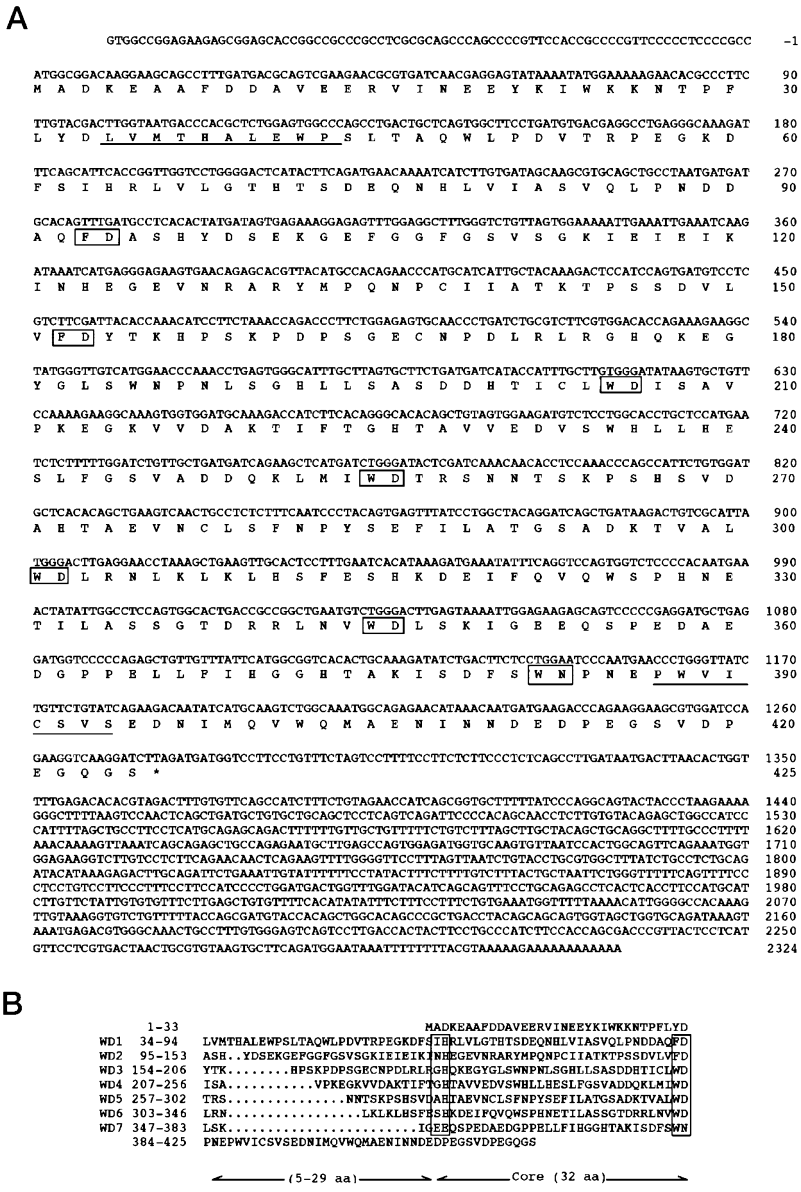

FIG. 1. **Nucleotide and deduced amino acid sequences of chicken chromatin assembly factor-1p48.** *A*, nucleotides are *numbered* in the 5′ to 3′ direction with the adenosine of the translation initiation codon designated as +1. The translation stop codon is indicated by an *asterisk*. The predicted amino acid sequence of chCAF-1p48 is presented in a *single-letter code*. The seven WD (including FD and WN) dipeptide motifs are *boxed*. Two regions corresponding to those in the human and mouse CAF-1p48s (15, 25), used as primer sequences, are *underlined*. *B*, the WD repeat motifs of chCAF-1p48. The characteristic GH and WD dipeptide motifs are *boxed*. Each WD repeat contains a variable region of 5–29 amino acid residues and a core of 32 amino acid residues.

pH 8.5, and the resultant eluate was concentrated with a Millipore membrane, followed by the addition of glycerol to a final concentration of 20%. The samples obtained were resolved by 10% SDS-PAGE, essentially as described (28).

GST Pulldown Affinity Assay—To produce [^{35}S]Met-labeled full-length chCAF-1p48 and chHDAC-1, -2, and -3, a set of truncated mutants of chCAF-1p48, and a set of truncated mutants of chHDAC-2, the TNT-coupled transcription-translation system (Promega) was used. *In vitro* binding assays were performed using 5 μl of [^{35}S]Met-labeled protein fractions and 4 μg of the GST fusion proteins or 6 μg of GST, prepared as described, in 200 μl of bead-binding buffer (50 mM potassium phosphate buffer, pH 7.5, 450 mM KCl, 10 mM $MgCl_2$, 10% glycerol, 1% Triton X-100, 1% BSA). After 1 h of standing, the reaction mixture was added to 20 μl of a 50% slurry of glutathione-agarose beads, containing 10 mg/ml BSA and 4 μg of EtBr, followed by gentle rotation for 1 h at 4 °C. The affinity beads were collected by centrifugation at 3,500 rpm for 2 min and then washed with 1 ml of the bead-binding buffer without BSA and EtBr but containing 0.1% phenylbenzosulfonyl fluoride five times. The beads were suspended in 30 μl of 2× SDS sample buffer and then boiled for 5 min. Aliquots (15 μl) of the resultant eluates were analyzed by 12% SDS-PAGE, and then the gels were stained, dried, and subjected to fluorography.

RESULTS

Cloning of cDNA Encoding chCAF-1p48—To determine unequivocally the identification of chCAF-1p48 as a novel subunit of CAF-1, we cloned and sequenced its cDNA. Based on conserved amino acid sequences (the corresponding sequences in the chicken homolog are *underlined* in Fig. 1*A*) in the mouse and human CAF-1p48s (15, 25), we prepared the 1082-base pair PCR fragment, corresponding to a part of cDNAs encoding the mammalian p48 subunits, by PCR using cDNAs from DT40 cells with degenerate primers, *i.e.* a sense primer and an antisense primer. Our screening, using the resultant PCR product, of a DT40 λZAP II cDNA library[2] yielded 11 positive cDNA clones. Sequence analysis of the largest cDNA insert of 2405 base pairs of the 11 cDNA clones revealed that the clone designated as 5 contained both an initiation codon and a 3′ poly(A) tail and thus appeared to contain the full-length sequence of the chicken CAF-1p48 cDNA. The nucleotide and deduced amino acid sequences are presented in Fig. 1*A*. The chicken protein comprises 425 amino acid residues including a putative initiation Met and contains only one amino acid alteration relative to the human and mouse CAF-1p48s (15, 25). The alteration in the amino acid sequence is Asn, instead of Tyr, at position 409. Thus, this protein exhibits 99.8%, 93.9%, and 90.4% identity in amino acid sequence to the human and mouse CAF-1p48s, human and mouse CAF-1p46s (15, 25), and *Dro-*

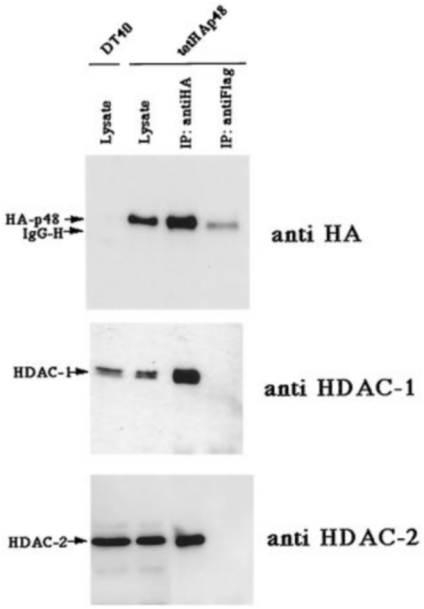

FIG. 2. **Western blotting of cell lysates and samples immunoprecipitated with anti-HA or anti-FLAG antiserum.** Aliquots of the lysates of DT40 and tetHAp48 cells and tetHAp48 samples immunoprecipitated with anti-HA or anti-FLAG antiserum were subjected to 12% SDS-PAGE. After being transferred to a nitrocellulose membrane, proteins were probed with anti-chHDAC-1 or -2 antiserum (*middle* and *lower panels*) or anti-HA antiserum (*upper panel*). *IP:antiHA*, the sample immunoprecipitated with anti-HA antiserum; *IP:antiFlag*, the sample immunoprecipitated with anti-FLAG antiserum; *HA-p48*, HA-tagged chCAF-1p48; *IgG-H*, mouse IgG heavy chain.

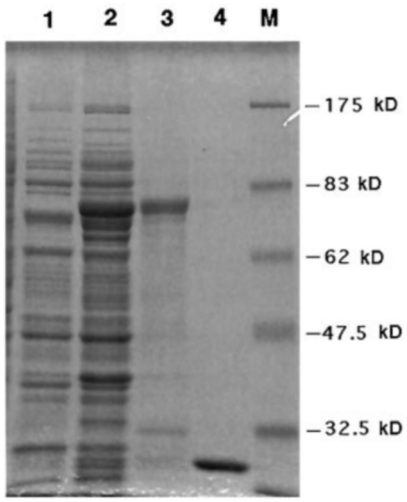

FIG. 3. **SDS gel electrophoretic patterns of GST-chCAF-1p48 fusion protein-containing fractions at different purification steps.** Protein samples prepared were subjected to 10% SDS-PAGE, followed by Coomassie Blue staining. *Lane 1*, whole cell lysate of BL-21 cells containing the pGEX-2TKchCAF-1p48 plasmid without induction by IPTG; *lane 2*, the lysate with induction by 50 μM IPTG; *lane 3*, the fraction purified with glutathione-agarose beads; *lane 4*, GST; *lane M*, molecular mass standards.

sophila CAF-1p55 (or NURF-55) (26, 27), respectively. Therefore, it is the chicken homolog of mammalian CAF-1p48s and is designated as chCAF-1p48 (GenBank^{TM} accession number AF097750).

Therefore, chCAF-1p48 is a member of the WD repeat protein family and possesses seven copies of the WD motif, a motif of 37–61 amino acid residues, including a WD, FD, or WN dipeptide (Fig. 1*B*). Like most proteins containing WD repeats, chCAF-1p48 is expected not only to physically associate with other proteins but also to act as a scaffold upon which multimeric complexes can be built.

In Vivo Interaction of chCAF-1p48 with chHDACs—To determine whether or not chCAF-1p48 binds to these chHDACs *in vivo*, Western blotting, using anti-chHDAC-1 and -2 antisera, against, respectively, recombinant chHDAC-1 and -2 C-terminal peptide-GST fusion proteins was carried out. DT40 cells were first co-transfected with ptet-HAchCAF-1p48 and ptTA-bleo, and the transfected cells that express the HA-tagged chCAF-1p48 were selected with phleomycin. A cell line (tetHAp48) overexpressing the HA-tagged protein in the absence of tetracycline was established. Proteins in the lysate of tetHAp48 cells were immunoprecipitated with anti-HA or anti-FLAG antiserum. The immunoprecipitated samples, together with the tetHAp48 lysate and the DT40 cell lysate, were subjected to Western blotting.

As shown in Fig. 2*A*, anti-HA antiserum recognized a protein species of the same size as the HA-tagged chCAF-1p48 present in the tetHAp48 cell lysate and the anti-HA antiserum-precipitated sample, but not the anti-FLAG antiserum-precipitated sample or the DT40 cell lysate. In the case of the immunoprecipitation with anti-HA antiserum, the band corresponding to the HA-tagged chCAF-1p48 migrated very closely to that of mouse IgG heavy chain present in the sample immunoprecipitated with anti-FLAG antiserum.

In contrast, anti-chHDAC-1 antiserum recognized the protein band corresponding to chHDAC-1 in the cell lysates of both tetHAp48 and DT40 cells (Fig. 2*B*). Furthermore, as expected, the same (chHDAC-1) band showed up for the sample immunoprecipitated with anti-HA antiserum but not that immunoprecipitated with anti-FLAG antiserum. Similarly, anti-chHDAC-2 antiserum recognized the band corresponding to chHDAC-2 in the lysates of both tetHAp48 and DT40 cells, as well as the sample immunoprecipitated with anti-HA antiserum (Fig. 2*C*). Reasonably, no band showed up for the sample immunoprecipitated with anti-FLAG antiserum. These results indicate clearly that both chHDAC-1 and -2 were present in the sample immunoprecipitated with anti-HA antiserum, suggesting that the two enzymes bind tightly to chCAF-1p48 *in vivo*.

Expression and Purification of GST-chCAF-1p48 Fusion Protein in E. coli—To construct a chimeric plasmid, pGEX-2TKchCAF-1p48, expressing the GST-chCAF-1p48 fusion protein, chCAF-1p48 cDNA was subcloned into the pGEX-2TK plasmid in frame. GST fusion proteins were synthesized in *E. coli*, extracted, and purified essentially as described above. As shown in Fig. 3, the electrophoretic patterns on SDS-PAGE of whole cell lysates before and after the induction with IPTG revealed that GST-chCAF-1p48 fusion proteins of approximately 74 kDa were dramatically accumulated in *E. coli* BL-21 cells containing the pGEX-2TKchCAF-1p48 plasmid. In addition, the GST-chCAF-1p48 fusion proteins were purified to more than 95% homogeneity, using glutathione-agarose beads (see *lane 3* in Fig. 3).

In Vitro Interaction of chCAF-1p48 with chHDAC-1, -2, and -3—We cloned and sequenced three cDNAs, encoding chHDAC-1, -2, and -3, respectively, which comprise 480, 488, and 428 amino acid residues, including a putative initiation Met.[2] Compared with chHDAC-1 and -2, the C-terminal region of chHDAC-3 is about 50 amino acids shorter. However, in the corresponding regions all three chHDACs exhibit extensive homology (95%). Furthermore, we recently developed *E. coli* systems with which all of chHDAC-1, -2, and -3 could be dramatically synthesized. These recombinant chHDAC-1, -2, and -3, like recombinant maize HDAC-2 expressed in *E. coli* (30), exhibited little enzymatic activity *in vitro* (data not shown). As

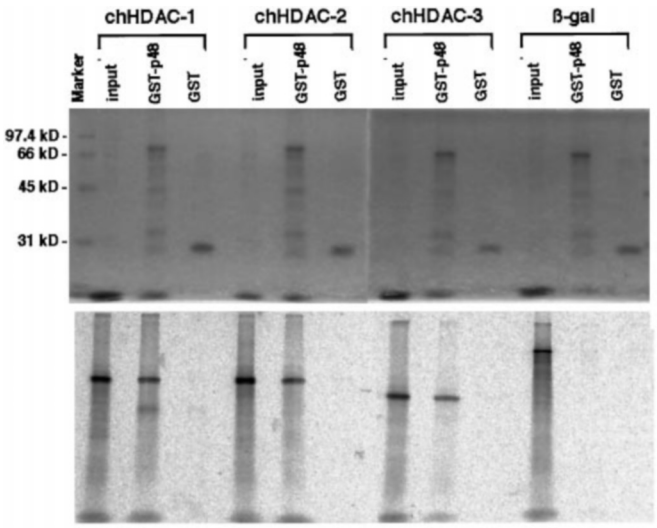

FIG. 4. ***In vitro* interaction of the GST-chCAF-1p48 fusion protein with chHDAC-1, -2, and -3.** chHDAC-1, -2, and -3 were each labeled with [^{35}S]Met with the *in vitro* translation system, and then their binding activities as to the GST-chCAF-1p48 fusion protein were examined by means of the GST pulldown affinity assay. Each sample was resolved on 12% SDS-PAGE, and then the proteins were stained with Coomassie Blue (*upper panel*), followed by fluorography (*lower panel*). β-Galactosidase (β-gal) was used as a control. *GST-p48*, GST-chCAF-1p48 fusion protein.

discussed previously (30), the problem may be due to (i) the fact that the chicken and maize enzymes are active only when assembled as correct complexes, (ii) the incorrect folding of recombinant HDACs, or (iii) the requirement of certain post-translational modifications, *e.g.* phosphorylation, for the active enzymes. Despite their lack of enzymatic activities, recombinant chHDAC-1, -2, and -3 probably have maintained the correct conformations to interact with chCAF-1p48.

To determine whether or not the p48 subunit binds to these three chHDACs, therefore, the GST pulldown affinity assay was carried out. chHDAC-1, -2, and -3 were translated *in vitro* in the presence of [^{35}S]Met and then assayed as to their abilities to interact with chCAF-1p48. Each sample was separated by 12% SDS-PAGE, and proteins were stained with Coomassie Blue, followed by fluorography. As shown in Fig. 4, the GST-chCAF-1p48 fusion protein bound to all of chHDAC-1, -2, and -3, whereas under the same conditions β-galactosidase did not bind to it, and GST alone also exhibited no interaction with these three chHDACs. These findings were confirmed by an immunoprecipitation experiment involving anti-chCAF-1p48 antiserum (data not shown), suggesting the existence of a region(s) conserved in chHDAC-1, -2, and -3 to which chCAF-1p48 binds.

Two Regions of chHDAC-2 Required for in Vitro Interaction with chCAF-1p48—To determine the putative binding region(s), which should be conserved in the three chHDACs, we first constructed a series of C-terminal deletion mutants of chHDAC-2 and studied their *in vitro* interaction with the GST-chCAF-1p48 fusion protein (Fig. 5). Three deletion mutant proteins, ΔchHDAC-2-(1–370), ΔchHDAC-2-(1–314), and ΔchHDAC-2-(1–180), exhibited similar binding activity toward the parental chHDAC-2 protein. On the other hand, two other mutant proteins, ΔchHDAC-2-(1–114) and ΔchHDAC-2-(1–81), exhibited no binding activity. These findings suggested that the region comprising amino acids 115–180 of chHDAC-2 is necessary for its binding to chCAF-1p48.

Next we constructed a series of mutants with simultaneous deletion of both the N-terminal and C-terminal regions of chHDAC-2 and assayed their binding activities essentially as described above. Fig. 5 shows that ΔchHDAC-2-(245–370), like ΔchHDAC-2-(82–370) and ΔchHDAC-2-(162–370), exhibited binding activity, although it even lacks the region comprising amino acids 115–180. In addition, ΔchHDAC-2-(315–488), lacking the N-terminal region comprising amino acids 1–314, exhibited no activity, whereas ΔchHDAC-2-(1–314) bearing the same N-terminal region exhibited the binding ability.

These results suggested that two possible binding regions of chHDAC-2 as to chCAF-1p48 are located between amino acids 115–180 and amino acids 245–314, respectively, and that either is enough as the binding region. Finally, to confirm these results we constructed two mutant proteins, ΔchHDAC-2-(82–180) and ΔchHDAC-2-(245–314), and studied their binding activities. As expected, the two regions definitely interacted with chCAF-1p48, indicating that either is enough for the *in vitro* interaction with chCAF-1p48. Moreover, the findings that both regions were extensively conserved within chHDAC-1 and -3,[2] as in homologs from other organisms, and that chCAF-1p48 bound to the two chHDACs (see Fig. 4) suggest the possible involvement of the two corresponding domains of all HDACs in their *in vitro* interactions with CAF-1p48.

Two WD Repeats of chCAF-1p48 Required for in Vitro Interaction with chHDAC-2—To clarify the putative binding domain(s) of chCAF-1p48 as to chHDAC-2, we first constructed a series of C-terminal truncated mutants of HA-tagged chCAF-1p48 and studied the *in vitro* interaction with the GST-chHDAC-2 fusion protein, essentially as described above. Two truncated proteins, ΔchCAF-1p48-(1–328) and ΔchCAF-1p48-(1–267), exhibited no binding activity, although the other one (ΔchCAF-1p48-(1–375)) exhibited binding activity similar to that of the parental chCAF-1p48 protein (Fig. 6). These findings suggested that the deletion of the region comprising amino acids 329–375, in addition to the C-terminal region comprising amino acids 376–425, resulted in the loss of the binding activity.

Next we constructed a series of N-terminal truncated mutants of HA-tagged chCAF-1p48 and studied their binding abilities as to chHDAC-2, essentially as described above. As shown in Fig. 6, the abilities of two truncated mutant proteins, ΔchCAF-1p48-(55–425) and ΔchCAF-1p48-(139–425), were the same as that of the parental chCAF-1p48 protein. On the other hand, two other truncated proteins, ΔchCAF-1p48-(181–425) and ΔchCAF-1p48-(268–425), did not exhibit the ability. These results indicated that the deletion of the region comprising amino acids 139–180, in addition to the N-terminal region comprising amino acids 1–138, resulted in the loss of the binding ability.

Moreover, we constructed two truncated mutants, respectively, with simultaneous deletion of both the N-terminal and C-terminal regions of chCAF-1p48 and assayed their binding activities. The results obtained indicated that ΔchCAF-1p48-(55–375) exhibited the activity but ΔchCAF-1p48-(139–375) had lost it, whereas both mutants similarly lacked the C-terminal region comprising amino acids 376–425.

Our analyses, like these, of a series of deletion mutant proteins of chCAF-1p48 revealed several noticeable features regarding its binding ability as to chHDAC-2 as follows. First the finding that ΔchCAF-1p48-(1–375) exhibited the binding activity but ΔchCAF-1p48-(1–328) did not revealed that a lack or disruption of two C-terminal WD repeats causes loss of the binding ability. Second, the finding that ΔchCAF-1p48-(139–425) possessed the binding activity but ΔchCAF-1p48-(181–425) had lost it revealed that a lack or disruption of two N-terminal WD repeats results in loss of the binding ability. Third, the finding that the two mutants, ΔchCAF-1p48-(139–

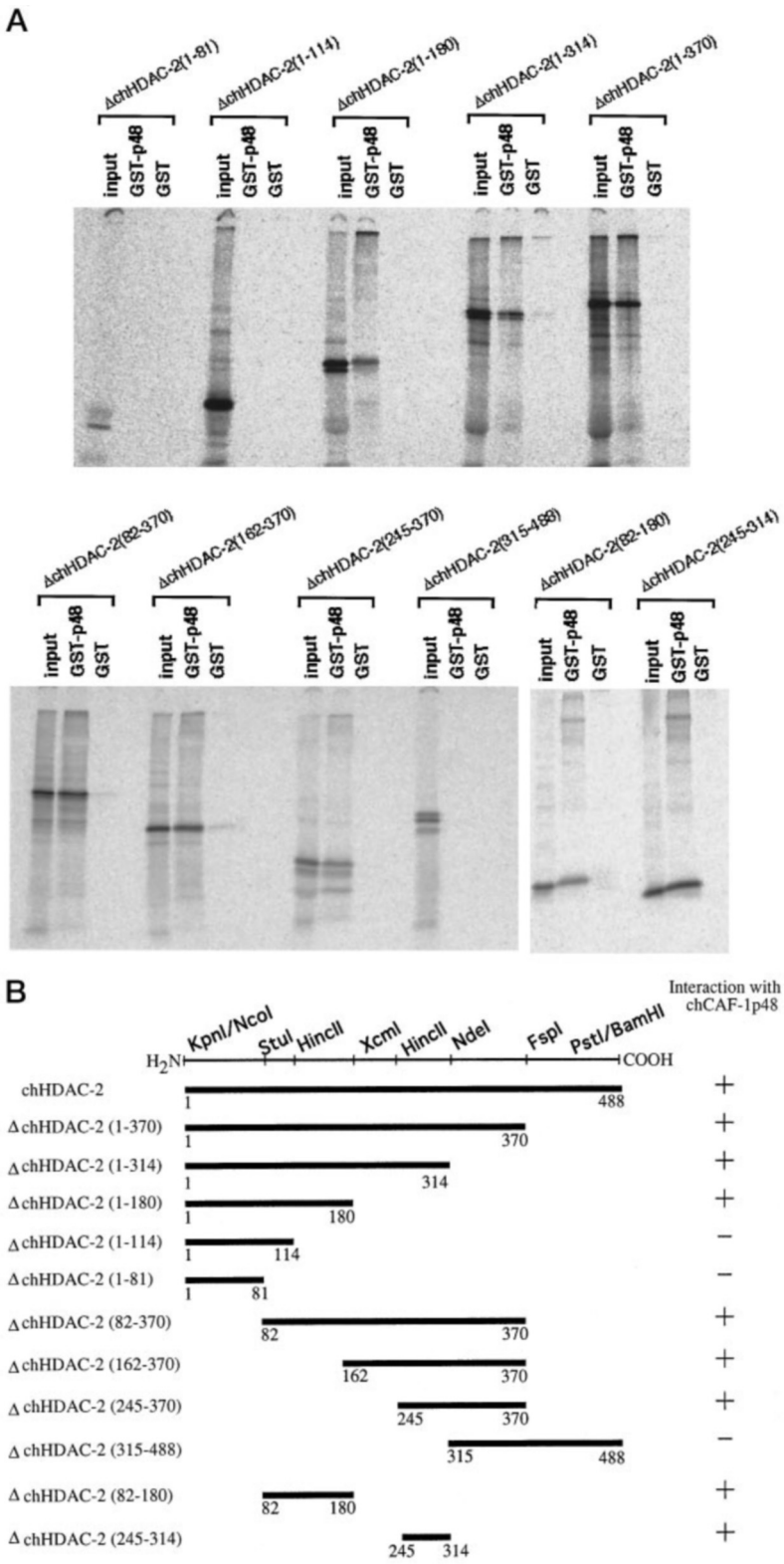

FIG. 5. ***In vitro* interaction of truncated mutant proteins of chHDAC-2 with the GST-chCAF-1p48 fusion protein.** *A*, a series of mutant proteins, respectively, devoid of the N-terminal, C-terminal, and both the N-terminal plus C-terminal regions of chHDAC-2 were constructed and their binding activities as to the GST-chCAF-1p48 fusion protein were examined as in Fig. 4. *B*, the results obtained for the *in vitro* interaction of chHDAC-2 and its truncated proteins with chCAF-1p48 are schematically presented.

425) and ΔchCAF-1p48-(55–375), exhibited the binding activity but ΔchCAF-1p48-(139–375) did not revealed that the simultaneous lack of an N-terminal WD repeat and a C-terminal one also causes loss of the binding ability. Taken together our results indicate that among the seven WD repeats of chCAF-1p48, two N-terminal, two C-terminal, or one N-terminal and one C-terminal, one is necessary for the *in vitro* interaction of the p48 subunit with chHDAC-2.

DISCUSSION

Eukaryotic HDACs, like acetylpolyamine amidohydrolases, are members of a deacetylase superfamily of proteins that not only recognize an acetylaminopropyl group and catalyze the removal of an acetyl group by cleaving a non-peptide amide bond but also that share nine blocks, 1 to 9, exhibiting considerable sequence homology (31). All of them are located in the approximately two-thirds N-terminal region of each of three

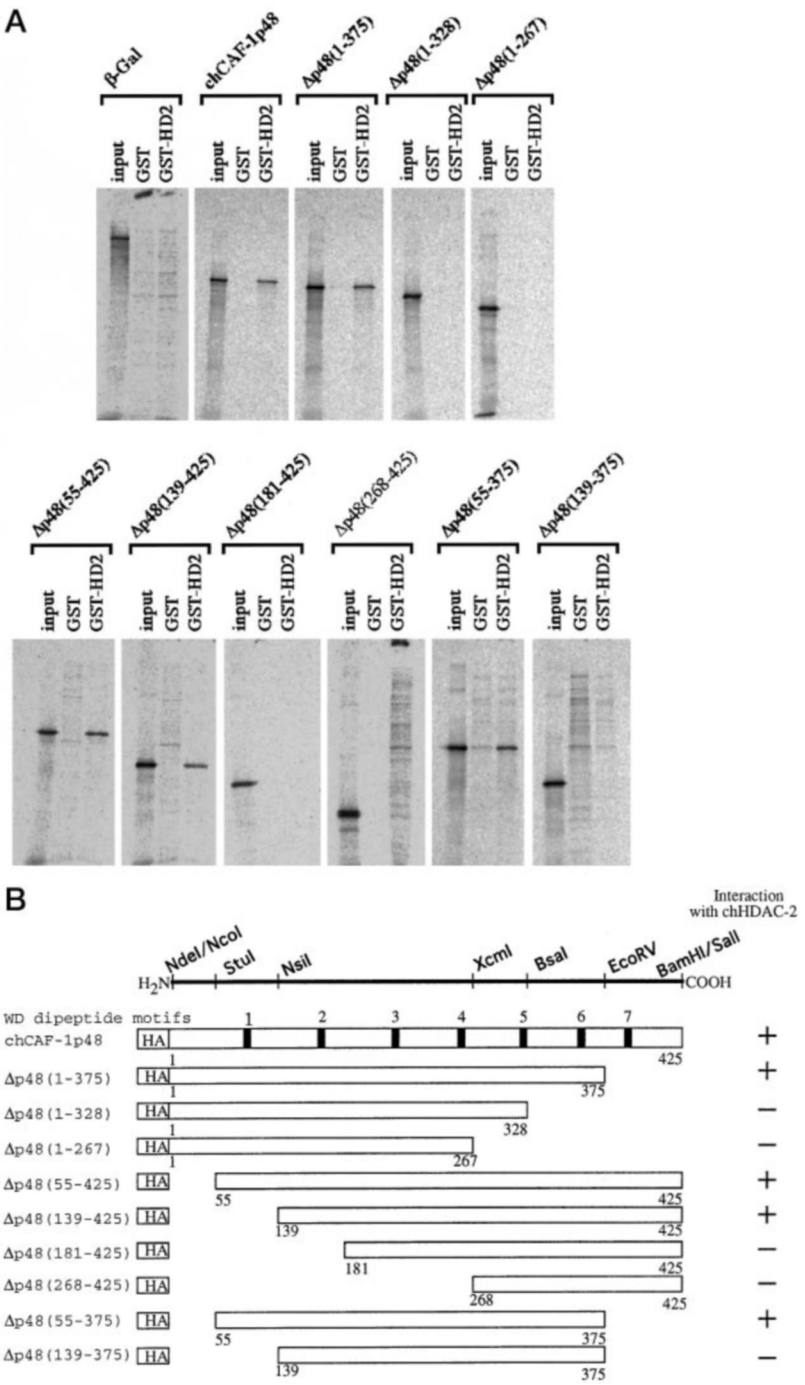

FIG. 6. ***In vitro* interaction of truncated mutant proteins of chCAF-1p48 with the GST-chHDAC-2 fusion protein.** *A*, a series of mutant proteins, respectively, devoid of the N-terminal, C-terminal, and both the N-terminal plus C-terminal regions of HA-tagged chCAF-1p48 were constructed, and then their binding activities as to the GST-chHDAC-2 fusion protein were examined as in Fig. 4. The mutant proteins used are denoted by appropriate abbreviations, *i.e.* Δp48(1–375) is the abbreviation for ΔchCAF-1p48-(1–375), as an example. *GST-HD2*, GST-chHDAC-2 fusion protein. *B*, the results obtained for the *in vitro* interaction of HA-tagged chCAF-1p48 and its truncated proteins with chHDAC-2 are schematically presented.

chHDACs.[2] Two binding domains, designated as BD1 and BD2, comprising amino acids 82–180 (in fact, probably 115–180) and amino acids 245–314 of chHDAC-2 (Fig. 5), respectively, contain blocks 3, 4, and 5 plus approximately half of the N-terminal region of block 6 and approximately half of the C-terminal region of block 8 plus block 9. BD1 and BD2 are also both located in the corresponding regions of chHDAC-1 and -3 as in those of other mammalian HDAC homologs (25, 32, 33). To determine how this interaction of BD1 and/or BD2 with chCAF-1p48 is involved in DNA-utilizing processes, such as replication, recombination, repair, and gene expression, further studies must be performed.

The amino acid sequence of chCAF-1p48 shows that it is a member of the superfamily of WD repeat proteins (Fig. 1). All of the seven WD repeats found in it well match those in the small subunits of CAF-1s in most eukaryotes (15, 25, 27). chCAF-1p48 exhibits binding ability as to chHDAC-2 (and chHDAC-1 and 3) when the second WD dipeptide motif and the shorter region of 13 amino acids preceding it remain, but the p48 subunit loses the ability when both the first and second WD repeats with the variable region of 27 amino acids just behind the second WD dipeptide motif are lacking (Fig. 6). On the other hand, the binding ability of chCAF-1p48 disappears when the region of 16 amino acids preceding the sixth WD dipeptide motif, together with this motif and the seventh one, is lacking. Conversely, the ability does not change even when the shorter region of 6 amino acids preceding the seventh WD dipeptide motif, together with both this motif and the C-terminal region of the protein, is lacking. Interestingly, even when the first WD repeat plus the region of 44 amino acids just behind the first FD dipeptide motif, or the shorter region of 6 amino acids preceding the seventh WN dipeptide motif plus the

C-terminal region is independently deleted, no influence is observed on the binding ability of chCAF-1p48; however, the simultaneous deletion of the two N-terminal and C-terminal portions abolishes the binding ability of chCAF-1p48 (Fig. 6). The findings made based on the *in vitro* assay system most likely reflected what happens *in vivo* because chCAF-1p48 also interacted with chHDAC-1 and -2 in the *in vivo* experiment (see Fig. 2).

Based on the results obtained for a series of truncated mutant proteins of chCAF-1p48, we propose a model for its interaction with chHDAC-2. As in most WD proteins (19), the WD repeat motifs of chCAF-1p48 should form a β-propeller structure, wherein each of the seven "blades" contains the amino acid residues of one WD repeat unit and probably function as a common platform for the protein-protein interaction involved in chromatin metabolism. At least two N-terminal WD repeats and two C-terminal WD repeats, respectively, are predominantly involved in maintaining the propeller structure. Deletion of the two N-terminal WD repeats with the shorter region preceding the second FD dipeptide motif or that of the two C-terminal WD repeats with the shorter region preceding the sixth WD dipeptide motif causes destruction of the proper surface loops and turns. This structural change does not allow the interaction of chCAF-1p48 with chHDAC-2, probably with BD1 and/or BD2. Similarly, simultaneous deletion of the first N-terminal WD repeat with the shorter region preceding the second FD dipeptide motif and of the last C-terminal WN dipeptide motif with the shorter region preceding the motif does not allow the propeller structure to be maintained properly, resulting in loss of the binding activity.

The overall picture concerning the *in vivo* interaction of chCAF-1p48 with chHDACs 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 (34) for a number of different genomic loci, including genes encoding histones (35–40) and histone deacetylases.[2]

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