J. Biochem. **139**, 91–97 (2006)
doi:10.1093/jb/mvj004

PKCI-W Forms a Heterodimer with PKCI-Z and Inhibits the Biological Activities of PKCI-Z *In Vitro*, Supporting the Predicted Role of PKCI-W in Sex Determination in Birds

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Received August 24, 2005; accepted October 3, 2005

The two chicken genes, *PKCI-W* on the W chromosome and *PKCI-Z* on the Z chromosome, belong to the gene family encoding the Hint (histidine triad nucleotide–binding protein)-branch proteins in the widely conserved HIT (histidine triad)-family. It has been speculated that PKCI-W is involved in the sex determination of birds by forming a heterodimer with PKCI-Z and inhibiting the function of PKCI-Z in female embryos. In this study, both PKCI-W and PKCI-Z were expressed in fusion [maltose-binding protein (MBP) or glutathione-S-transferase (GST)] and tagged [$(His)_6$ or FLAG] forms (FT-forms) in *Escherichia coli* and purified. Formation of homodimers of PKCI-W-containing or the PKCI-Z–containing FT-protein and the formation of a heterodimer between the PKCI-W–containing and the PKCI-Z–containing FT-proteins were demonstrated by Western blotting after GST-pulldown or binding to and elution from the Co^{2+}-resin. The homodimer of PKCI-Z, but not PKCI-W, bound to an N^6-(3- aminopropyl) adenosine affinity column and hydrolyzed adenosine 5′-monophosphoramidate. Both of these activities were inhibited *in vitro* in a dominant-negative manner by the formation of a heterodimer containing PKCI-W. These *in vitro* experimental results support the predicted role of PKCI-W in the process of sex determination in birds.

Key words: chicken, dominant-negative inhibition, heterodimer, PKCI/Hint, sex chromosome.

The *ASW* (*1*) and *Wpkci* (*2*) genes, now designated as *PKCI-W* according to Ellegren (*3*), were discovered independently on the chicken W chromosome, a female-specific sex chromosome, and were found to be identical to a gene encoding a protein belonging to the Hint (histidine triad nucleotide–binding protein)-branch of the HIT (histidine triad protein) superfamily that is present in a wide variety of organisms (*4*). A counterpart gene, *chPKCI* (now designated as *PKCI-Z*) was found and located on the chicken Z chromosome, a sex chromosome common to males and females (*2*). Among the five genes; *CHD1-W*, *ATP5A1-W*, *SPIN-W*, *ASW/PKCI-W and FET-1* identified and located on the chicken W chromosome, the first four genes have counterparts that have been identified on the Z chromosome (*5*). However, it is not known whether a counterpart gene of *FET-1* exists on the Z chromosome. FET-1 may be involved in the differentiation of the ovary, but it is rather unlikely that FET-1 has a triggering role in female determination, because its expression is evident in the developing left gonad in 4.5 to 6-day female embryos (*6*). *ASW/PKCI-W* is the only gene whose sequence is substantially different from that of its counterpart gene on the Z chromosome (*2*). The overall amino acid sequence identity of *PKCI-W* and *PKCI-Z*, as deduced from their cDNA sequences, is about 65%, but PKCI-W is unique among the Hint branch proteins in that it lacks the HIT motif and contains a unique Leu, Arg-rich sequence (*2*). It has been shown that PKCI/Hint forms a homodimer and exhibits high-affinity binding for a column containing adenosine bound through the N^6-position and adenosine 5′-monophosphoramidase activity (*7*). High-resolution X-ray crystallographic analysis of the human and rabbit PKCI-1 proteins demonstrates that the protomer has a five-stranded anti-parallel β-sheet and two α-helices, and that two protomers come together to form a ten-stranded anti-parallel β-sheet with contacts between one of the α-helices (helix B or α2) of each protomer and between the carboxyl terminal Gly-126 of each protomer or between Arg-119 of one protomer and Gly-126 of another protomer (*8*). Interestingly, the α2 region and the C-terminal region encompassing the HIT domain in PKCI-Z are well conserved in PKCI-W. Based on these properties of the deduced sequences, a model was proposed in which PKCI-W forms a heterodimer with PKCI-Z and thereby inhibits the biological functions of PKCI-Z, consequentially triggering a gene expression cascade toward female sex determination (*1, 2*). However, this model has not been verified at the protein level. The only feature suggested at the protein level is that a GFP-fusion form of PKCI-W was found to localize in nuclei while a similar form of PKCI-Z was distributed in both cytoplasm and nuclei when their respective cDNA constructs were expressed in chicken embryonic fibroblasts (*2*).

Here we show that fusion and tagged (FT-) forms of PKCI-W and PKCI-Z expressed in *E. coli* and purified could form a heterodimer *in vitro*, and that this leads to an inhibition of the biological activities of PKCI-Z in a

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dominant-negative manner. These *in vitro* experimental results support the predicted roles of PKCI-W in sex determination in birds.

EXPERIMENTAL PROCEDURES

Construction of Recombinant Vectors and Expression, Purification of FT-Proteins—The entire coding sequences of PKCI-W and PKCI-Z were obtained by PCR from their respective cDNA clones, pWpkci-8 and pchPKCI-3. The coding sequences for RGS-$(His)_6$ and FLAG were obtained by PCR from pIVEX2.3-MCS (Roche) and YEpFLAG-1 (SIGMA), respectively. For the former construct, the linker sequence preceding the coding sequence for $(His)_6$ was changed from GGGGGTTCT encoding Gly Gly Ser to AGGGGTTCT encoding Arg Gly Ser. The PKCI-W- or PKCI-Z-encoding sequence and the sequence encoding a tag were ligated in frame and further ligated into pMALc2E (New England Biolabs) for the expression of a maltose-binding protein (MBP)–fusion protein or into pGEX6P-1 (Amersham Biosciences) for the expression of a glutathione S-transferase (GST)–fusion protein.

The production of GST-PKCI-Z-FLAG in *E. coli* BL-21 and purification of the product were carried out according to the manufacturer's protocol with GSTrap FF (Amersham Biosciences). After purification with GSTrap, the protein was purified by diethylaminoethyl (DEAE) anion-exchange column chromatography (HiTrap DEAE FF, 1 ml; Amersham Biosciences) with Tris-buffer (20 mM Tris-HCl, pH 7.4, containing 0–0.5 M NaCl), and purified by passage through a Superose 12 10/300 GL gel-filtration column (Amersham Biosciences) with TBS buffer (50 mM Tris-HCl, pH 7.4, 138 mM NaCl and 2.7 mM KCl).

The production of MBP-fusion proteins in *E. coli* and purification of the products were carried out according to the manufacturer's protocol except that *E. coli* XL-1 (STRATAGENE) was used as a host strain. After purification with amylose resin (New England Biolabs), MBP-PKCI-Z-His was purified on a TALON column (CLONTECH) according to the TALON manual. FT-PKCI-W proteins were purified with HiTrap DEAE FF with bis-Tris buffer (20 mM bis-Tris [pH 6.5]) containing 0–0.5 M NaCl. MBP-PKCI-W-FLAG was purified by Superose 12 chromatography, as described above.

Protein bands were reduced, alkylated, and subjected to in-gel tryptic digestion according to Kikuchi *et al.* (*9*), and the peptides were identified by nano liquid chromatography electro spray ionization mass spectrometry/mass spectrometry (nano LC-ESI-MS/MS).

Antibodies and Western Blotting—Rabbit anti-MBP antiserum (New England Biolabs), goat anti-GST antibody (Amersham Biosciences), mouse anti–RGS-His antibody (QIAGEN) and mouse anti–FLAG M2 monoclonal antibody (SIGMA) were used to detect fused proteins and tagged peptides. The anti–PKCI-W-peptide antibody and anti–PKCI-W-peptide antibody were raised in female New Zealand White rabbits by immunizing them with synthetic peptides: CTRSALRSMIFHRK for PKCI-W, where the N-terminal C was not present in the original sequence but was added for the purpose of conjugation as described below, and CLAFHDISPQAPTH for PKCI-Z. These peptides were conjugated with the *m*-maleimidobenzoyl derivative of keyhole limpet hemocyanin under the standard conditions. The antisera were subjected to affinity purification by binding to the antigen peptides coupled to epoxy-activated Sepharose 6B (Amersham Biosciences) and eluted with 0.1 M glycine-HCl (pH 2.5). Western blotting was detected by the reaction with alkaline phosphatase–conjugated anti-goat, anti-mouse or anti-rabbit IgG (Promega) and the chemiluminescence detection reagent CDP-Star (Amersham Biosciences).

Mixture and Dialysis of Formed Dimers—A purified FT-protein or a mixture of two different proteins in TBS buffer was adjusted with TBS containing 6 M guanidine-HCl to achieve a 2 M concentration of guanidine-HCl, and then immediately dialyzed against TBS for 12 h at 4°C.

Pulldown Assay—Aliquots (0.5 nmol) of either protein were mixed and dialyzed. The dialyzed material was mixed with a 10-μl bed volume of Glutathione-Sepharose 4B (Amersham Biosciences) and washed with TBS. Then the bound material was eluted with GST elution buffer (TBS containing 10 mM reduced glutathione) (GST pulldown). Alternatively, the dialyzed material was mixed with a 10-μl bed volume of TALON resin (CLONTECH) and washed with TBS, after which the bound material was eluted with elution buffer (TBS containing 150 mM imidazole) (His-tag pulldown). The eluted materials were subjected to SDS-PAGE and Western blotting with the anti–RGS-His antibody (GST pulldown) or the anti–FLAG M2 antibody (His-tag pulldown). Pulldown experiments were performed at 4°C.

Affinity Chromatography on an N^6-(3-Aminopropyl) Adenosine–Conjugated Column—N^6-(3-Aminopropyl) adenosine was synthesized and 8 mg of which was coupled to 2 ml Affi-Gel 10 (Bio-Rad) according to Gilmour *et al.* (*11*) except that the reaction between 6-chloropurine riboside and 1,3-diaminopropane was carried out at room temperature for 2 h. Homodimers of MBP-PKCI-Z-His or MBP-PKCI-W-FLAG (2.0 nmol each) or heterodimers formed between 2.0 nmol of MBP-PKCI-Z-His and 2.0 or 4.0 nmol of MBP-PKCI-W-FLAG were applied to a 1 ml affinity column in TBS, and the column was washed successively with 10 ml of TBS and 5 ml of TBS containing 200 μM cAMP at 4°C. The bound material, if any, was eluted with 5 ml of TBS containing 200 μM adenosine. The wash and the eluate were collected in 2.5 ml fractions, concentrated to 1 ml, and subjected to SDS-PAGE and Western blotting.

Adenosine 5′-Monophosphoramidase Activity—Homodimers or heterodimers prepared as described above were assayed for hydrolyzing activity toward adenosine 5′-monophoshoramidate ($AMPNH_2$, SIGMA) according to Bieganowski *et al.* (*7*) except that the reaction buffer was TBS. Homodimers of MBP-PKCI-Z-His or MBP-PKCI-W-FLAG (0.1 nmol) or heterodimers formed between 0.1 nmol of MBP-PKCI-Z-His and 0.03 to 0.2 nmol of MBP-PKCI-W-FLAG were adjusted to 200 μl TBS containing 200 μM $AMPNH_2$. The samples were incubated at 37°C for 60 min, and the reaction material was diluted 10-fold with distilled water. In the case of material not dialyzed against 2 M guanidine-HCl, the incubation time was 10 min. $AMPNH_2$ and the product, AMP, were separated by the anion-exchange chromatography on a HiTrap Q FF column (1 ml) (Amersham Biosciences) with a linear gradient of 0–0.25 M NH_4HCO_3, pH 8. The elution profile was monitored at A_{254}.

RESULTS

Expression, Purification, and Immunological Detection of PKCI-Z and PKCI-W as FT-Proteins—Two different fusion partners, glutathione S-transferase (GST) and maltose-binding protein (MBP), and two different peptide tags, RGS-$(His)_6$ peptide (His) and FLAG peptide (FLAG), were utilized to construct recombinant expression vectors to produce four different FT-proteins: GST-PKCI-Z-FLAG, MBP-PKCI-Z-His, MBP-PKCI-W-FLAG and MBP-PKCI-W-His (Fig. 1A). These constructs were expressed in *E. coli*, and the FT-proteins were purified from the lysates as described in Experimental Procedures. The purity of each FT-protein was demonstrated by silver-staining after SDS–polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 1B). Each FT-protein was specifically detected by Western blotting with an antibody against MBP, GST, RGS-$(His)_4$ (abbreviated as anti-His) or FLAG, or with the antibody against PKCI-Z-peptide or PKCI-W-peptide (Fig. 1C). The peptides used as immunogens for the latter two antibodies were selected from the Leu, Arg-rich region of PKCI-W or its corresponding region in PKCI-Z, which are the least homologous regions between the two proteins (*2*). The amino acid sequences of PKCI-Z and PKCI-W in their FT-proteins were identified by nano LC-ESI-MS/MS (data not shown). The GST-fusion PKCI-W was expressed in *E. coli* but could not be solubilized.

Evidence for the Formation of Homodimers and a Heterodimer of FT-Proteins—Different combinations of mixtures of the purified FT-proteins (Fig. 1) were dialyzed against TBS buffer containing 2 M guanidine-HCl and then against the same buffer without guanidine-HCl. The dialyzed sample was either bound to and eluted from glutathione-Sepharose 4B beads (GST-pulldown) or bound to and eluted from Co^{2+} resin (TALON) (His-tag pulldown). The eluted material was separated by SDS-PAGE and subjected to Western blotting with either an anti-His antibody or anti-FLAG antibody (Fig. 2).

When MBP-PKCI-Z-His and GST-PKCI-Z-FLAG were mixed and treated as above for GST-pulldown, the presence of MBP-PKCI-Z-His in the eluate was demonstrated by the reaction with the anti-His antibody (Fig. 2A, lane 1). MBP-PKCI-Z-His alone did not bind to GST (Fig. 2A, lane 2) nor to the glutathione-Sepharose beads (Fig. 2A, lane 3). Conversely, when GST-PKCI-Z-FLAG and MBP-PKCI-Z-His were treated as above and subjected to His-tag pulldown, GST-PKCI-Z-FLAG was present in the eluate, as demonstrated by the reaction with the anti-FLAG antibody (Fig. 2B, lane 1). GST-PKCI-Z-FLAG alone did not bind to Co^{2+} resin (Fig. 2B, lane 2). Similarly, when the mixture of MBP-PKCI-W-FLAG and MBP-PKCI-W-His was treated as above and subjected to His-tag pulldown, MBP-PKCI-W-FLAG was detected in the eluate with the anti-FLAG antibody (Fig. 2C, lane 1). MBP-PKCI-W-FLAG alone did not bind to Co^{2+} resin (Fig. 2C, lane 2). These results indicate that two types of FT-proteins, each containing PKCI-Z or PKCI-W, formed homodimers in terms of PKCI-Z or PKCI-W. The former results are consistent with those of X-ray crystallographic studies of mammalian PKCI/Hint, which demonstrated the formation of a homodimer (*8, 10*).

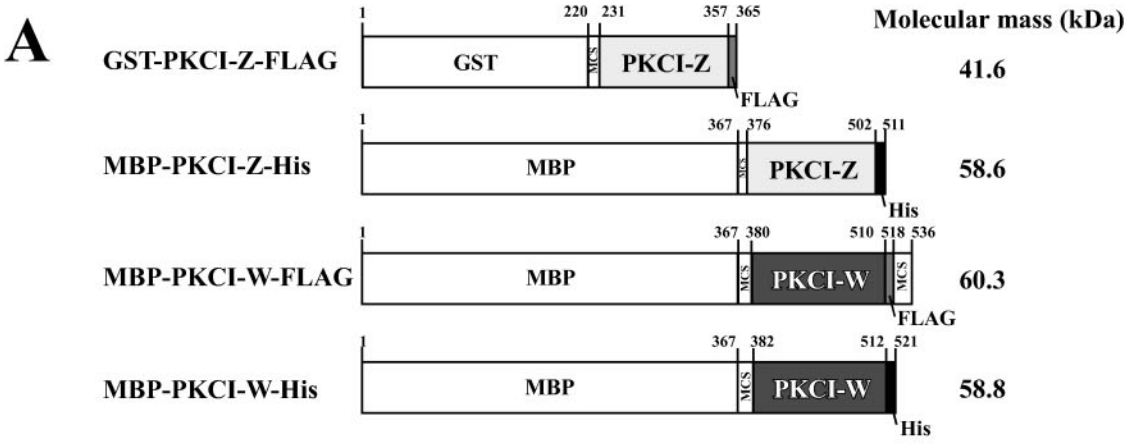
Next, the mixture of GST-PKCI-Z-FLAG and MBP-PKCI-W-His was subjected to sequential dialysis and GST-pulldown. MBP-PKCI-W-His was detected in the eluate by the reaction with the anti-His antibody (Fig. 2D, lane 1). MBP-PKCI-W-His alone bound to neither GST (Fig. 2D, lane 2) nor glutathione-Sepharose (Fig. 2D, lane 3). Conversely, when the mixture of GST-PKCI-Z-FLAG and MBP-PKCI-W-His was treated as above and subjected to His-tag pulldown, GST-PKCI-Z-FLAG was detected in the eluate by the reaction with the anti-FLAG antibody (Fig. 2E, lane 1). GST-PKCI-Z-FLAG alone did not bind to the Co^{2+} resin (Fig. 2E, lane 2). Similarly, when MBP-PKCI-W-FLAG and MBP-PKCI-Z-His were treated as above and subjected to His-tag pulldown, MBP-PKCI-W-FLAG was detected in the eluate with the anti-FLAG antibody (Fig. 2F, lane 1). MBP-PKCI-W-FLAG alone did not bind to the Co^{2+} resin (Fig. 2F, lane 2). These results indicate that a heterodimer was formed between FT-protein-containing PKCI-Z and PKCI-W.

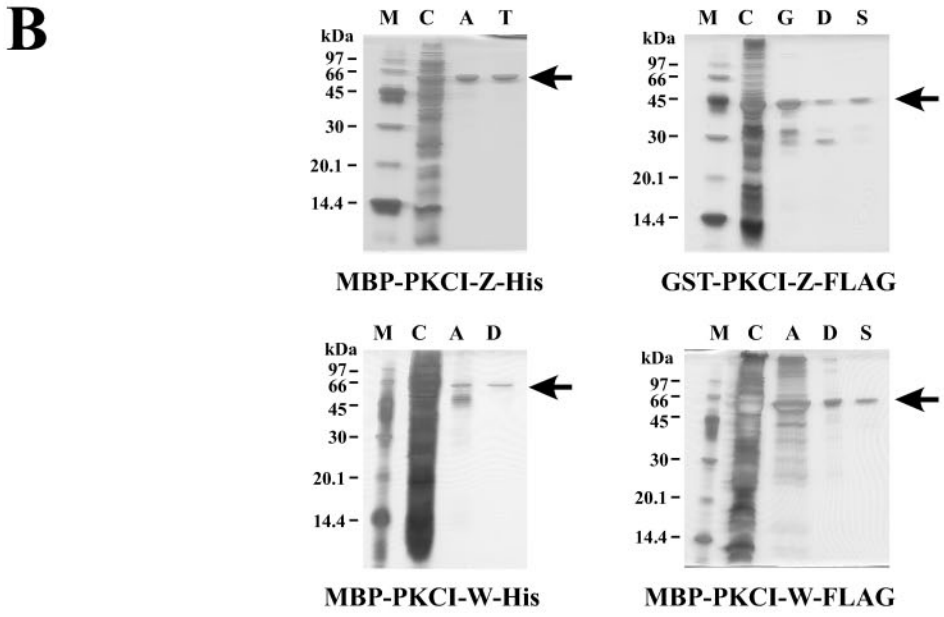
High-Affinity Binding of FT-PKCI-Z to Adenosine and Its Dominant-Negative-Type Inhibition by the Formation of a Heterodimer with FT-PKCI-W—It was demonstrated that the rabbit ortholog of PKCI/Hint, p13.7, shows high-affinity binding with adenosine and comparable affinity for IMP and GMP (*11*). To examine if chicken PKCI-Z in its FT-form exhibits a similar activity, we tested its ability to bind to an N^6-(3-aminopropyl) adenosine–conjugated Affi-Gel column. In these experiments, either a purified FT-protein or a mixture of the two different FT-proteins was first dialyzed against 2 M guanidine-HCl and then dialyzed against buffer without guanidine-HCl. Treatment with 6 M guanidine-HCl resulted in the loss of the biological activities of FT-PKCI-Z.

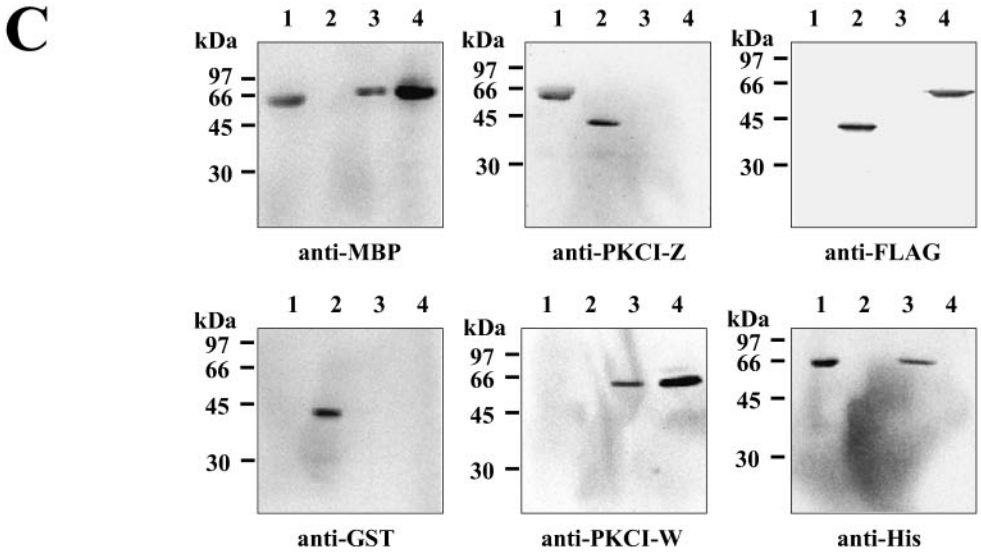
MBP-PKCI-Z-His bound to the affinity column effectively and was eluted with 200 μM adenosine but not with the same concentration of cAMP as judged from the results of SDS-PAGE and Western blotting (Fig. 3A). On the other hand, MBP-PKCI-W-FLAG did not bind to the affinity column (Fig. 3B) as expected from the absence of the HIT motif in PKCI-W (*2*).

Next, the effect of the formation of a heterodimer between FT-PKCI-Z and FT-PKCI-W on the binding of FT-PKCI-Z to adenosine was examined. MBP-PKCI-Z-His and MBP-PKCI-W-FLAG were mixed in a 1:1 or 1:2 molar ratio, and each mixture was subjected to sequential dialysis against 2 M guanidine-HCl and buffer without guanidine-HCl, followed by adenosine affinity chromatography, SDS-PAGE, and Western blotting as described above (Fig. 3C). When MBP-PKCI-Z-His and MBP-PKCI-W-FLAG were mixed in a 1:2 molar ratio, binding was lost (Fig. 3C). These results demonstrate that the high affinity binding of the FT-protein containing PKCI-Z to adenosine was inhibited by the formation of a heterodimer with FT-protein–containing PKCI-W.

Adenosine 5′-Monophosphoramidase Activity of FT-PKCI-Z and Its Dominant-Negative-Type Inhibition by the Formation of a Heterodimer with FT-PKCI-W—It has been demonstrated that the two Hint-branch proteins, rabbit Hint and yeast Hnt1, can hydrolyze adenosine 5′-monophosphoramidate ($AMPNH_2$) to AMP and a presumptive ammonia leaving group (*7*). The enzymatic activity of yeast Hnt1 is dependent on the presence of an intact HIT motif (*7*). We examined the adenosine 5′-monophosphoramidase activity of chicken FT-PKCI-Z and FT-PKCI-W, both of which were pre-treated with







2 M guanidine-HCl and dialyzed against the buffer without guanidine-HCl, by analyzing the formation of AMP from $AMPNH_2$ using HiTrap Q FF anion-exchange column chromatography. MBP-PKCI-Z-His showed a significant $AMPNH_2$ hydrolysis activity (Fig. 4A), whereas MBP-PKCI-W-FLAG exhibited no detectable activity (Fig. 4A), as expected from the absence of the HIT motif in PKCI-W.

Next, the enzymatic activity of heterodimers formed between MBP-PKCI-Z-His and MBP-PKCI-W-FLAG at different molar ratios was examined. The $AMPNH_2$-hydrolyzing activity of MBP-PKCI-Z-His became nearly undetectable when the molar ratio of FT-PKCI-Z: FT-PKCI-W was 1:2 or 1:3 (Fig. 4B). These results suggest that the adenosine 5′-monophosphoramidase activity of FT-PKCI-Z is inhibited in a dominant-negative manner by the formation of a heterodimer with FT-PKCI-W.

Importantly, when a mixture of MBP-PKCI-Z-His and MBP-PKCI-W-FLAG was not treated with 2 M guanidine-HCl, but co-dialyzed against the buffer without guanidine, the adenosine 5′-monophosphoramidase activity of MBP-PKCI-Z-His was not at all inhibited (Fig. 4A), implying that the dissociation of homodimers and reassociation to heterodimers may be the basis of the observed dominant-negative-type inhibition.

DISCUSSION

In contrast to the mammalian *PKCI*/*Hint* genes, which are linked to autosomes (*4*, *12*), a unique feature of chicken *PKCI*/*Hint* genes is their linkage to the sex chromosomes: *PKCI-Z* on the Z chromosome and *ASW*/*PKCI-W* on the W chromosome. The chicken *PKCI-Z* gene has been regarded as the ortholog of mammalian *PKCI*/*Hint* genes, because its deduced amino acid sequence is about 90% identical to that of mammalian PKCI/Hint (*2*). The present results showing that the FT-protein-containing PKCI-Z has a distinct affinity for adenosine and a strong adenosine 5′-monophosphoramidase activity confirm this notion. Therefore, in male chickens having ZZ sex chromosomes, two doses of the *PKCI-Z* gene are present, a situation similar to that in male and female mammals having the autosome-linked *PKCI*/*Hint* genes. It is likely that both *PKCI-Z* genes on the two Z chromosomes in male chickens are transcribed, because both alleles of the five Z-linked genes examined in male chickens were demonstrated to be transcribed, implying that the mechanism for Z chromosome inactivation resulting in the virtual silencing of genes on one of the Z chromosome is absent in male chickens (*13*).

Chicken PKCI-W is a unique member of the Hint-branch proteins of the HIT family, because it contains neither the HIT motif [H(110)-V-H-L-H(114)-V-L in rabbit Hint] nor His-51 (*2*). It has been shown that the HIT motif forms a part of the phosphate binding loop (*10*) and that His-51 cooperates with the two His residues in the HIT motif in the binding of zinc (*8*). As expected from these features of PKCI-W, FT-proteins containing PKCI-W show neither affinity toward adenosine nor adenosine 5′-monophosphoramidase activity.

The level of mRNA expression of both *PKCI-Z* and *PKCI-W* genes is particularly high in female chicken embryos before Hamburger-Hamilton stage 30 (*14*), that is, before the onset of morphological differentiation of the gonads. These mRNAs are detected in several embryonic tissues, including both the left and right genital ridges. The relative level of *PKCI-W* mRNA per whole chicken embryo at stage 29 is about 10 times higher than that of *PKCI-Z* mRNA (*2*). It is conceivable that the higher level of *PKCI-W* mRNA is caused by the approximately 40-fold tandem reiteration of the *PKCI-W* genes in contrast to the single-copy nature of the *PKCI-Z* gene. The reiteration of the *PKCI-W* gene is observed in various species of Carinatae birds (*2*). The higher level of *PKCI-W* mRNA may lead to a higher concentration of the PKCI-W protein than of the PKCI-Z protein in early female embryos, which may be favorable for the formation of heterodimers between PKCI-W and PKCI-Z.

The present *in vitro* study demonstrates that the FT-proteins–containing PKCI-Z or PKCI-W form homodimers and a heterodimer, and that the homodimer of the FT-protein–containing PKCI-Z exhibits the biological activities reported for PKCI/Hint. When FT-PKCI-Z and FT-PKCI-W were mixed in a 1:2 molar ratio, the heterodimer formed between them exhibited no detectable affinity for adenosine nor any adenosine 5′-monophosphoramidase activity, indicating the dominant-negative effect of PKCI-W. This dominant-negative effect of PKCI-W may be predicted from structural considerations; *i.e.*, Gln-127 of PKCI-W might pull His-114 at the C-terminus of the HIT motif (HVHLH) of PKCI-Z out of its active conformation (*4, 15, 16*).

The biological functions of PKCI/Hint are not yet fully understood. At the moment, the correlation between the affinity for adenosine or the hydrolysis of $AMPNH_2$ by PKCI/Hint and the sex-determining mechanism in birds does not seem to be readily explainable. However, an interesting aspect of PKCI/Hint function, *i.e.*, the interaction of PKCI/Hint with other protein partners, has been suggested. The results of a study involving a yeast two-hybrid system and co-immunoprecipitation experiments suggested that PKCI-1 could interact with the mammalian transcription factor micropthalmia (*mi*), and that this interaction represses *mi*-mediated transcriptional activation (*17*). Similar experimental approaches have also produced results suggesting that the human PKCI-1

Fig. 1. **Purification and immunological detection of FT (fusion and tagged)-forms of PKCI-Z or PKCI-W expressed in *E. coli*.** A: Overall model of four FT-proteins. Numbers indicate amino acid residues. GST, glutathione S-transferase; MBP, maltose-binding protein; MCS, peptide expressed from the multiple cloning site; FLAG, FLAG-tag; and His, RGS-$(His)_6$-tag. B: Expressed and purified FT-proteins (MBP-PKCI-Z-His, GST-PKCI-Z-FLAG, MBP-PKCI-W-His and MBP-PKCI-W-FLAG) subjected to SDS–polyacrylamide gel electrophoresis (15% acrylamide) and silver staining. Lane M, protein and peptide molecular weight marker LMW (Amersham Biosciences); lane C, soluble supernatant of the crude extract from *E. coli* expressing FT-proteins; lane A, after purification on an amylose resin column; lane T, after a TALON resin column; lane G, after a GST affinity column GSTrap FF; lane D, after an anion-exchange column HiTrap DEAE FF; lane S, after a gel-filtration column Superose 12 10/300 GL. The purification methods are described under "EXPERIMENTAL PROCEDURES." Arrows indicate proteins identified by gel band excision and nano LC-ESI-MS/MS. C, Western blotting, lane 1, MBP-PKCI-Z-His; lane 2, GST-PKCI-Z-FLAG; lane 3, MBP-PKCI-W-His; lane 4, MBP-PKCI-W-FLAG. Each protein was specifically detected with antibodies or antiserum as indicated.

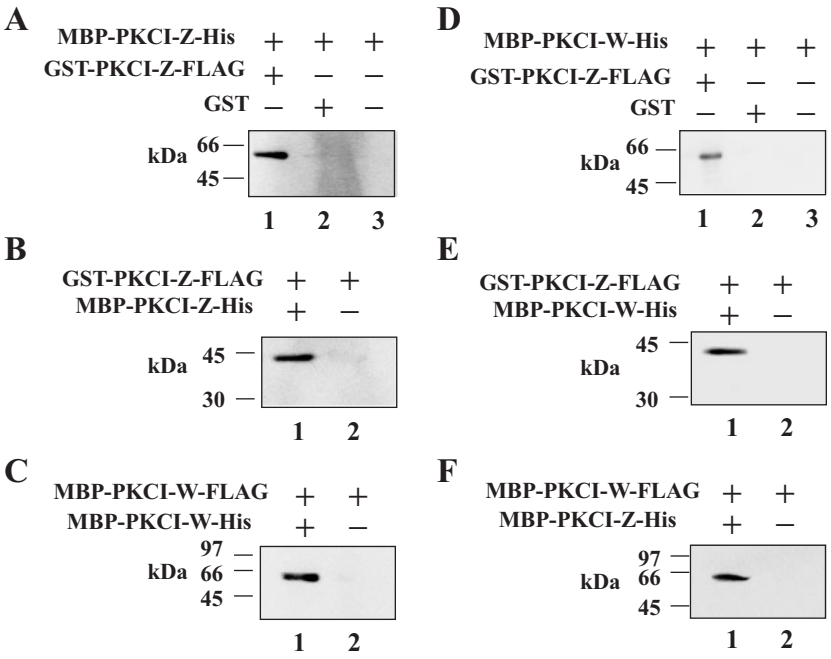


Fig. 2. **Results of pulldown assay support the interaction between FT-PKCI-Z and FT-PKCI-W.** Before the pulldown assay, the proteins were mixed and adjusted in TBS buffer containing 2 M guanidine-HCl, and then dialyzed against TBS. Panels A and D, show the results of GST-pulldown assay and detection by Western blotting with anti-His antibody; B, C, E, and F show the results of His-tag pulldown assay and detection with anti-FLAG antibody.

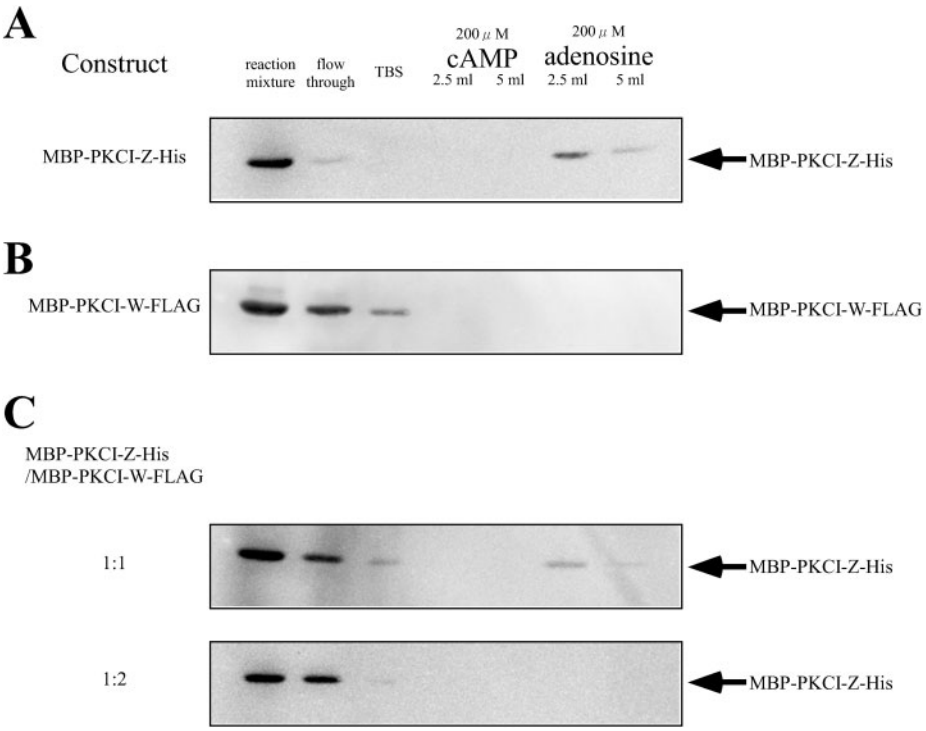


Fig. 3. **Affinity of FT-PKCI-Z but not FT-PKCI-W for adenosine, and inhibition of the former affinity by the formation of a heterodimer.** MBP-PKCI-Z-His (A), MBP-PKCI-W-FLAG (B) or heterodimers of MBP-PKCI-Z-His and MBP-PKCI-W-FLAG mixed in a 1:1 or 1:2 molar ratio (C). Each sample was applied to a 1 ml affinity column, and the flow through fraction was collected. The column was washed with 10 ml of TBS and 5 ml of TBS containing 200 μM cAMP, and the fractions were collected. The bound material was eluted with 5 ml of TBS containing 200 μM adenosine, and fractions were collected. All fractions were subjected to SDS-PAGE and Western blotting.

may interact with cyclin-dependent kinase 7 (Cdk7) and, similarly, that yeast Hnt1 may interact with Kin28, an ortholog of Cdk7 (*18*). Later, it was shown in yeast that the loss of Hnt1 enzyme activity causes hypersensitivity to mutations at *Kin28*, *Ccl1* and *Tfb3*, each of which encodes a protein constituting the TFIIK kinase subcomplex of the general transcription factor TFIIH, and also to mutations of *Cak1* encoding a protein kinase that phosphorylates Kin28 (*7*). These results imply that if a novel protein partner of PKCI-Z is found in early chicken embryos and if their association is blocked by heterodimer formation between PKCI-Z and PKCI-W in female embryos, a clue to link the PKCI-Z/PKCI-W system with the sex determination pathway may be obtained.

In summary, we have demonstrated for the first time the possible functions of chicken PKCI-W and PKCI-Z by using these recombinant proteins. In this *in vitro* model system, we treated the FT-proteins with guanidine-HCl to prepare

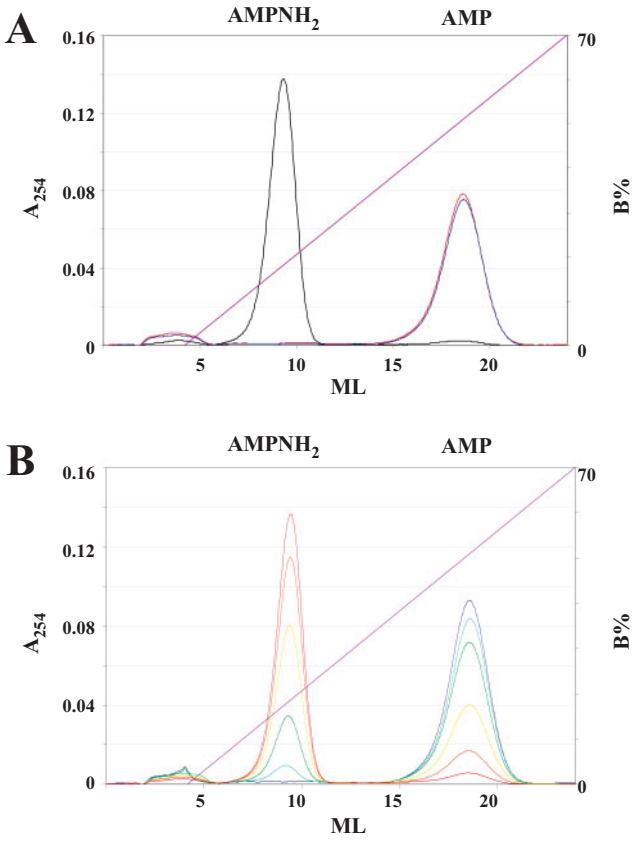


Fig. 4. **Adenosine 5′-monophosphoramidase activity of FT-PKCI-Z and its inhibition by the formation of a heterodimer with FT-PKCI-W.** Adenosine 5′-monophosphoramidate ($AMPNH_2$) and adenosine 5′-monophosphate (AMP) were separated by a HiTrap Q FF with a linear gradient of 0–0.25 M NH_4HCO_3, pH 8 (pink line). A: MBP-PKCI-Z-His (blue), MBP-PKCI-W-FLAG (black) or a mixture of MBP-PKCI-Z-His and MBP-PKCI-W-FLAG in a 1:3 molar ratio (red). These materials were not treated with guanidine but dialyzed against TBS buffer. B: MBP-PKCI-Z-His alone (blue) or mixed it with MBP-PKCI-W-FLAG in a 1:0.3 (light blue), 1:0.5 (green), 1:1 (yellow), 1:2 (orange) or 1:3 (red) molar ratio. These samples were treated with guanidine and dialyzed against TBS buffer.

functional FT-protein monomers to form heterodimers. Co-expression of *PKCI-W* mRNA and *PKCI-Z* mRNA was detected in the undifferentiated gonads of female embryos (*2*), and the level of expression of the *PKCI-W* gene was much higher than that of the *PKCI-Z* gene. Taken together, PKCI-W may inhibit the function of PKCI-Z by forming heterodimers in cells during the differentiation of chicken female embryo. Although further studies are now underway to elucidate the function of both the PKCI-W and PKCI-Z proteins in embryogenesis *in vivo*, the present biochemical data obtained by studying FT-proteins *in vitro* support a model for the possible function of the PKCI-Z/PKCI-W system in sex determination in birds (*2*).

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