Molecular and Cellular Biochemistry **227:** 99–105, 2001.
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A gene located at 72A in *Drosophila melanogaster* encodes a novel zinc-finger protein that interacts with protein kinase CK2

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Abstract

Drosophila melanogaster protein kinase CK2 (DmCK2) is a Ser/Thr protein kinase composed of catalytic α and regulatory β subunits associated as an $\alpha_2\beta_2$ heterotetramer. Using the two hybrid system, we have screened a *Drosophila* embryo cDNA library in order to identify proteins that interact with DmCK2α. One of these cDNAs encodes a novel previously undescribed zinc-finger protein, which we call ZFP47. ZFP47 interacts with DmCK2α but not with DmCK2β, indicating that this interaction is specific for the catalytic subunit of CK2. *In situ* hybridization to polytene chromosomes indicates that the corresponding gene is located at the 72A interval of chromosome III. Sequence analysis indicates that ZFP47 contains a consensus site for phosphorylation by CK2, 4 C_2H_2-type zinc-fingers, and a bipartite nuclear localization signal. Consistent with the prediction of a site for phosphorylation by CK2, we demonstrate that ZFP47 is phosphorylated by CK2 purified from *Drosophila* embryos. These studies demonstrate that ZFP47 is a new physiological partner and substrate of CK2. (Mol Cell Biochem **227**: 99–105, 2001)

Key words: protein kinase, CK2, *Drosophila*, two hybrid

Introduction

Protein kinase CK2 is a highly conserved enzyme that is ubiquitous among eukaryotes. CK2 is composed of catalytic (α) and regulatory (β) subunits that combine to form an $\alpha_2\beta_2$ holoenzyme [1]. CK2 from most organisms is composed of two distinct α subunit isoforms. The only exceptions identified to date are *D. melanogaster* [2], *Caenorhabditis elegans* [3], and *Schizosaccharomyces pombe* [4], which contain a single α subunit. On the other hand, β subunit heterogeneity has so far been documented in *Saccharomyces cerevisiae* [5], *D. melanogaster* [6], and *Arabidopsis thaliana* [7].

Studies utilizing synthetic peptides suggests that the sequence S/T-x-x-D/E defines a minimal consensus for phosphorylation by CK2, and that acidic residues at the n+1 and n+2 positions relative to the phosphoacceptor dramatically stimulate phosphorylation [8, 9]. In addition, CK2 has also been reported to phosphorylate yeast Fpr3 at a Tyr residue [10], although it has been suggested that structural features, rather than the sequence *per se*, favor this non-canonical phosphorylation [11]. Consistent with the preference of CK2 for hyperacidic domains, a number of proteins critical for transcription, cell-cycle progression, and signal transduction contain such sites, and are known to be phosphorylated by the enzyme *in vitro* and *in vivo* (reviewed in [1]. Comparisons between the monomeric α subunit and the $\alpha_2\beta_2$ holoenzyme demonstrate that the β subunit increases the V_{max} approximately 5–50-fold with marginal effects on the k_m for the protein substrate [12–14]. These results suggest that substrate specificity is likely to be integral to the α subunit. CK2 is stimulated by polybasic compounds such as polylysine and protamine [15, 16], and inhibited by polyacidic compounds such as polyAsp/Glu [17], but the *in vivo* relevance of these observations remains unknown.

Genetic analyses in yeast have demonstrated that CK2 activity is essential for cell cycle progression [18], cytokinesis

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[19], and cell polarity [20]. In addition, overexpression of CK2 elicits leukemias and lymphomas in transgenic mice [21, 22] suggesting that the enzyme is a potential oncoprotein. In contrast to these model systems, analysis of *D. melanogaster* CK2 (DmCK2) has lagged despite the fact that the cDNAs encoding DmCK2 were the first to be isolated [2], and the enzyme from this organism has been subjected to extensive biochemical [23–26] and molecular [6, 27] analysis. This deficiency is due to the lack of mutations in the genes encoding DmCK2 subunits. However, an alternative strategy that has been successful applied, is the identification of CK2-interacting proteins via the two hybrid approach. Because many of these proteins appear to be substrates, misexpression of transgenes encoding non-phosphorylated and/or constitutively phosphorylated variants via the Gal4-system [28], followed by phenotypic analysis have begun to clarify functions of DmCK2. Studies along these routes have demonstrated that the Homeobox protein ANTP is negatively regulated by CK2 during embryogenesis [29], and that transcriptional repressors derived from the *Enhancer of Split Complex* such as m5, m7, and m8, which regulate neurogenesis, and the segment polarity protein, Dishevelled, are targets of this enzyme [30, 31]. These results suggest that CK2 is likely to be a critical regulator of embryogenesis as well as cell-fate determination.

We describe here the isolation of a cDNA that encodes a novel zinc-finger protein, which we call ZFP47. Sequence analysis suggests that ZFP47 has a predicted mass of 47 kDa, and contains 4 C_2H_2 zinc-fingers, a bipartite nuclear localization signal, and a consensus site for phosphorylation by CK2. Consistent with the latter prediction, we demonstrate that ZFP47 is phosphorylated by the holoenzyme purified from *Drosophila* embryos. These studies suggest that ZFP47 is a new physiological partner and substrate of DmCK2.

Materials and methods

Construction of two hybrid plasmids and Gal4-based yeast two hybrid screening

The construction of plasmids expressing DmCK2α and DmCK2β as C-terminal fusions with the DNA-binding (DB), and activation-domain (AD) of *S. cerevisiae* Gal4 has been described previously [6]. Two hybrid plasmids suitable for expressing the α, α′, β, and β′ subunits of yeast CK2 were made by PCR amplification of the respective open reading frames, and subcloning in frame with Gal4AD contained in the vector, pGAD424 [32]. The PCR amplified open reading frames encoding all 4 subunits of yeast CK2 were completely sequenced on an Applied Biosystems, Inc. model 373A DNA sequencer using the Prism Dye Terminator Cycle sequencing kit (Applied Biosystems) using custom primers to ensure that no errors were introduced.

Yeast strain HF7C [33] expressing Gal4DB-DmCK2α (the bait) was used to screen a 3–18-h *D. melanogaster* embryo two hybrid cDNA library. A total of 2×10^6 transformants were plated on glucose dropout medium lacking tryptophan, leucine, and histidine [34], and colonies exhibiting rapid growth were counterscreened for expression of *LacZ* [35]. The library plasmids were recovered from His+/LacZ+ clones, and used to retransform HF7C expressing Gal4DB-alone, GAL4DB-Dmα and GAL4DB-Dmβ. Those cDNAs which induced expression of *HIS3* and *LacZ* only in response to GAL4DB-Dmα were identified by sequencing. One of these cDNAs, DmA35, encodes a novel zinc-finger protein, which we call ZFP47. This cDNA and its encoded protein are the subject of this study.

In situ *hybridization to polytene chromosomes*

In situ hybridization to polytene chromosomes was carried out as described [36]. Briefly, the DmA35 cDNA was labeled by nick translation with biotin-dUTP (BioRad Laboratories) and hybridized at high stringency to salivary gland squashes prepared from third instar larvae of *D. melanogaster*. Bound probe was visualized with a streptavidin-alkaline phosphatase conjugate (BioRad Laboratories). Slides were examined by phase-contrast microscopy and photographed at 400 ×.

Phosphorylation of ZFP47 by CK2

ZFP47 was expressed as a fusion protein with *Schistosoma japonicum* glutathione-S-transferase (GST), and purified as previously described [30]. Following purification, the fusion protein was exchanged into storage buffer (50 mM Tris, pH 8.0, 0.5 mM EDTA, 10% glycerol, 200 mM NaCl, 1 mM PMSF) using a Biomax-10K centrifugal filter device (Millipore). Phosphorylations of GST-ZFP47 fusion proteins were carried out in 50 mM Tris, pH 8.5, 100 mM NaCl, 10 mM $MgCl_2$, 10 μM ATP, 5 μCi [γ-^{32}P]ATP in a total vol. of 40 μl, at 25°C. The reactions were initiated by the addition of 5 μl of the enzyme at a concentration of 4 μg/ml. For studying the effects of poly(DL)lysine on phosphorylation, reactions were supplemented at a final concentration of 100 μg/ml. The reactions were terminated by adding 10 μl of 5 × sample buffer [37], boiled for 5 min, and subjected to electrophoresis on 12% acrylamide gels containing sodium dodecylsulfate. Gels were stained with Coomassie Blue, and exposed to Kodak XAR-5 film at room temperature.

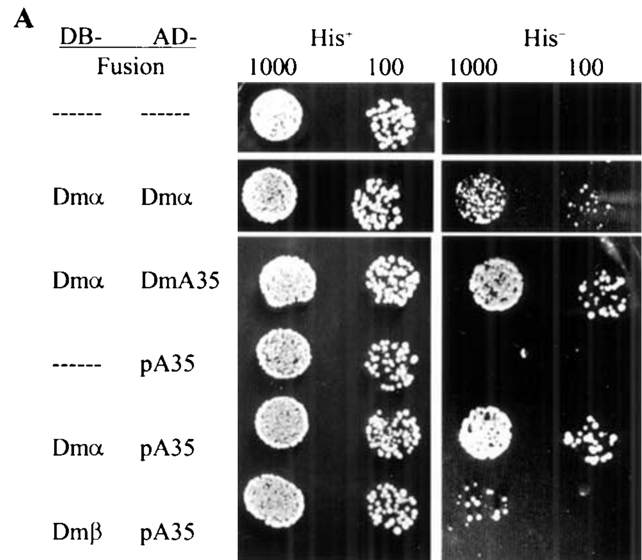
Results and discussion

Isolation of cDNAs encoding ZFP35

The yeast strain HF7C expressing Gal4DB-DmCK2α as a bait was used to screen a *D. melanogaster* embryonic two hybrid cDNA library. From approximately 2 × 10^6 transformants, 15 clones that activated transcription of *HIS3* and *LacZ* were recovered. All 15 clones induced the two reporter genes only when co-transformed with DmCK2α (data not shown). Sequencing of the cDNAs revealed that seven of the clones encode DmCK2β [27], one encodes DmCK2α [6], two encode DmCK2β′, a novel isoform of the β subunit [6], three encode the neurogenic transcriptional repressor, E(spl)m7 [30], one (DmA24) encodes a potential transcription factor, and one (DmA35) encodes a novel zinc-finger protein (this report).

The library plasmid was recovered from the yeast clone DmA35 (henceforth referred to as pA35), and retested for its ability to induce transcription of the reporter genes, *HIS3* and *LacZ*. As expected, no observable induction of the *HIS3* or *LacZ* genes was observed when HF7C expressed pA35 by itself (Figs 1A and 1B). On the other hand, a robust transcription of *HIS3* and *LacZ* was observed when cells coexpressed pA35 and DmCK2α, suggesting that reporter gene induction was dependent on the presence of a Gal4DB-fusion protein. Surprisingly, coexpression of pA35 and DmCK2β weakly elicited transcription of the *HIS3* gene (based on colony size on minimal medium lacking histidine, see Fig. 1A), whereas that of *LacZ* was undetectable using either ONPG or Xgal as a substrate (Fig. 1B). At face value, the former result would imply that the protein encoded by the pA35 cDNA directly interacts, albeit weakly, with DmCK2β. We, however, consider this possibility unlikely for a number of reasons. Explicit testing of the interactions between the subunits of DmCK2 via the two hybrid system indicates that the heterotypic interaction, i.e. α vs. β, is very robust [6]. On the other hand, the α-α homotypic interaction appears to elicit *HIS3* gene expression (see Fig. 1A), but a counterscreen for *LacZ* activity is negative with ONPG, and exhibits a weak blue color with Xgal as a substrate (Se Fig. 1B). This apparent discrepancy in the transcriptional readout of the two reporter genes is attributable to a lower threshold requirement of the *HIS3* gene product for growth of yeast in the absence of histidine, combined with our observation that Xgal is at least 10-fold more sensitive than ONPG for detection of *LacZ* activity (Trott and Bidwai, unpublished). Furthermore, the histidine-independent growth of an α-α strain is eliminated by 1 mM 3-aminotriazole, an inhibitor of the *HIS3* gene product, whereas growth of the α-β combinations is unaffected at concentrations exceeding 20 mM (data not shown). Consistent with this, the His-independent growth of a strain expressing ZFP47 +

A



B

DB-	AD-	*LacZ* Activity	
Fusion		ONPG	Xgal
------	------	0	White
Dmα	Dmα	0	Light Blue
Dmα	DmA35	2.3±0.1	Blue
------	pA35	0	White
Dmα	pA35	2.2±0.3	Blue
Dmβ	pA35	0	White

C

Interaction of *Drosophila* and yeast CK2 subunits

	Scα	Scα'	Scβ	Scβ'
Dmα	N.D.	N.D.	+ +	+++++
Dmβ	++	+++++	N.D.	N.D.

Fig. 1. Isolation of ZFP47 and interaction with DmCK2 subunits. (A) *S. cerevisiae* strain HF7C was transformed with plasmids expressing the indicated fusions with GAL4DB or GAL4AD. Untransformed HF7C was used as a control; a dashed line indicates the absence of a plasmid. Following growth in dropout medium, aliquots corresponding to 1000 and 100 cells were plated onto complete (His^+) or minimal media lacking histidine (His^-) and incubated at 29°C for 3–4 days. (B) Transformants expressing the indicated fusion proteins were grown in glucose medium, and *LacZ* activity was determined in triplicate as described [35], and is expressed in Miller units. In addition, a qualitative assessment of *LacZ* activity was determined via a filter-based assay using Xgal as a substrate. (C) Yeast strain HF7C was transformed with plasmids expressing the *Drosophila* CK2 subunits as fusions with GAL4DB, and yeast CK2 subunits as fusions with GAL4AD. Transformants were selected, and tested for induction of *HIS3* expression by plating on minimal medium lacking histidine, whereas induction of *LacZ* was tested qualitatively using Xgal, and quantitatively using ONPG as substrates. The interaction data is represented qualitatively, and N.D. indicates not determined.

Dmβ is more sensitive to this inhibitor than one expressing ZFP47 + Dmα (data not shown). In addition, we have observed that Dmβ interacts modestly with the yeast α subunit, but very strongly with the α′ subunit in the two hybrid system, whereas Dmα exhibits a modest interaction with the yeast β subunit, and a strong interaction with yeast β′ (Fig. 1C). The former results are in line with genetic analysis demonstrating that overexpression of Dmβ suppresses the phenotype associated with loss of yeast β/β′ [38]. Taking into account these observations, it is, therefore, conceivable that the apparent weak interaction of pA35 and Dmβ may be bridged by the yeast α subunits. The only rigorous way to exclude this possibility would be to carry out the analysis in a strain lacking α and α′, but such a strain cannot be constructed because CK2 activity is essential for viability [39]. In any event, the analysis demonstrates that pA35 exhibits a robust interaction with DmCK2α and, perhaps, a weak interaction with DmCK2β, although the precise mechanism may be confounded by the interplay of the α/β subunits of yeast CK2.

Nucleotide sequence of the pA35 cDNA, and cytological location and genomic organization of the corresponding gene

The cDNA contained in the plasmid pA35 was completely sequenced as described in Materials and methods. This cDNA (deposited as GenBank Accession No. U44206) is 1251 bp long, contains a single open reading frame of 1230 bp, and based upon the absence of a polyA-tail is lacking its 3' untranslated region (Fig. 2). The putative initiation codon is located within the context CAAA**ATG**, which is an excellent match to the start site consensus for *D. melanogaster*, and satisfies the requirement for an adenosine residue at the n-3 position relative to the initiation codon [40]. Conceptual translation suggests that this cDNA encodes a 410 amino acid polypeptide (which we call ZFP47), with a calculated molecular mass of 47 kDa and a theoretical pI of 8.2.

The ZFP47 cDNA was subsequently used to localize the corresponding gene via *in situ* hybridization to polytene chro-

A: Nucleotide sequence of ZFP47.

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ATTTACCCAAAATGACAGAAGCGGAAATTTGCAAACTATGCAAGGATAACCTAGCAATTGAGATCGATCTCGAGAAGCCCAAATTCAAAT
           M  T  E  A  E  I  C  K  L  C  K  D  N  L  A  I  E  I  D  L  E  K  P  K  F  K
CGGCCAATAAACTGCTGCAAAGACTTTTCGATTGGTACAAAATTGATGCTGCTATCCTGGGGGAAAATTACTGCATTTGCGAGCCCTGCT
S  A  N  K  L  L  Q  R  L  F  D  W  Y  K  I  D  A  A  I  L  G  E  N  Y  C  I  C  E  P  C
TTGGAGAGACTTTGCGCATAAGCGAATCCCTGGAGAAGTGGGGCACGGCCCAGGACGAGTTTCACCGGAGGCCCATCGAAATAGACGACT
F  G  E  T  L  R  I  S  E  S  L  E  K  W  G  T  A  Q  D  E  F  H  R  R  P  I  E  I  D  D
CCACTGCATTTCAGATCAAGTTGGAGGTGACATCAAACAAAGAAGAGGTAGAAACGGAAAAGAATGATGAGGAGAATTCTTTCGATCAGC
S  T  A  F  Q  I  K  L  E  V  T  S  N  K  E  E  V  E  T  E  K  N  D  E  E  N  S  F  D  Q
ATATCGCGGAGCCAGTGAAGGAAGATCCATTGGAAAAAAACGAGCATATGGAGATCGTAGTCGAAGAATCTTTGAAAACCGCGTCCGTGG
H  I  A  E  P  V  K  E  D  P  L  E  K  N  E  H  M  E  I  V  V  E  E  S  L  K  T  A  S  V
ATTTCTTCAGCGTGGGATTGTCACGCGATGGCTTGGCAGTCACCAAGTTCTTCAAGGACAAGACCCAAGCCACTCGGATGCTGTGCACCT
D  F  F  S  V  G  L  S  R  D  G  L  A  V  T  K  F  F  K  D  K  T  Q  A  T  R  M  L  C  T
GTTGCGGCCATGTGCTCGAAATCCTGACCCACATACGCATGCATTCTTGTAAACCCCGCCCGAATCCGGTCTACTATTGCCGCGATTGTG
C  C  G  H  V  L  E  I  L  T  H  I  R  M  H  S  C  K  P  R  P  N  P  V  Y  Y  C  R  D  C
GTTCAGAGTTCCACAAACTACACGAACTACATGAGCACATGAAGACGATGAAACACAACAAGACCATGTGCGGCGCAAAGGATTTGGAAT
G  S  E  F  H  K  L  H  E  L  H  E  H  M  K  T  M  K  H  N  K  T  M  C  G  A  K  D  L  E
TTCTGTGCCTCAGATGCAACCAGATATTCCCACGCTATTTCGACGTTATCCGCCACGAGAACAGCGTTCACAATGTATCCGATTTCATCT
F  L  C  L  R  C  N  Q  I  F  P  R  Y  F  D  V  I  R  H  E  N  S  V  H  N  V  S  D  F  I
GCGTGGAGTGCAACATGTCCTTCGCTAACCAAGGATCCTACAGGAGGCACACGAGGAGCCATAAAGTCAAATTCACGCAGCAGCGATTTT
C  V  E  C  N  M  S  F  A  N  Q  G  S  Y  R  R  H  T  R  S  H  K  V  K  F  T  Q  Q  R  F
ATGAGTGCCCATATAAGGACTGCACCTCAAGTTATCGAGTGTGGGCGCGCTTAATGTCGCACATGAGTTCCCACGGCCTCTTTTCTGGAT
Y  E  C  P  Y  K  D  C  T  S  S  Y  R  V  W  A  R  L  M  S  H  M  S  S  H  G  L  F  S  G
TCAAAAGCAAAGTGCCGTCTACCCAACAGGAACGACGTCTTCTGGAGGGCAAGTTTATGACGCTGGACCCCATGAAACGGGTTGAACCCG
F  K  S  K  V  P  S  T  Q  Q  E  R  R  L  L  E  G  K  F  M  T  L  D  P  M  K  R  V  E  P
ATCGCCGAAAGTTGCCATACCGCAAGAAAAGCTTTTACGCTAAGGCAGATTTCCCGGAATCCCCGACGAAATGTACCAACGGAGGAGAGT
D  R  R  K  L  P  Y  R  K  K  S  F  Y  A  K  A  D  F  P  E  S  P  T  K  C  T  N  G  G  E
TCATCAGCCTGCCCAAGAAAATTTCCCTGCAGAAGGGAAAATACATCAATCTTTCGGACAATTCAAGACCTTAGGGTAAAT
F  I  S  L  P  K  K  I  S  L  Q  K  G  K  Y  I  N  L  S  D  N  S  R  P  *  410
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B: Intron Exon Structure of the ZFP47 Gene.

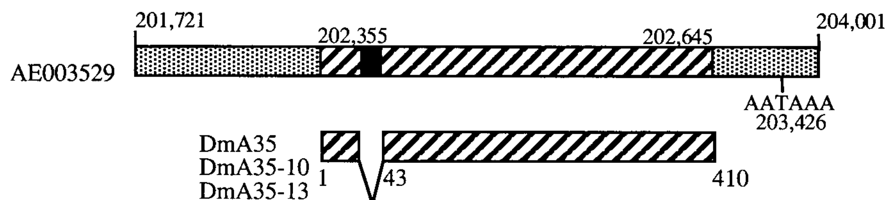

Fig. 2. Nucleotide sequence of DmA35 cDNA, and intron/exon structure of the corresponding gene. (A) The nucleotide sequence of the 1251 bp cDNA (deposited as GenBank Accession # U44206) is shown above the deduced amino acid sequence of ZFP47 (shown in one letter code). The potential Kozak consensus sequence 5' to the initiation codon, ATG, is underlined, and the dotted region at the 3' end of the cDNA indicates the absence of the polyA-addition signal and the polyA-tail. (B) The start and end points of the cDNA sequence are shown as a crosshached box within the context of bp 202,021–203,520 of a genomic clone (GenBank Accession # AE003529, shaded box). The open reading frame encoding ZFP47 is encoded on two exons (crosshatched boxes), separated by an intron (black box), and numbers indicate the amino acids of the protein encoded on the respective exons. The location of the the most likely polyA addition signal AATAAA in the genomic sequence is indicated.

mosomes of third instar larvae. The analysis suggests that the ZFP47 gene is located on the left arm of chromosome III at the 72A polytene interval (data not shown). This result is in full agreement with data from the *Drosophila* genome sequencing project demonstrating identical cytology, and indicating that the ZFP47 gene is single copy. With the recent completion of the *Drosophila* genome sequence, we have used the ZFP47 cDNA sequence to identify the corresponding genomic sequence and its organization. To this end, we have identified a 250 kb genomic clone (Gen Bank Accession # AE003529) that harbors sequences corresponding to the ZFP47 gene from nucleotide positions 202,355–202,645 (see Fig. 2B). No additional genomic clones corresponding to ZFP47 were identified in this analysis, suggesting that this gene is, in fact, single copy in the *Drosophila* genome, in agreement with our cytological analysis (see above). A comparison of this region of the genomic clone to the isolated cDNAs suggests that the ZFP47 protein is encoded on two exons (Fig. 2B). The first exon encompassing amino acids 1–42, and the second exon encompassing amino acids 43–410 are separated by a single 57 bp intron which satisfies the requirement for GT at the donor splice site, and an AG at the acceptor site (data not shown). In addition, the genomic sequence contains a single polyA-addition signal, AATAAA, that is 781bp 3' to the end of the coding region. In this regard, neither the two hybrid clones (pA35), nor two additional clones isolated from a commercially available *Drosophila* embryonic library (DmA35-10 and DmA35-13) contain a polyA-tail. It is, therefore, difficult to predict whether the polyA-addition signal in the corresponding genomic sequence is the preferred site *in vivo*. In addition, the 3' region of the genomic sequence does not exhibit the presence of polyA-like sequences, thus precluding the possibility that the ZFP47 polypeptide is encoded by a processed pseudogene that arose via retrotransposition during *Drosophila* evolution.

Motifs in ZFP47 and phosphorylation by CK2

The deduced amino acid sequence of ZFP47 was used to conduct a BlastP analysis against all available sequences (GenBank release of March 2001). The closest similarity of ZFP47 observed by this analysis was to the 4 zinc-finger domains of a human [41] and murine [42] kruppel-like protein, PLZF, that has been proposed to encode the promyelocytic leukemia associated zinc-finger protein (Gen Bank Accession # AAD03619 and AAB33814, respectively). This conservation is, however, limited to the putative zinc-finger-domains, both with regards to the zinc-ligands and the highly conserved aromatic residue, Phe/Tyr, between ligand 1 and

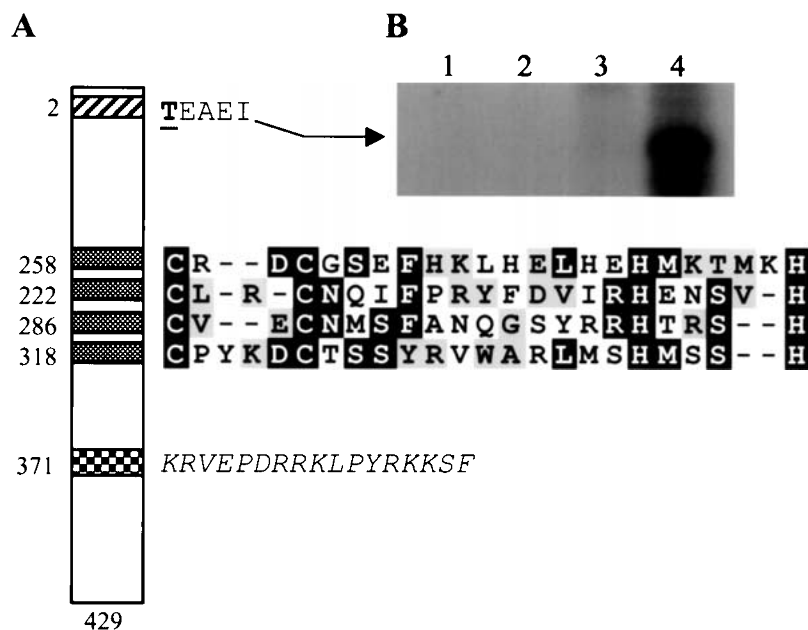

Fig. 3. Functional motifs in ZFP47 and phosphorylation by CK2. (A) The amino acid sequence of ZFP47 was analyzed for the presence of functional motifs using algorithms available via the world wide web. The motifs contained in ZFP47 are an N-terminal consensus site for phosphorylation by CK2 (cross-hatched box), 4 C_2H_2-type zinc-finger domains (shaded boxes), and a bipartite nuclear localization signal (checkerboard box). The numbers next to the boxes correspond to the position of the motifs relative to the predicted N-terminus. (B) GST-ZFP47 fusion protein was purified, and subjected to phosphorylation using monomeric α subunit (lanes 1 and 2), and embryo CK2 (lanes 3 and 4). Samples were phosphorylated in either the absence (lanes 1 and 3), or presence of 100 μg/ml poly(DL)lysine (lanes 2 and 4). Samples were electrophoresed in 12% SDS-polyacrylamide gels, stained with Coomassie blue, and autoradiographed. The arrow indicates the mobility of full-length GST-ZFP47.

2 (data not shown). Because zinc-finger motifs are, in general, highly conserved with regard to the spacing of the putative zinc-ligands, Cys and/or His [43], and that the conservation of the intervening residues is weak, suggests that the ZFP47 gene encodes a novel zinc-finger protein. In the absence of a clear homolog in other organisms that could be used to predict a possible function for ZFP47, we have used the deduced amino acid sequence of this protein to predict the presence of functional and/or structural motifs. This analysis suggests that the encoded polypeptide contains 3 such motifs (Fig. 3A). The first of these is N-terminal sequence, M[1]**T**EAE, which fits the consensus for phosphorylation by CK2 and satisfies the requirement of the enzyme for a phosphoacceptor (Ser/Thr) with acidic residues at the n+1, and n+3 positions [11]. The second motif, present at 4 copies, is predicted to form a C_2H_2-type zinc-finger, and satisfies the requirement for an aromatic residue, in this case Phe/Tyr, between the second (Cys) and third (His) ligands that are predicted to coordinate the zinc atom [43]. The third motif, K[318]RVEPDRRKLPYRKKSF, is highly homologous to bipartite nuclear localization sequences [44].

Analysis of the primary sequence of ZFP47 suggests the presence of a consensus site for phosphorylation by CK2. In order to ascertain whether ZFP47 is, in fact, phosphorylated by CK2, we have bacterially expressed this protein as a GST-fusion, purified it to homogeneity, and subjected it to phosphorylation using two isoforms of DmCK2, i.e. the monomeric α subunit purified from a yeast expression system [24], and the $\alpha_2\beta_2$ holoenzyme purified from *Drosophila* embryos [26]. Although the former isoform is closer to the scenario of the two hybrid interaction, the latter isoform may reflect more accurately the situation *in vivo*. We have not explicitly tested for phosphorylation of unfused GST, as we have previously demonstrated that GST is not a substrate for either isoform of CK2 [30]. The results demonstrate that ZFP47 does not appear to be phosphorylated by Dmα (Fig. 3B, lane 1), or by the holoenzyme purified from embryos (Fig. 3B, lane 3). We, therefore, tested whether phosphorylation of ZFP47 by either isoform was responsive to poly(DL)lysine. The results demonstrate that while phosphorylation by the monomeric α subunit remains unresponsive to this polybasic activator, phosphorylation by the holoenzyme is markedly stimulated in its presence (Fig. 3B, compare lanes 2 and 4). The phosphorylation of GST-ZFP47 in a poly(DL)lysine-dependent manner is not due to a non specific phosphorylation, perhaps, of the GST affinity tag itself, as we have previously demonstrated that neither BSA [12] nor GST [30], are targeted by CK2 upon polybasic activation. This result appears consistent with the proposal that the β subunit of CK2 largely mediates interaction with polybasic activators [12, 45]. Therefore, analogous to our previous result with Calmodulin [12], and the actin bundling protein, Sac6 (Bidwai, unpublished), phosphorylation of ZFP47 appears to be dependent on the presence of the regulatory (β) subunit of DmCK2, and maximal phosphorylation appears to also be dependent on prior activation of the enzyme by poly(DL)lysine. ZFP47 thus represents a substrate whose phosphorylation is regulated by the β subunit in a polylysine-dependent manner. However, its role in modulating physical association of CK2 with ZFP47 is somewhat unclear for reasons outlined above. Given the very weak interaction of ZFP47 with Dmβ, and a relatively robust interaction with Dmα, it is conceivable that the interaction of this protein with CK2 may be greatly enhanced when tested against the holoenzyme, i.e. the protein may contain regions of interaction that involve both subunits. However, such analysis is currently precluded by the unavailability of a yeast strain that produces DmCK2 holoenzyme suitable for two hybrid analysis. That CK2 interacts with, and phosphorylates this protein is also consistent with the observation that this kinase exists in a complex with many of its *in vivo* substrates such as Topoisomerase II [46], HSP90 [47], ANTP [29], m5/7/8 [30], and Dishevelled [31] to name a few. However, unlike the case with ANTP, Topoisomerase II, etc., the consequences of CK2-mediated phosphorylation of ZFP47 on its *in vivo* functions are difficult to predict *a priori*, because no mutations that colocalize at the 72A interval have so far been reported in this organism. However, the recently initiated effort to generate systematic mutations in all of the genes in this model organism, should elaborate on the biological functions of this gene, and will permit a functional analysis of the phosphorylation of this protein by CK2 *in vivo*.

Acknowledgements

We thank Claiborne Glover for providing valuable resources. This work was supported by American Cancer Society Grant (RPG-9918901-DDC) to A.P.B.

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