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Interaction of casein kinase II with ribosomal protein L22 of *Drosophila melanogaster*

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Abstract

The ubiquitous eukaryotic protein kinase CKII (casein kinase II) has been found to interact with a number of cellular proteins, either through the catalytic subunit or the regulatory subunit. Using the yeast two-hybrid screening method, we found that the catalytic subunit of *Drosophila melanogaster* CKII (DmCKII) interacts with *Drosophila* ribosomal protein L22 (rpL22). This interaction was also observed in vitro with a glutathione-*S*-transferase (GST)-rpL22 fusion protein. The predicted full-length *Drosophila* rpL22 protein has an N-terminal extension rich in alanine, lysine, and proline that appears to be unique to *Drosophila*. Deletion mapping revealed that the conserved core of rpL22 is responsible for the interaction with CKII. Moreover, purified DmCKII can phosphorylate a GST-L22 fusion protein at the C-terminal end, suggesting that this protein may be a substrate of CKII in *Drosophila*.

Keywords: Protein kinase; Phosphorylation; Ribosomal protein

Protein kinase CKII (casein kinase II) is a highly conserved, apparently messenger-independent, Ser/Thr protein kinase that is ubiquitous in eukaryotes [1–4]. The enzyme is composed of a catalytic α subunit and a regulatory β subunit that combine to form an $\alpha_2\beta_2$ holoenzyme, the crystal structure of which has recently been determined [5]. CKII is unusual in being acidic-directed, with acidic residues on the C-terminal side of the modified residue being important determinants [6]. The enzyme is constitutively active as isolated but can be inhibited in vitro by polyanions, which compete with the protein substrate, and stimulated by polycations, which exert their effect via the β subunit. CKII has a broad substrate specificity [2] and is involved in multiple cellular functions, including cell cycle progression, cell proliferation, cell growth, metabolism, etc. CKII is essential for viability and required for cell cycle progression in *Saccharomyces cerevisiae* [3] and is capable of functioning as an oncogene in mouse [7].

Several results suggest that CKII is involved in rDNA transcription, ribosome biogenesis, and protein synthesis. Systematic analysis of protein complexes in *S. cerevisiae* places CKII in both nuclear and nucleolar complexes involved in transcription, chromatin structure, and RNA metabolism [8], and CKII copurifies with both mammalian [9] and *Xenopus* [10] RNA polymerase I complexes. Phosphorylation of the rDNA upstream binding factor (UBF) by CKII is necessary for full stimulation of rDNA transcription by UBF in vitro [11]. CKII physically interacts with and phosphorylates nucleolin, a multifunctional protein required for ribosome biogenesis [12], as well as Nopp140, implicated in ribosome assembly and nuclear transport of nucleolar components [13,14]. Several protein synthesis factors have also been shown to be CKII substrates [2,15]. For example, CKII phosphorylates yeast translation initiation factor eIF2α both in vivo and in vitro [16].

Ribosomal protein L22 (rpL22) is a eukaryotic ribosomal-nucleolar protein that was originally identified as Epstein–Barr virus-encoded RNA (EBER)-associated protein (EAP), an endogenous host cell protein that physically associates with EBER1 during latent

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transformation of human B lymphocytes by Epstein–Barr virus (EBV) [17]. rpL22/EAP also physically associates with the herpes simplex 1 (HSV-1) regulatory proteins ICP4 [18] and ICP22 [19] as well as with the equine herpesvirus 1 (EHV-1) immediate-early (IE) protein [20], a homolog of ICP4. Relocation of rpL22/EAP occurs during viral infection in each of these systems, and a regulatory role in viral gene expression, at either the transcriptional or translation level, appears likely in each case as well [17–20]. In uninfected cells, rpL22 is localized in both the cytoplasm and nucleoli [17], consistent with potential roles in ribosome biogenesis, transport, and/or translation, but the physiological role of rpL22 in uninfected cells remains to be defined.

To better understand the biological function of CKII, we have used the yeast two-hybrid system to identify proteins that interact with the *Drosophila melanogaster* CKIIα subunit. A putative *Drosophila* ortholog of rpL22 has been isolated in this screen. *Drosophila* rpL22 has a long N-terminal extension that is not seen in any other known rpL22 proteins. The site of interaction with CKII maps to the conserved core of rpL22. Using an affinity-purified rpL22 fusion protein, we show that rpL22 interacts with homogeneous CKII holoenzyme in vitro. Recombinant rpL22 serves as an efficient substrate of CKII in vitro and the phosphorylation site has been mapped to the C-terminal region of the protein.

Materials and methods

Two-hybrid screening. The *Drosophila melanogaster* CKIIα (DmCKIIα) gene was amplified by PCR using two primers, 5′-GG GGATCCTTATTGCTGATTATTGGGAT-3′ and 5′-GGGAATTC ATGACACTTCCTAGTGCGGC-3′, containing *Bam*HI and *Eco*RI sites, respectively. The PCR was carried out as follows: 92 °C for 45 s, 40 °C for 45 s, and 72 °C for 2 min, for a total of 25 cycles. The PCR product was subcloned into the plasmid pGBT9 (gift of Dr. Stanley Fields, SUNY, Stony Brook) which contains the DNA-binding (DB) domain of the *S. cerevisiae* transcription factor Gal4. The resulting constructs express DmCKIIα as a C-terminal fusion with the DB domain (amino acids 1–147) of Gal4. The insert was completely sequenced on an Applied Biosystems DNA Sequencer 373A to guarantee the correctness of the sequence.

Yeast strain HF7c expressing the GAL4DB-DmCKIIα fusion was used as a host strain to screen a 3–12 h *Drosophila* embryo cDNA library (provided by S.J. Elledge, Baylor College of Medicine, Houston) as recommended by the supplier of the plamids and strain (Clontech). Briefly, the two-hybrid cDNA library (approximately 1×10^7–1×10^8 total recombinants) inserted in the plasmid pACT was transformed into the host strain. pACT utilizes the constitutive *ADH1* promoter for expression of cDNA-encoded protein as a C-terminal fusion with the activation domain of Gal4 (GAL4AD). An estimated 2×10^7 transformants were plated on medium lacking Trp, Leu, and His [21]. Large colonies showing higher growth rate were tested for the expression of β-galactosidase [22]. Plasmids were recovered from His- and lacZ-positive clones by rescue in *Escherichia coli* and retransformed into HF7c and into HF7c expressing GAL4DB-DmCKIIα or GAL4DB-DmCKIIβ to verify the specificity of two-hybrid interaction. Those cDNAs that induced the reporter genes in a bait-specific manner were identified by DNA sequencing. Twelve clones encoded a *Drosophila*

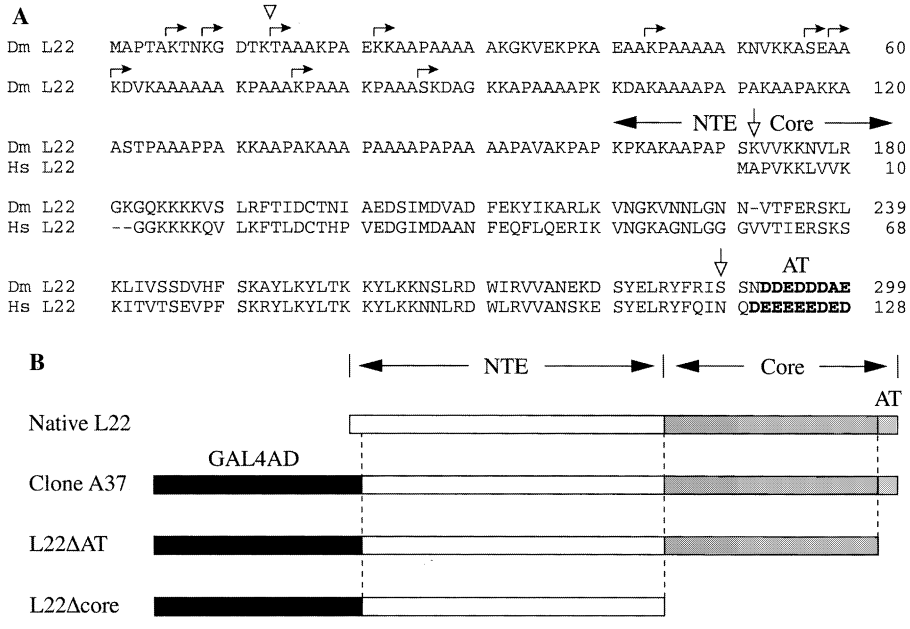

Fig. 1. Sequence and features of rpL22. (A) Sequence alignment of the rpL22 polypeptides from *Drosophila* (Dm L22; GenBank Accession No. P50887) and human (Hs L22; GenBank Accession No. P35268). The dash represents a gap introduced to maintain alignment. The N-terminal extension (NTE) is unique to *Drosophila*; the remainder of the protein (Core) is highly conserved among all eukaryotic species. The acidic tail (AT) of the core region is shown in bold. Open arrows indicate the position of two residues replaced by stop codons in mutants rpL22ΔAT and rpL22Δcore. Right-angle arrows indicate the location the 10 fusion junctions observed in the two-hybrid screen; the junction indicated by the open arrowhead (clone A37) was employed in all other fusion constructs described in this paper. (B) Scale diagram of full-length rpL22 protein and selected derivatives. GAL4AD: activation domain of GAL4 present in the two-hybrid fusion proteins.

homolog of rpL22. Ten of these displayed unique fusion junctions (Fig. 1).

Cloning of full-length cDNA of the putative rpL22 protein. Because all of the clones from the two-hybrid cDNA library lacked the N-terminal methionine, a lambda Uni-ZAP XR library of *Drosophila* cDNAs (Catalog No. 937602, Stratagene) was screened to identify full-length cDNA encoding the putative rpL22. A ^{32}P-labeled probe was generated from the insert of clone A37 (Fig. 1) using the Stratagene Random Hexamer kit. Labeled probe was incubated with nitrocellulose replicas of phage plaques and putative rpL22 clones were identified by autoradiography [21]. After three rounds of screening, purified phage were converted to plasmid using the ExAssist strategy (Stratagene) and the cDNA inserts were sequenced. Two clones (37.3 and 37.4) contained the potential initiating methionine codon. The composite cDNA sequence of *Drosophila* rpL22 has been deposited in GenBank Accession No. U42587.

In vitro mutagenesis. Two C-terminal truncations of rpL22 were made in clone A37 using the Stratagene QuickChange Site-Directed Mutagenesis System. Two pairs of mutagenic primers, 5′-CTTGACCA CCTAGCTGGGAGCTG-3′ and 5′-CAGCTCCCAGCTAGGTGGT CAAG-3′, or 5′-CGTCGTTGGATCAGATTCTGAAG-3′ and 5′-CT TCAGAATCTGATCCAACGACG-3′, were used to generate a stop codon following amino acids S171 and I289, respectively. Subsequently, these two mutants were transformed into an HF7c strain containing the GAL4DB-DmCKIIα plasmid to test the effect of the mutations on the interaction of the truncated peptides with the CKIIα subunit.

GST-fusion protein expression. The putative *Drosophila* rpL22 cDNA and its mutant derivatives were isolated by digestion of clone A37 and its derivatives with *Bgl*II and subcloned into the *Bam*HI site of the *E. coli* expression vector pZEX (gift of Z. Paroush, Hebrew University, Jerusalem). pZEX was modified from pGEX (Pharmacia) to accommodate the multiple cloning site and reading frame of the library-encoded cDNAs, which are expressed under IPTG induction as C-terminal fusions with *Schistosoma japonicum* glutathione-*S*-transferase (GST). The expressed fusion proteins were then purified on glutathione–Sepharose 4B matrix as suggested by the manufacturer (Pharmacia).

In vitro binding of DmCKII holoenzyme to GST-L22. Purified GST or GST-L22 was mixed with 40 μl glutathione–Sepharose 4B matrix (Pharmacia Biotech). After incubation at 4 °C for 30 min, samples were washed four times with 100 μl phosphate-buffered saline (PBS). Approximately 100 μl purified *Drosophila* CKII holoenzyme [23] was mixed with the samples and incubated overnight at 4 °C. After washing four times with 100 μl PBS, 40 μ l glutathione elution buffer (10 mM reduced glutathione in 50 mM Tris–HCl, pH 8.0) was added to each sample and the samples were incubated at room temperature for 15 min. The first wash (unbound CKII) and the glutathione eluate (bound CKII) were analyzed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) as described below. To detect the CKII subunits, a parallel gel was subjected to Western blotting by standard techniques [21] using an antibody to *Drosophila* CKII holoenzyme [23].

Phosphorylation of recombinant rpL22 by CKII. Reactions were carried out in 50 mM Tris–HCl, pH 8.0, 100 mM NaCl, 10 mM $MgCl_2$, 1 μM ATP, and 1 μCi [γ-^{32}P]ATP in a total volume of 30 μl. Affinity-purified GST, GST-L22 fusion, or its mutant derivatives were used as substrates. The reactions were initiated by the addition of 7.5 μl of 4 μg/ml CKII holoenzyme in 20 mM Tris, pH 8.0, 0.5 mM EDTA, 200 mM NaCl, 0.5 mM DTT, 10% glycerol, and 0.05% Triton X-100. The reactions were incubated for 15 min and were terminated by adding an equal volume (30 μl) of 2× SDS sample buffer [24]. Aliquots of each reaction were electrophoresed on 12% polyacrylamide gels containing sodium dodecyl sulfate as described [24]. Gels were stained with Coomassie brilliant blue R-250 (0.05%) and destained thereafter. The destained gels were exposed to Kodak X-OMAT XAR-5 film overnight at room temperature.

Results and discussion

Drosophila CKII two-hybrid screening

A two-hybrid screen for proteins that interact with the α subunit of *Drosophila* CKII identified at least five distinct polypeptides. One of these, the putative *Drosophila* rpL22 protein (Fig. 1), is described here. Except for the long N-terminal extension (NTE), the sequence of this protein shares a high degree of sequence identity with rpL22 from other species (Fig. 1 and data not shown). The rpL22 protein interacted strongly with DmCKIIα in the yeast two-hybrid system (Table 1). The interaction was specific for DmCKIIα since GAL4AD-L22 did not induce reporter gene expression when placed in the cell alone or with other unrelated GAL4DB-fused proteins such as DmCKIIβ.

Because none of the 10 distinct rpL22 clones isolated from the two-hybrid library contained the initiating methionine (Fig. 1), we screened a lambda Uni-ZAP cDNA library with a probe derived from one of the two-hybrid rpL22 clones. Sequencing of positive clones yielded two apparently full-length clones of rpL22, both of which also have the unusual NTE. The isolation of multiple distinct cDNAs from two different libraries confirms that the NTE is not an artifact and confirms that *Drosophila* has a unique form of rpL22 relative to other species. Full-length cDNA encoding Dm rpL22 has also been isolated in a screen for proteins that interact with the automodification domain of *Drosophila* poly(ADP-ribose) polymerase [25].

A search of the completed *Drosophila* genome revealed a second potential rpL22 gene, designated CG9871. Interestingly, the predicted product of this gene contains an NTE similar to that found in rpL22. The two paralogs are located at distinct positions on the polytene map (rpL22 at 1C4 and CG9871 at 59D8-9; FlyBase documentation). A BLAST search of available *Drosophila* ESTs retrieved over 500 ESTs encoding rpL22 versus three encoding CG9871, suggesting that the two genes are expressed at very different levels. Moreover, the rpL22 ESTs were isolated from head or larval libraries, whereas all three CG9871 ESTs were isolated from a testis library. Two recessive lethal mutations have recently been identified in the rpL22 locus, suggesting that it encodes an essential function [26]. Collectively, these data suggest that the gene isolated here represents the principal rpL22 gene in *Drosophila*, whereas CG9871 may encode a testis-specific isoform.

The NTE of Dm rpL22 (and CG9871) is highly enriched in Ala, Lys, and Pro residues. As noted by Koyama et al. [25], this region is similar to the C-terminal region of histone H1, suggesting a possible role in DNA binding. A very similar N-terminal sequence is found on *Drosophila* rpL23a and again this extension is unique to *Drosophila* [25].

Table 1
CKIIα interacts with the putative *Drosophila* rpL22 in the yeast two-hybrid system[a]

	Gal4AD-CKIIα	Gal4AD-CKIIβ	Gal4AD
Gal4DB-CKIIβ	+++	–	–
Gal4DB-L22	++++	–	–
Gal4DB-L22ΔAT	++++	–	–
Gal4DB-L22Δcore	–	–	–

[a] CKIIβ and rpL22 or its mutant variants were fused with the Gal4 DNA-binding domain (Gal4DB) and co-transformed with various Gal4 transcription activation domain (Gal4AD) fusions into yeast strain HF7c. Two-hybrid interactions were tested by both β-galactosidase activity and yeast growth on selective medium. A "+" indicates $lacZ^+$ His^+ and "−" indicates $lacZ^-$ His^-. The number of "+'s" represents the strength of the reporter signals. L22ΔAT and L22Δcore stand for the deletion of the acidic tail and the C-terminal half of the rpL22protein, respectively.

Mapping of the rpL22 region that interacts with CKII

The two-hybrid fusion junctions we observed all map in the first half of the NTE (Fig. 1). The first 85 residues of the protein (the first half of the NTE) are thus dispensable for interaction with CKII. To further localize the region of rpL22 responsible for the interaction with CKII, we constructed a series of C-terminal truncation mutants (Fig. 1). We specifically wished to test the importance of (1) the NTE, (2) the conserved region that is homologous to rpL22 from other species (core), and (3) a highly acidic sequence (acidic tail, AT) located at the extreme C-terminal end of the core. The lysine-rich NTE could potentially interact with the β subunit as a polybasic activator while the acidic region might interact with the active site of the α subunit, either as a substrate (in *Drosophila*; see below) or as a substrate analog/inhibitor (in other species).

Truncations of the acidic C-terminal sequence (rpL22ΔAT) or the entire core of rpL22 (rpL22Δcore) were made by site-directed mutagenesis. Plasmids containing these mutant GAL4AD-L22 derivatives were transformed into HF7c harboring GAL4DB-CKIIα to test the ability of mutant rpL22 to interact with CKIIα. As shown in Table 1, truncation of the acidic C-terminal stretch (acidic tail) of rpL22 did not affect the two-hybrid interaction with CKII. Although the acidic tail of rpL22 in *Drosophila* is part of a potential CKII recognition motif (see below), the results of this experiment indicate that the interaction between CKII and rpL22 is not due to an enzyme-substrate recognition process. In contrast, deletion of the entire core of rpL22 completely eliminated the two-hybrid interaction, indicating that the region between the NTE and the acidic tail is essential for the interaction. Since this region is highly conserved among different species, this result suggests that the interaction between rpL22 and CKII may be conserved among species.

In vitro binding between rpL22 and CKII

To verify the yeast two-hybrid result, the binding of *Drosophila* CKII to rpL22 was investigated in vitro. To this end, a GST-L22 fusion protein was expressed in *E. coli* and cell lysates were applied to a glutathione–Sepharose 4B matrix. After washing to eliminate unbound material, purified *Drosophila* CKII holoenzyme [27] was applied. Unbound CKII was removed by washing and potential rpL22-CKII complexes were then eluted by the addition of reduced glutathione. Eluted proteins were separated by SDS–PAGE and the presence of CKII in the resolved proteins was visualized by Western blotting with a polyclonal anti-CKII antibody. As shown in panel B of Fig. 2, a sizable fraction of the input CKII was bound to a GST-L22 fusion protein (lane 4), whereas only a trace was bound to GST protein alone (lane 2). The strength and specificity of this interaction are underscored by the relatively small amount of rpL22 fusion protein employed in these experiments, relative to GST alone (panel A).

While we cannot entirely rule out the possibility that the CKII–rpL22 interaction is mediated by other molecules (including RNA), the direct binding observed between purified CKII and bacterially expressed and purified GST-L22 makes this less likely.

Phosphorylation of rpL22 by CKII

The cloned *Drosophila* rpL22 has two potential CKII phosphorylation sites, Ser289 and Ser290, which are located immediately upstream of the acidic residues of the AT. To determine if these sites can be phosphorylated by CKII, we performed in vitro kinase assays with homogeneous CKII and affinity-purified GST, GST-L22, or GST-L22ΔAT, the latter lacking the two putative CKII sites. Reaction products were analyzed by SDS–PAGE followed by autoradiography. As shown in Fig. 3, rpL22 protein can be phosphorylated by CKII either in the absence (lane 2) or the presence (data not shown) of the CKII activator protamine. Furthermore, the loss of phosphorylation signal in the rpL22ΔAT mutant (Fig. 3, compare lanes 2 and 3) implies that phosphorylation by CKII occurs at one or both Ser residues located in the C-terminal tail. No incorporation was seen in the absence of added CKII (data not shown).

The ability of CKII to phosphorylate *Drosophila* rpL22 raises the possibility that this modification regu-

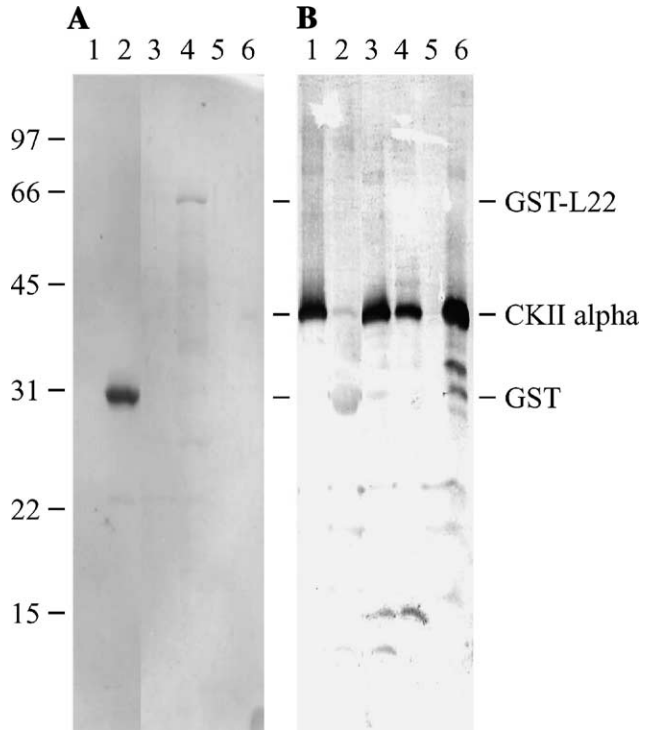

Fig. 2. In vitro binding of purified CKII holoenzyme to recombinant GST-L22. Affinity-purified GST-L22 protein was immobilized on glutathione–Sepharose 4B beads and in vitro binding of purified *Drosophila* CKII holoenzyme was determined as described in Materials and methods. Panel A: Coomassie blue-stained gel; panel B: Western blot of an identical gel with anti-CKII antibody. Lane 1: GST plus CKII (unbound fraction); lane 2: GST plus CKII (bound fraction); lane 3: GST-L22 plus CKII (unbound fraction); lane 4: GST-L22 plus CKII (bound fraction); lane 5: blank; lane 6: purified CKII control. Note that CKIIβ did not strongly react with the antibody in this experiment. The mobility of molecular mass markers (in kDa) is indicated at the left.

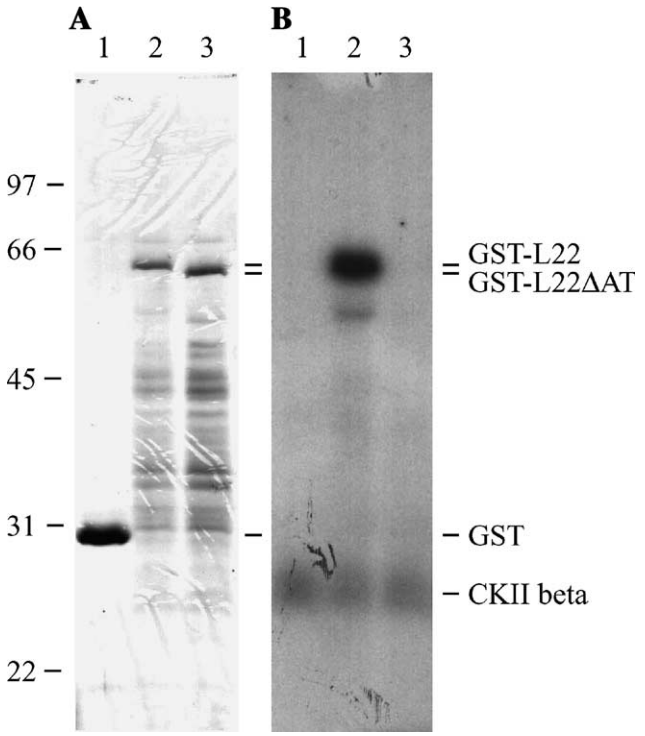

Fig. 3. Mapping of the CKII phosphorylation site in *Drosophila* rpL22. Purified GST, GST-L22, or GST-L22ΔAT was mixed with purified CKII. Phosphorylation reactions were carried out as described in Materials and methods, and the products were analyzed by SDS–PAGE and autoradiography. Panel A: Coomassie blue-stained gel of the reaction products; panel B: autoradiograph of the gel. Lane 1: GST; lane 2: GST-L22; lane 3: GST-L22ΔAT. The mobility of molecular mass markers (in kDa) is indicated at the left.

lates rpL22 function in this species. The C-terminal acidic region of human rpL22 has recently been implicated in intranuclear trafficking of the rpL22 protein from the nucleoplasm to the nucleolus [28]. Specifically, the authors define a competition between residues 1–9 of human rpL22 and the C-terminal tail, in which the AT functions as a nucleoplasmic retention signal that is overridden by interaction of the AT with the N-terminal residues. Since both motifs are well conserved in rpL22 from diverse species, including *Drosophila* (Fig. 1), this behavior may be general. Given the strategic location of S289/290 adjacent to the AT, CKII phosphorylation could regulate this interaction and hence rpL22 partitioning. Whatever the possible function of rpL22 phosphorylation, it is important to emphasize that the two serine residues in *Drosophila* are not conserved in other organisms nor in the *Drosophila* paralog CG9871 (Fig. 1 and data not shown). Therefore, any regulation of rpL22 function by CKII phosphorylation would be restricted to *Drosophila* rpL22, whereas interaction with CKII would be generally applicable to all rpL22 homologs (see above).

Two other integral ribosomal proteins have been shown to interact with CKII, rpL5 [29] and rpL41 [30], both isolated in a two-hybrid screen using the β subunit as bait. Like rpL22, rpL5 is a substrate of CKII in vitro and phosphorylation of the protein decreases its 5S rRNA binding activity [31]. Interestingly, rpL5 has also been shown to interact with protein phosphatase-1 (PP-1) [32], raising the possibility that PP-1 and CKII regulate the function of a common target within the ribosome. Although rpL41 modulates the activity of CKII against topoisomerase IIα in vitro [30] and rpL5 modulates the enzymatic activity of PP-1 [32], attempts to evaluate the influence of rpL22 on CKII activity failed to show any consistent effects (data not shown). Nevertheless, we cannot exclude the possibility that rpL22 affects CKII activity in vivo, possibly via a third component.

The physical association between CKII and rpL22 described here supports a role for CKII in ribosome biogenesis and/or translation on mature ribosomes. As noted in the introduction, CKII is present in nuclear and nucleolar complexes [8] and interacts with and/or

phosphorylates diverse nucleolar proteins involved in rDNA expression, ribosome biogenesis, and ribosome transport [9–14]. The physical association of CKII with rpL22 may facilitate one or more of these processes. CKII is also physically associated with mature ribosomes [33,34]. Known targets within the ribosome include the highly conserved stalk proteins, P0, P1, and P2, which appear to mediate interactions with translation elongation factors [34,35]. In addition, both initiation and elongation factors are known targets of CKII [2,15] and could potentially be modified by ribosome-bound CKII.

rpL22 is co-opted by a number of vertebrate viruses, interacting either with virally encoded proteins, as in the case of HVS-1 [18,19] and EHV-1 [20], or with virally encoded RNA, as in the case of EBV virus [17]. The association of CKII with rpL22 may thus be important in the life cycle of such viruses. Interestingly, the region of HVS-1 ICP4 and its homologs that interacts with rpL22 maps to a small region that includes the serine-rich tract (SRT), a polyserine sequence followed by 5–7 acidic residues [18,20]. Because a phosphoserine can also serve as an acidic determinant for CKII [36], the SRT could be multiply phosphorylated by CKII. If so, rpL22 might serve as a link between kinase and substrate in these viral systems. Surprisingly, although the existence of the CKII motif has been often noted, whether CKII phosphorylates the motif and what the consequences might be appear not to have been explored. The association of CKII with rpL22 may also be relevant to EBV-induced lymphocyte transformation. Such a possibility is particularly intriguing given the stochastic production of lymphomas observed in response to dysregulated expression of CKIIα in lymphocytes of transgenic mice [7]. Further analysis of the interaction between CKII and rpL22 could provide novel approaches to interfering with viral replication and/or transformation.

Acknowledgments

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