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Interaction of the β Subunit of Casein Kinase II with the Ribosomal Protein L5

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Casein kinase II (CKII) usually exists as a heterotetramer with $\alpha_2\beta_2$, $\alpha\alpha'\beta_2$, or $\alpha'_2\beta_2$. The α or α' subunits catalyze protein phosphorylation, whereas the function of the β subunit remains unclear. One of the possible functions of the β subunit may be to mediate the interaction of the catalytic subunit with target proteins. To identify proteins capable of associating with the β subunit *in vivo*, we have used a two-hybrid system. One protein identified is human ribosomal protein L5. The protein L5 does not interact with the α or α' subunits of CKII, supporting the idea that the β subunit can determine a substrate specificity of CKII. These results furthermore suggest a novel role for CKII in ribosomal L5 phosphorylation, in ribosomal assembly, or ribosomal transport in the intact cells. The protein L5 may act as a regulator of the activity or subcellular localization of CKII.

Casein kinase II (CKII), located in both nucleus and cytoplasm, is a serine/threonine kinase present in all eukaryotes (1). The primary sequences of the enzyme are highly conserved among distantly related species from yeast to human. CKII preferentially phosphorylates serine/threonine residues followed by a stretch of acidic residues on the immediate C-terminal side. *In vitro*, the catalytic activity of CKII is inhibited by acidic compounds such as heparin, whereas activity is stimulated by polybasic compounds such as polyamines (2). The enzymes from most sources exist as a stable heterotetramer with an $\alpha_2\beta_2$, $\alpha\alpha'\beta_2$, or $\alpha'_2\beta_2$ structure. The α and α' subunits (35-44 kDa) are the catalytic subunits that are the products of distinct genes. In *Saccharomyces cerevisiae*, simultaneous distruption of the genes encoding the α and α' subunits is lethal, indicating that CKII performs a critical function in cellular survival (3).

The physiological role of the β subunit (24-29 kDa) is not clear. However, it has been reported that the β subunit might be multifunctional. The β subunit is a regulatory subunit that is required for maximal catalytic activity of CKII (4-5). It mediates the tetramer formation by both the self-association and the interaction of the β subunit with the α or α' subunits (6). In *Schizosaccharomyces pombe*, the β subunit is absolutely necessary for full activation of the catalytic activity of CKII *in vivo* (7). The β subunit may determine the substrate specificity of CKII (4, 8). Stimulation of CKII activity, by polybasic compounds, is mediated by the β subunit (8). In addition, the β subunit can be autophosphorylated and transphosphorylated (9).

CKII catalyzes the phosphorylation of a number of proteins involved in nucleic acid metabolism and in cell growth, including DNA topoisomerases and ligase, RNA and DNA polymerases, and oncogene products such as Myc, Myb, Jun, Fos, p53, and Rb (1, 10). Phosphorylation of cytoskeletal proteins such as tubulin by CKII might control the morphological changes which occur during mitosis and cytokinesis (11). Among these substrate proteins, DNA topoisomerase II (12), p53 (13), pp49 (14), and hsp90 (15) are copurified or interact with CKII *in*

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vitro. Several *in vitro* studies have suggested that the β subunit is required for mediating the interaction of the α subunit with specific substrates and peptides (4, 8). The association of p53 with CKII is also mediated by the β subunit of CKII (13). However, the physiological significance of complex formation between these proteins and CKII is not understood. We hypothesized that the β subunit might interact with either already known substrate proteins or yet-unidentified target proteins *in vivo.* We sought, therefore, to identify proteins that interacted with the β subunit *in vivo,* using a two-hybrid cloning strategy (16). We report here that human ribosomal protein L5 associates with the β subunit of CKII, but not with the α or α' subunits *in vivo.*

MATERIALS AND METHODS

Strains and media. E. coli XL1-Blue (F′::Tn10 proA$^+$B$^+$ lacI^q Δ(lacZ) M15/recA1 endA1 gyrA96 (Nal^r) thi-1 hsdR17 (rk$^-$mk$^+$) supE44 relA1 lac) was the transformation recipient for all plasmid construction. *E. coli* HB101 (F^{-1} Δ(gpt-proA)62 leuB6 supE44 ara-14 galK2 lacY1 Δ(mcrC-mrr) rspL20 (Str^r) xyl-5 mtl-1 recA13) was used to recover library plasmids from yeast. *S. cerevisiae* HF7C (MATa ura3-52 his3-200 ade2-101 lys2-801 trp1-901 leu2-3,112 gal4-542 gal80-538 LYS2::GALI$_{UAS}$ ALI$_{TATA}$-HIS3 URA3::GAL4$_{17mers(\times 3)}$ −CyCI$_{TATA}$-1acZ) was used for a reporter strain (17). For drug selection, LB media plates were supplemented with ampicillin (50 μg/ml). *E. coli* HB101 for selecting library plasmids was grown on M9 minimal media plates containing proline (86 μg/ml) and ampicillin (50 μg/ml). Yeast cultures were grown in either YPAD media (1% yeast extract, 2% peptone, 0.004% adenine sulfate, 2% glucose) or synthetic minimal media with appropriate supplements (18).

Plasmids. In order to insert the complete open reading frame of the β subunit of CKII into the vector pGBT9, the entire coding sequence of the β subunit was PCR amplified from pT7II-CKIIβ (19). The forward and reverse oligonucleotide primers used in PCR had the following sequences: 5′ primer, 5′-GCTGACCCGGGGATGAGCAGC-TCAGAGGAGGTG-3′ and 3′ primer, 5′-TGGGGGTVGACATCAGCAATGTCTTGACTGG-3′. The *Sma*I and *Sal*I sites are underlined. The PCR incubations were carried out 25 cycles of denaturation at 94 °C for 1 min, annealing at 48 °C for 2 min, and extension at 72 °C for 2 min. The PCR products were digested with *Sma*I and *Sal*I, purified, and cloned into the *Sma*I and *Sal*I sites of pGBT9. For the PCR amplification of α, the following sequences were used: 5′ primer, 5′-AACCGGATCCACATGTCGGGACCCGTGCCAAGC-3′ and 3′ primer, 5′-ATACTGCAG-TTACTGCTGAGCGCCAGCGGC-3′. For the PCR amplification of α', the following sequences were used: 5′ primer, 5′-GTCCGGATCCCCATGCCCGGCCCGGCCGCGGGC-3′ and 3′ primer, 5′-TTCCTGCAGTCATCGTGCTGC-CGTGAGACC-3′. The *Bam*HI and *Pst*I sites are underlined. Similarly, the α and α' sequences of CKII were amplified and cloned into the *Bam*HI and *Pst*I sites of pGBT9. A reading frame of all hybrid plasmids was confirmed by DNA sequencing.

Two-hybrid screen. Both pGBT9-CKIIβ and pGADGH-cDNA were co-transformed into *S. cerevisiae* HF7C. Transformants were plated on synthetic media lacking tryptophan, leucine, and histidine. After 3-5 days of growth, His$^+$ colonies were assayed for β-galactosodase activity by a qualitative method. Positive colonies showed blue color in several hours. Plasmid DNAs were recovered from positive colonies and transformed into *E. coli* HB101. Transformants carrying the library plasmid grow on minimal media plate lacking leucine. Primary positives were tested for target specificity by retransformation with the control plasmid pGBT9-Ras into the reporter strain. Only library plasmids that did not express the reporter gene in the presence of Ras were studied further.

β-Galactosidase activity assay. For qualitative assay, yeast colonies were transferred onto Whatman 50 filters, frozen in liquid nitrogen for 10 seconds, and thawed at room temperature. Filters were then overlaid on Whatman 1 filters saturated with β-galactosodase assay buffer (60 mM NaH_2PO_4, 60 mM Na_2HPO_4, 10 mM KCl, 1 mM $MgSO_4$, 0.5% glucose, 50 mM β-mercaptoethanol, 0.01% 5-bromo-4-chloro-3-indolyl-β-D-galactoside) and incubated at 30°C. For quantitative assay, yeast cells were grown overnight in sythetic media lacking tryptophan and leucine. The cells were pelleted, resuspended in fresh sythetic media, and were then shaken for 5 hours. The cell pellets were collected, frozen in liquid nitrogen for 2 min, and thawed at room temperature. β-galactosidase activity was measured using o-nitrophenyl-β-D-galactopyranoside, as a substrate (18).

DNA sequencing analysis. The sequencing was carried out by the dideoxy chain termination method. In the search for sequence similarities, the GenBank Sequence Data Library was employed.

RESULTS

Isolation of CKII β-Interacting Proteins

To screen for human proteins that interact with the β subunit of CKII, we employed the two-hybrid cloning procedure. We fused the complete open reading frame of each subunit of

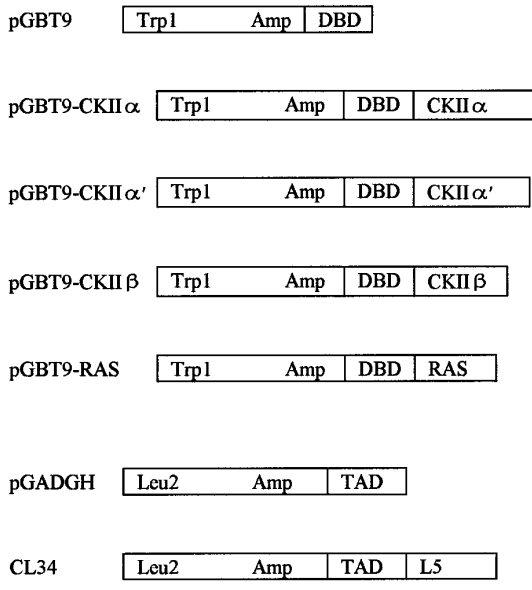

FIG. 1. Plasmids used in this study. pGBT9 and pGADGH encode the DNA binding domain (DBD) and the transcriptional activation domain (TAD) of the Gal4 protein. The complete open reading frames of each CKII subunit and human Ras were fused in frame to DBD of pGBT9. CL34 is one of positive clones, obtained from this cDNA library screen.

human CKII to the GAL4 DNA-binding domain in pGBT9 (Fig. 1). Yeast reporter strains co-transformed with pGBT9-CKIIβ and the control GAL4 transcriptional activation domain vector pGADGH were unable to activate transcription of the lacZ reporter gene. However, when the reporter strains were co-transformed with pGBT9-CKIIβ and pGADGH-CKIIα or $-$CKIIα', transactivation of the lacZ gene occurred, indicating that these fusion proteins were expressed correctly and that the β subunit interacted with either the α or the α' subunits (data not shown). Thus, we concluded that this system permitted the detection of an interaction between CKIIβ and its target proteins.

Yeast reporter strains were co-transformed with pGBT9-CKIIβ and pGADGH-human cDNA library. The construction of this library will be described elsewhere. Approximately, 1.3×10^6 independent transformants were screened. Thirty-eight His^+ colonies were blue when tested for β-galactosidase activity by a qualitative assay as described in Materials and Methods. Library plasmids were recovered from each of these primary positive strains and used to retransform the reporter strain in combination with either the original target or control plasmid pGBT9-Ras. Twenty colonies were identified that induced the expression of lacZ only in the presence of pGBT9-CKIIβ (Fig. 2).

Ribosomal Protein L5 as a CKIIβ-Associated Protein

Sequence analysis of one of the positive clones, CL34, revealed that it contained a 522 nucleotide insert with an adaptor sequence of 8 nt, a reading frame of 439 nt, and a 3′ noncoding region of 54 nt, followed by a poly(A) tract. When the nucleotide sequence was compared with reported sequences using the GenBank data base, the insert was identical to the ribosomal protein L5 (20). The insert encodes a polypeptides of 145 amino acids, which is the C-terminal portion (153-297) of the ribosomal protein L5 (Fig. 3).

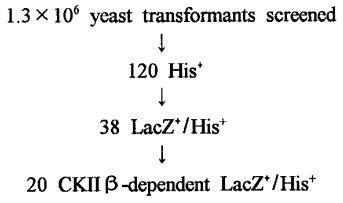

FIG. 2. Summary of human cDNA library screen. A detailed description is provided in text.

Specific Interaction of Ribosomal Protein L5 and the β Subunit of CKII

To test whether the protein L5 interacts specifically with only the β subunit among three CKII subunits, we co-transformed the reporter strains with CL34 and each CKII subunit-fused DNA binding domain vector. The interactions were tested by either qualitative or quantitative assay. A series of controls were undertaken to ensure that positive β-galactosidase activity reflected interactions between CKIIβ and L5 protein. These included co-transformation with one or both vectors lacking inserts, and transformation with only one vector. Without exception, control transformations resulted in extremely low levels of β-galactosidase activity by either qualitative or quantitative assay (Table 1). In contrast to control transformations, when the L5 and CKIIβ hybrid proteins were co-expressed in the reporter cells, they interacted strongly, resulting in β-galactosidase activity that was readily detected either qualitatively or quantitatively. These results demonstrate that L5 protein interacts with the β subunit, but not with either the α or α' subunits of CKII.

DISCUSSION

We have used a two-hybrid screen strategy to identify cDNAs that encode proteins capable of associating with the β subunit of CKII. One of several proteins identified is the ribisomal protein L5. Our analysis using the two-hybrid system also indicates that the protein L5 interacts with the β subunit, but not with either the α or α' subunits.

CKII forms a heterotetrameric structure composed of two catalytic (α and/or α') and two β subunits (1). The β subunit mediates the tetramer formation by both the self-association and the interaction of the β subunit with the α (or α') subunits (6). In the tetrameric structure the β subunit confers to the catalytic subunit a fully active and functional enzyme, possibly by mediating the interaction of the catalytic subunit with the specific substrates (4, 5, 8). p53 interact with CKII *in vitro*, and the association of p53 with CKII is mediated by the β subunit (13). Since the protein L5 interacts with only the β subunit among the three subunits of CKII here, the present study indicates that the β subunit mediates the interaction of CKII with its target proteins *in vivo*. This study also suggests the possibility that ribosomal protein L5 may act as a regulator of the activity or subcellular localization of CKII.

CKII preferentially phosphorylates serine/threonine residues followed by a stretch of acidic residues on the immediate C-terminal side. Among these acidic residues, the third position after the phosphoacceptor is the most important determinant. In some cases, however, the acidic residue locates at position +1 or +2 instead of position +3 from the phosphoacceptor (21). In this sense the L5 protein has a putative CKII phosphorylation site, YDSESKE (at position 38 downstream from N-terminal in Fig. 3). Furthermore, a recent two-hybrid screen demonstrated that chicken ribosomal protein L5 interacted with the protein phosphatase PP1, suggesting that the protein L5 might be a target protein for dephosphorylation by PP1 (22). These results raise the possibility that L5 may exist as a phosphoprotein in the intact cells. Although it is not known whether the protein L5 is phosphorylated in the cells, CKII is a

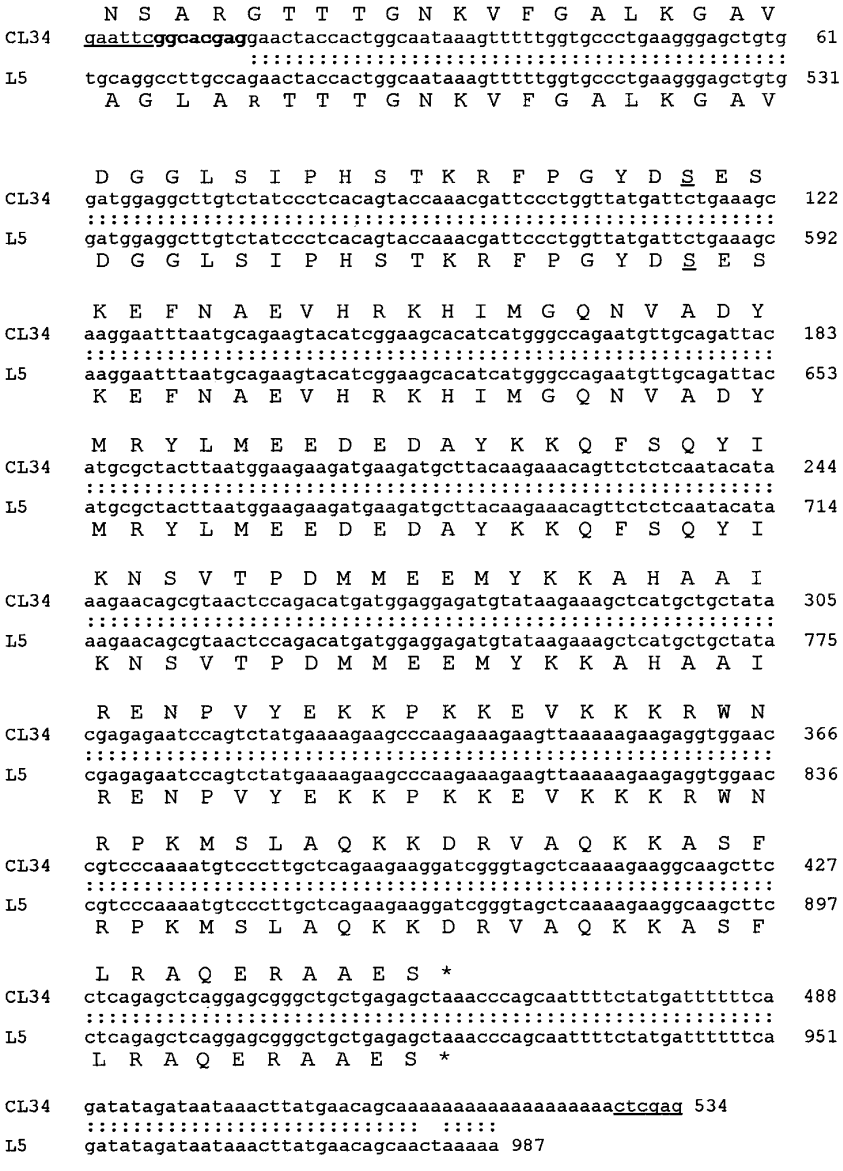

FIG. 3. The nucleotide and deduced amino acid sequences of CL34. A comparison of the nucleotide and amino acid sequences of CL34 with human ribosomal L5 protein (20) is shown. The nucleotide sequence is given in lowercase letters and the amino acid sequence is in capital letters. Colons indicate identical nucleotides. An asterisk denotes the end of the open reading frame. Underlined nucleotides indicate the insertion sites, *Eco*RI and *Xho*I, of cDNA into pGADGH. The adaptor sequence, used in the construction of the pGADGH cDNA library, is indicated in bold face. Underlined amino acids correspond to a putative CKII-phosphorylation residue.

possible protein kinase for the protein L5 phosphorylation. Whether CKII phosphorylates the protein L5 *in vitro* is under study.

The protein L5 forms a stable complex with 5S rRNA during ribosome assembly in mammalian cells (23). And the transport of cytoplasmic 5S rRNA to the nucleolus depends on the formation of the complex with L5 protein (24). Thus, the present data suggest that the β

TABLE 1
The Interaction of CKIIβ and L5 Proteins

DNA binding domain plasmid	Activation domain plasmid	Color	β-Galactosidase activity[a]
pGBT9-CKII β		White	0.7 ± 0.4
pGBT9-CKII α		White	ND
pGBT9-CKII α'		White	ND
pGBT9-RAS		White	ND
	pGADGH	White	0.5 ± 0.3
	CL34	White	0.8 ± 0.4
pGBT9-CKII β	CL34	Blue	7.9 ± 1.5
pGBT9-CKII α	CL34	White	0.4 ± 0.2
pGBT9-CKII α'	CL34	White	0.3 ± 0.1
pGBT9-RAS	CL34	White	ND

β-Galactosidase activity was determined by either a qualitative or quantitative assay for yeast strains containing the indicated plasmids, as described in Materials and Methods.

[a] Values reported are the mean of duplicate assays of two independent transformants. ND, not determined.

subunit itself or the CKII enzyme may regulate ribosome assembly through the physical association or the phosphorylation of L5 protein.

Recently it has been reported that the L5 protein is associated with both the mdm-2 protein and the mdm-2-p53 complex (25). Previous works have shown that the β subunit of CKII is associated directly with p53 (13). The β subunit has been shown here to be associated with L5 protein. All these data lead to the possibility that the β subunit participates in the formation of various ternary and quaternary complexes such as CKIIβ-L5-mdm-2, CKIIβ-L5-mdm-2-p53, L5-mdm-2-p53-CKIIβ, and/or CKIIβ-L5-mdm-2-p53-CKIIβ. While the physiological significance of these complexes is not clear, they may associated with cell proliferation.

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