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Identification of Mutations in Protein Kinase CKII β Subunit That Affect Its Binding to Ribosomal Protein L41 and Homodimerization

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Protein kinase CKII is composed of two catalytic (α or α') subunits and two regulatory (β) subunits. The CKIIβ subunit is thought to mediate the tetramer formation and interact with other target proteins. However, its physiological function remains obscure. In this study, point mutants of CKIIβ that are defective for the L41 binding were isolated by using the reverse two-hybrid system. A sequence analysis of the point mutants revealed that Asp-26, Met-52, and Met-78 of CKIIβ are critical for L41 binding; Asn-67 (and/or Lys-139) and Met-52 are important for CKIIβ homodimerization. Two point mutants, R75 and R83, of CKIIβ interacted with L5, topoisomerase IIβ, and CKBBP1/SAG, but not with the wild-type CKIIβ. This indicates that CKIIβ homodimerization is not a prerequisite for its binding to target proteins. These CKIIβ point mutants may be useful in exploring the biochemical physiological functions of CKIIβ.

Keywords: Protein kinase CKII, Protein-protein interaction, Random PCR mutagenesis, Reverse two-hybrid system

Introduction

Protein kinase CKII (CKII) is a ubiquitous and highly conserved Ser/Thr kinase, which was found in all of the eukaryotes that were examined and in various subcellular compartments. CKII catalyzes the phosphorylation of a large number of both cytoplasmic and nuclear proteins (including DNA binding proteins, nuclear oncoproteins, and transcription factors), and the phosphorylation modulates the activities of these proteins either positively or negatively (Pinna, 1990, 1997; Issinger, 1993). The overexpression of CKIIα leads to tumorigenesis in mice overexpressing myc (Seldin and Leder, 1995). An important role of CKII in cell cycle control has been demonstrated in the yeast *S. cerevisiae*. The analysis, using temperature-sensitive mutants for the CKII gene, showed that CKII is required for cell cycle progression in both the G1 and G2/M phases of the cell cycle (Padmanabha *et al*., 1990; Hanna *et al*., 1995). In addition, recent studies demonstrated that CKII may have anti-apoptotic functions (Litchfield, 2003). These observations suggest that CKII plays a critical role in cell growth, proliferation, and apoptosis; however, its complete physiological role and regulatory mechanism remains obscure.

CKII is a heterotetrameric enzyme that is composed of two catalytic (α or α') subunits and two regulatory (β) subunits (Pinna, 1990, 1997; Issinger, 1993). The regulatory role of the β subunit (CKIIβ) is complex. Upon binding to the CKIIβ subunit, the α subunit (CKIIα) changes catalytic activity and substrate specificity (Lin *et al*., 1991; Jakobi and Traugh, 1992). Allosteric regulation, such as the stimulation of CKII activity by polybasic compounds, is also mediated by CKIIβ (Meggio *et al*., 1992). The CKIIβ subunit mediates the tetramer formation by both the β-β homodimerization and α-β heterodimerization (Gietz *et al*., 1995). In addition, CKIIβ is capable of interacting with other proteins, including ribosomal proteins L5 (Kim *et al*., 1996) and L41 (Lee *et al*., 1997), DNA topoisomerase IIα and IIβ (Park *et al*., 2001), p53 (Appel *et al*., 1995), A-Raf (Boldyreff and Issinger, 1997; Hagemann, 1997), Mos (Chen *et al*., 1997), CKBBP1/SAG (Son *et al*., 1999; Kim *et al.,* 2002), and CD5 (Raman *et al*., 1998). The activity of A-Raf is stimulated by CKIIβ binding (Hagemann, 1997), whereas Mos activity is inhibited (Chen *et al*., 1997). These results suggest that CKIIβ serves as a multifunctional regulator by forming complexes with different classes of proteins. Recently, we mapped the subregions of CKIIβ that are involved in intersubunit contacts, as well as the

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Abbreviations: CKII, protein kinase CKII; CKIIα, the α subunit of CKII; CKIIβ, the β subunit of CKII; PCR, polymerase chain reaction; 3-AT, 3-amino-triazole; FOA, 5-fluoroorotic acid.

interaction with target proteins, including the ribosomal proteins L5 and L41, DNA topoisomerase IIβ, CKBBP1/SAG (Ahn *et al*., 2001).

In this study, we identified and characterized the point mutations in CKIIβ that disrupt the complex formation with L41 and CKIIβ homodimerization using the reverse two-hybrid system. The present results indicate that Asp-26, Met-52, and Met-78 within CKIIβ are critical for L41 binding, and that Asn-67 (and/or Lys-139) and Met-52 are important for CKIIβ homodimerization.

Materials and Methods

Materials The transformation recipient for all plasmid constructions was *E. coli* DH5α. *S. cerevisiae* HF7c (*MATa ura3-52 his3-200 ade2-101 lys2-801 trpl-901 leu2-3,112 gal4-542 gal80-538 LYS2::GAL1*$_{UAS}$-*GAL1*$_{TATA}$-*HIS3 URA3::GAL4*$_{17mers(x3)}$-*CyC1*$_{TATA}$-*lacZ*) and *S. cerevisiae* MaV103 (*MATa leu2-3,112 trp1-901 his3Δ200 ade2-101 gal4Δ gal80Δ SPAL::URA3 GAL1::LacZ GAL1::HIS3@LYS2 can1*^{R} *cyh2*^{R}) were used for the yeast two-hybrid system and reverse two-hybrid system, respectively (Feilotter *et al*., 1994; Vidal *et al*., 1996). The 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 (Rose *et al*., 1990).

pGBT9-CKIIα and pGBT9-CKIIβ are the yeast shuttle vector plasmids pGBT9 containing the complete open-reading frame of the human CKIIα and CKIIβ subunits, respectively (Kim *et al*., 1996). pGADGH-CKIIα and pGADGH-CKIIβ are the yeast shuttle vector plasmids pGADGH containing the complete open-reading frame of the human CKIIα and CKIIβ subunits, respectively (Kim *et al*., 1998).

PCR mutagenesis and reverse two-hybrid system Mutagenic PCR was a minor modification of that which was described by Leung *et al*. (1989). The reaction mixture contained 100 ng of pGBT9-CKIIβ, 50 μM each dATP, dTTP, and dGTP, 10μM dCTP, 1 μM of 5' primer (5'-GCTTCAGTGGAGACTGATATGCCT-3') and 3' primer (5'-TGACCTACAGGAAAGAGTTACTCA-3'), 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 1 μg/μl BSA, 1.5 mM $MgCl_2$, and 5 units of Taq DNA polymerase. After 10 cycles of PCR (94°C for 1 min, 50.6°C for 2 min, and 72°C for 2 min), $MnCl_2$ was added to 300 μM and the reaction was continued for 30 cycles. The resultant PCR product was purified and introduced into *S. cerevisiae* MaV103, along with pGADGH-L41 and pGBT9-CKIIβ that were linearized with *Eco*RI (Vidal *et al.,* 1996). Two-hybrid transformations were plated onto synthetic media that lacked tryptophan and leucine, but were supplemented with 0.25% 5-fluoroorotic acid (FOA). The second selection for the CKIIβ-L41 interaction was then performed on synthetic media that lacked tryptophan and leucine, but was supplemented with 30 mM 3-amino-triazole (3-AT) (Vidal *et al*., 1996).

DNA sequencing The nucleotide sequencing was carried out by the dideoxy chain termination method.

Yeast two-hybrid assay The reporter strain *S. cerevisiae* HF7c was co-transformed with various combinations of hybrid plasmids containing a DNA binding domain or transcriptional activation domain. The transformants were plated on synthetic media that lacked tryptophan and leucine. After 4 days of growth, the transformants were patched on selective media that lacked tryptophan, leucine, and histidine, but included 2 mM 3-AT. They were then incubated for 4 days at 30°C. Interactions between the hybrid proteins were monitored by either growth on the selective media or β-galactosidase activity assay (Fields and Song, 1989; Park and Bae, 2001).

Results and Discussion

Isolation of CKIIβ point mutants defective for L41 binding To isolate the point mutants of CKIIβ which fail to bind to ribosomal protein L41, we randomly mutagenized the CKIIβ sequences by the biased-pool PCR method, as described in Materials and Methods. This mutagenized CKIIβ pool was introduced into the reporter strain MaV103 along with pGADGH-L41 and linearized pGBT9-CKIIβ, and the resultant transformants were analyzed for the reporter gene expression. From a total of 1100 colonies that were analyzed, thirty clones showed no detectable reporter gene expression, which indicates that these clones contain mutation(s) within the L41-binding domain of CKIIβ (Fig. 1A). To eliminate the clones that contained the frame-shift or nonsense mutation, pGBT9-CKIIβ DNAs were recovered from eight primary positive colonies and used to retransform the yeast strain MaV103 with pGADGH-CKIIα. Because the CKIIα-binding domain of CKIIβ localizes within its C-terminal region, the colonies that were positive for the CKIIα-CKIIβ interaction were presumed to contain an open-reading frame (ORF) throughout the full-length of CKIIβ. Among the eight cDNAs that were rescreened, five clones were identified that interacted with CKIIα (Fig. 1B), but not with L41 (Fig. 1A).

Sequencing of CKIIβ point mutants defective for L41 binding A sequence analysis of these five clones revealed that two (R59 and R83) contained single amino acid changes in CKIIβ, two (R16 and R26) contained two amino acid residue mutations, and one (R75) contained four amino acid residue mutations (Table 1). Consistent with the results from the deletion mutant analysis of the previous results (Ahn *et al*., 2001), the point mutations that affected the L41 binding were localized throughout a large region of the CKIIβ protein. Because residues 1-167 within CKIIβ were shown to be sufficient for the L41 binding (Ahn *et al*., 2001), it was unlikely that Gly-189, Met-195, and Thr-213 were involved in the L41 binding. These results suggest that Asp-26, Met-52, and/or Met-78 within CKIIβ are critical for the L41 binding (see below).

The amino acids 55-70 of CKIIβ contain clusters of acidic residues, which are responsible for an intrinsic negative

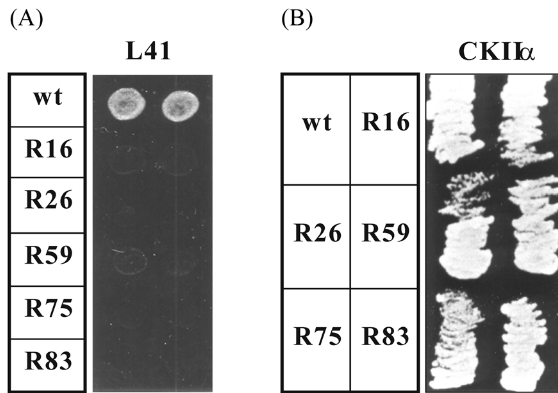

Fig. 1. Isolation of CKIIβ point mutants defective for L41 binding. (A) The randomly mutagenized pool of CKIIβ was co-transformed with pGADGH-L41 into the reporter strain MaV103. Co-transformants were selected on synthetic media lacking tryptophan and leucine but supplemented with 0.25% FOA, and then on synthetic media lacking tryptophan and leucine but supplemented with 30 mM 3-AT. Thirty clones that showed no detectable reporter gene expression were isolated. Of the eight cDNAs that were rescreened for an interaction with CKIIα, five clones were identified that did not interact with L41. (B) The reporter strain HF7c was co-transformed with pGADGH-CKIIα wild-type and pGBT9-CKIIβ mutants. Double transformants were patched for 4 days at 30°C on selective media that lacked tryptophan, leucine, and histidine, but included 2 mM 3-AT. All of the constructs were also screened against the empty expression vectors, pGBT9 and pGADGH, in order to control the autonomous activation of the hybrid proteins. wt, wild-type.

Table 1. Identification of mutations in human CKIIβ defective for binding to L41

Mutant	Amino acid change
R16	Asp-26→Gly; Gly-189→Cys
R26	Leu-39→Pro; Lys-147→Met
R59	Met-78→Thr
R75	Asn-67→Ile; Lys-139→Arg; Met-195→Val; Thr-213→Ala
R83	Met-52→Ile

regulation of CKII activity and for the interaction with the polybasic compounds, such as polylysine and spermine (Boldyreff *et al.*, 1994; Meggio *et al.*, 1994). However, the present results indicate that this acidic stretch of CKIIβ is probably not important for the binding to the highly basic protein, L41. Rather, our findings suggest that the L41-binding site on CKIIβ may be different from either the polylysine- or spermine-binding site. This might explain why the stimulation of the CKII activity by L41 was not detected with β-casein or the synthetic peptide substrate (RRRDDDSDDD) in our previous study (Lee *et al.*, 1997).

Identification of amino acids important for CKIIβ homodimerization To determine if the point mutants that were selected actually affect the self-association of CKIIβ, each CKIIβ mutant construct was introduced into the reporter stain HF7c with pGADGH-CKIIβ, and the interaction between the two hybrid proteins was detected by the expression of the reporter genes, *HIS3* and *lacZ*. Among the five mutants that were examined, three (R26, R75, and R83) failed to interact with CKIIβ, and two (R16 and R59) interacted with wild-type CKIIβ (Fig. 2). Although our previous study showed that residues 19-167 are sufficient for the CKIIβ homodimerization (Ahn *et al.*, 2001), the results presented here indicate that Asp-26 and Met-78 of CKIIβ are not necessary for homodimerization, and that Asn-67 and/or Lys-139 and Met-52 may be important for self-association.

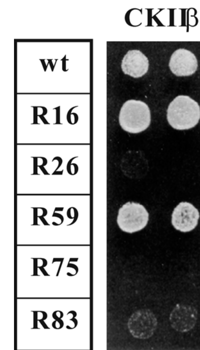

Fig. 2. Interaction of the CKIIβ point mutants with wild-type CKIIβ. The reporter strain HF7c was co-transformed with the pGADGH-CKIIβ wild-type and pGBT9-CKIIβ wild-type or pGBT9-CKIIβ mutants. Double transformants were patched for 4 days at 30°C on selective media that lacked tryptophan, leucine and histidine, but included 30 mM 3-AT. wt, wild-type.

Table 2. Summary of interactions between the CKIIβ point mutants with CKII subunits or target proteins

Mutant	CKIIα	CKIIβ	L5	TopoIIβ	CKBBP1
R16	+	+	+	+	+
R26	+	-	-	-	-
R59	+	+	+	+	+
R75	+	-	+	+	+
R83	+	-	+	+	+

The interaction between the CKIIβ point mutants and wild-type CKIIα and β, L5, topoisomerase IIβ (TopoIIβ), or CKBBP1/SAG was examined by the yeast two-hybrid system. The protein-protein interactions were detected by the expression of the reporter genes, *HIS3* and *lacZ*. + indicates that the reporter genes were activated; - indicates that the reporter genes were not activated.

Interaction of the CKIIβ point mutants with its target proteins These CKIIβ mutants were also used to identify the amino acids that are important for the binding to ribosomal protein L5, topoisomerase IIβ, and CKBBP1/SAG. Four mutants (except R26) still retained the binding ability for L5, topoisomerase IIβ, and CKBBP1/SAG in the yeast two-

hybrid system (Table 2). The result indicates that at least Asp-26, Met-52, Met-78, Asn-67, and Lys-139 of CKIIβ are not critical for the binding to L5, topoisomerase IIβ, and CKBBP1/SAG. Importantly, the fact that R75, as well as R83, interacts with L5, topoisomerase IIβ, and CKBBP1/SAG but not with CKIIβ suggests that CKIIβ homodimerization is not a prerequisite for binding to these target proteins (Table 2).

Based on the present data alone, we cannot distinguish between the residues of CKIIβ that are contact sites for target proteins, and the residues whose change results in an altered CKIIβ conformation, which is then no longer capable of binding target proteins. However, we suggest that the substitution of Leu with Pro at position 39 of CKIIβ may adversely affect the overall structure of the CKIIβ protein with the exception of the C-terminal region, because R26 interacted with CKIIα but not with L41, wild-type CKIIβ, L5, topoisomerase IIβ, and CKBBP1/SAG. In contrast to R26, the three-dimensional conformation of R16, R59, R75, and R83 is suspected to be almost like that of the native protein, because these CKIIβ mutant proteins still interacted with the CKIIβ-target proteins, including L5, topoisomerase IIβ, and CKBBP1/SAG.

In summary, we identified point mutations in CKIIβ that disrupt the complex formation with L41 and CKIIβ homodimerization. These results indicated that Asp-26, Met-52, and Met-78 of CKIIβ are critical for L41 binding, and that Asn-67 (and/or Lys-139) and Met-52 are important for CKIIβ homodimerization. The introduction into cells of these CKIIβ mutants that are defective in binding to L41 or in homodimerization may reveal the physiological significance of the interaction of CKIIβ with L41 and the CKII tetramer formation. The biochemical differences between the CKII dimer (αβ) and CKII tetramer ($\alpha_2\beta_2$) are being studied.

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