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The Ring-H2 Finger Motif of CKBBP1/SAG Is Necessary for Interaction with Protein Kinase CKII and Optimal Cell Proliferation

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Protein kinase CKII (CKII) is required for progression through the cell division cycle. We recently reported that the β subunit of protein kinase CKII (CKIIβ) associates with CKBBP1 that contains the Ring-H2 finger motif in the yeast two-hybrid system. We demonstrate here that the Ring-H2 finger-disrupted mutant of CKBBP1 does not interact with purified CKIIβ *in vitro*, which shows that the Ring-H2 finger motif is critical for direct interaction with CKIIβ. The CKII holoenzyme is efficiently co-precipitated with the wild-type CKBBP1, but not with the Ring-H2 finger-disrupted CKBBP1, from whole cell extracts when epitope-tagged CKBBP1 is transiently expressed in HeLa cells. Disruption of the Ring-H2 finger motif does not affect the cellular localization of CKBBP1 in HeLa cells. The increased expression of either the wild-type CKBBP1 or Ring-H2 finger-disrupted CKBBP1 does not modulate the protein or the activity levels of CKII in HeLa cells. However, the stable expression of Ring-H2 finger-disrupted CKBBP1 in HeLa cells suppresses cell proliferation and causes the accumulation of the G1/G0 peak of the cell cycle. The Ring-H2 finger motif is required for maximal CKBBP1 phosphorylation by CKII, suggesting that the stable binding of CKBBP1 to CKII is necessary for its efficient phosphorylation. Taken together, these results suggest that the complex formation of CKIIβ with CKBBP1 and/or CKII-mediated CKBBP1 phosphorylation is important for the G1/S phase transition of the cell cycle.

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Abbreviations: CKII, protein kinase CKII (formerly casein kinase II); CKBBP, CKIIβ-binding protein; FACS, fluorescence-activated cell sorter; GFP, green fluorescent protein; PCR, polymerase chain reaction; PMSF, phenylmethylsulfonyl fluoride; SAG, sensitive-to-apoptosis gene; SDS, sodium dodecyl sulfate.

Keywords: Cell proliferation, CKBBP1/SAG, Protein-protein interaction, Protein kinase CKII, Ring-H2 finger motif

Introduction

Protein kinase CKII (CKII) is a ubiquitous and highly-conserved Ser/Thr kinase. It is found in all of the eukaryotes that were examined, as well as in various subcellular compartments (Pinna, 1990; Issinger, 1993; Allende and Allende, 1995). CKII is a calcium-independent and cyclic nucleotide-independent enzyme that utilizes either ATP or GTP as a phosphate donor. CKII is a heterotetramer of two catalytic (α and/ or α') and two regulatory (β) subunits. The α and α' subunits are different gene products, and exhibit the catalytic activity of the enzyme. The β subunit is a regulatory subunit that modulates the catalytic activity of the α subunit. It also mediates the tetramer formation and substrate recognition (Lin *et al*., 1991; Jakobi and Traugh, 1992). The CKII expression level is greatly enhanced in proliferating cells and tumor tissues (Mûnstermann *et al*., 1990; Issinger, 1993), and the overexpression of the α subunit of CKII causes lymphomas in transgenic mice (Seldin and Leder, 1995; Kelliher *et al*., 1996). CKII phosphorylates a broad spectrum of substrates that are involved in cell growth and proliferation. These include DNA-binding proteins, nuclear oncoproteins, and transcription factors (Pinna, 1990; Issinger, 1993; Allende and Allende, 1995). Genetic analyses in yeast have demonstrated that CKII is essential for the cell-cycle progression in G1 and G2/M (Hanna *et al*., 1995). These observations suggest that CKII plays a significant role in the cell proliferation and cell division cycle; however, its precise physiological role and regulatory mechanism remain largely unknown.

In order to study the physiological role and regulatory mechanism of CKII, we identified human cellular proteins

that interact with CKII using the yeast two-hybrid system (Park and Bae, 2001). In our previous study, we reported that a protein called CKBBP1 (CKIIβ-binding protein 1) is a cellular interaction partner of the β subunit of CKII (Son *et al*., 1999). This protein was also identified by others and given the name SAG (sensitive to apoptosis gene) (Duan *et al*., 1999). CKBBP1/SAG is a Ring-H2 finger motif-containing protein with a molecular weight of 12.6 kDa, which is localized in both the nucleus and cytoplasm of cells. ROC1 (also called Rbx1 and Hrt1), a CKBBP1 homologue, is a component of the SCF (Skip1-Cullin-F-box protein) E3 ubiquitin ligase complex, as well as of the von Hippel-Lindau (VHL) tumor suppressor complex (Kamura *et al*., 1999; Ohta *et al*., 1999; Skowyra *et al*., 1999).

In the present study, we constructed a Ring-H2 finger-disrupted mutant form of CKBBP1. We show here that the Ring-H2 finger motif of CKBBP1 is necessary for efficient binding to CKIIβ, as well as for optimal cell proliferation.

Materials and Methods

Antibodies A polyclonal anti-CKBBP1 antibody was raised against recombinant CKBBP1, as described elsewhere (Son*et al*., 1999). The polyclonal anti-CKIIα and monoclonal anti-CKIIβ antibodies were obtained from Calbiochem (La Jolla, USA).

Site-directed mutagenesis and plasmid constructions The bacterial expression vector pET14b-CKBBP1, which expresses the full-length CKBBP1 with a hexahistidine (His) tag to the amino terminus, was described previously (Son *et al*., 1999). To replace both Cys 80 and His 82 within the Ring-H2 finger motif of CKBBP1 with Ala, an overlap extension PCR was performed as described (Ho *et al*., 1989). The desired mutations were incorporated into oligonucleotide primers. Two initial PCRs were performed with either the N-terminal forward (N) primer (5'-CGGCCATATGGCCGACGTGGAAGACGG-3') and mutagenic reverse primer (5'-AGTTGTGGAAGGATGCATTTGCTTCTCCC CAGA-3'), or the mutagenic forward primer (5'-TCTGGGGA GAAGCAAATGCATCCTTCCACA-3') and C-terminal reverse (C) primer (5'-CTAACTCGAGTCATTTGCCGATTCTTTGGA-3'). Equal amounts of these two PCR products were mixed, denatured, reannealed, then applied to a second PCR using the N and C primers. After digestion of the PCR products with *Nde*I/*Xho*I, the fragment was ligated into the *Nde*I/*Xho*I sites of pET14b.

To generate a Myc-His-tagged CKBBP1 expression construct, the CKBBP1 cDNA was PCR-amplified using the primers 5'-CCGGAATTCCGCCATGGCCGACGTGGAAGA-3' and 5'-GCG GGATCCATTTGCCGATTCTTTGGACCA-3'. The PCR fragment was digested with *Eco*RI and *Bam*HI, then subcloned into the *Eco*RI- and *Bam*HI-digested pcDNA3.1/Myc-His vectors (Invitrogene, Carlsbad, USA). To generate a green fluorescent protein (GFP)-tagged CKBBP1 expression construct, the CKBBP1 cDNA was PCR-amplified using the primers 5'-CGCGGCCTCGA GATGGCCGACGTGGAAG-3' and 5'-CTATCGGATCCCCTTT GCCGATTCTTTG-3'. The PCR fragment was digested with *Xho*I and *Bam*HI, then subcloned into *Xho*I- and *Bam*HI-digested pEGFP-N1 vectors (Clontech, Palo Alto, USA). The construct sequences were confirmed by nucleotide sequencing.

Purification of CKII and CKBBP1 Human CKII holoenzyme and CKIIβ were expressed and purified in *E. coli,* as described previously (Kim *et al*., 1998). Human CKBBP1 was expressed and purified in *E. coli,* as described previously (Son *et al*., 1999).

Cell culture and establishment of stable cell line The HeLa cells were grown in Dulbecco's modified Eagle's media that was supplemented with 10% fetal bovine serum at 37°C in 5% CO_2. To transiently express the Myc-His-tagged CKBBP1 in cells, the HeLa cells were transfected with the Myc-His-tagged CKBBP1 constructs using Lipofectamine (Qiagen, Hilden, Germany), as described by the manufacturer. At 12 h after the transfection, the medium was changed, and the cells were grown for another 36 h before being harvested. To establish CKBBP1-expressing stable cell lines, the HeLa cells were transfected with the Myc-His-tagged CKBBP1 constructs or the vector control by Lipofectamine. One day later, the cells were cultured in the presence of 1 mg/ml G418. After 2 weeks, the clones were picked and grown in the same medium in the presence of 100 μg/ml G418. Stable clones were examined for protein expression by Western blotting.

Preparation of HeLa cell extract For the Ni pull-down assay, approximately 1×10^6 HeLa cells in 100 mm-dishes were washed with ice-cold phosphate-buffered saline (PBS), collected by scraping with a rubber policeman, and lysed in 100μl of an ice-cold RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.5 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin). For the CKII activity assay, the cells were lysed in a lysis buffer (50 mM Tris-HCl, pH 8.0, 20 mM NaCl, 1 mM $MgCl_2$, 1 mM EDTA, 1% Nonidet P-40, 0.5 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM sodium orthovanadate, 1 mM sodium pyrophosphate, and 4 mM p-nitrophenyl phosphate) by sonication. The particulate debris was removed by centrifugation at 12,000 × *g*. The supernatant volumes were adjusted to give an equal protein concentration.

Western blotting The proteins were separated on a 12 or 15% polyacrylamide gel in the presence of SDS, then transferred electrophoretically to a nitrocellulose membrane (Yi *et al*., 2001). The membrane was blocked with 5% skim milk in TBST (20 mM) Tris-HCl, pH 7.4, 150 mM NaCl, and 0.05% Tween 20) for 2 h, then incubated with antibodies that are specific to CKBBP1, CKIIβ, or CKIIα at a 1 : 500 dilution in 1% skim milk for 1 h. The membrane was washed 3 times in TBST, then treated with the ECL system (Amersham Corp, Buckinghamshire, UK).

Pull-down assays *In vitro* binding assays were performed by incubating Ni-NTA agarose beads with 500 ng of purified His-CKBBP1 and CKIIβ in 200 μl of a binding buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 1 mM PMSF). The reaction was allowed to proceed for 1 h while rocking at 4°C. After the beads were washed three times with a washing buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM PMSF, and 20 mM imidazole), the bound proteins were eluted with an elution buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM PMSF, and 200 mM

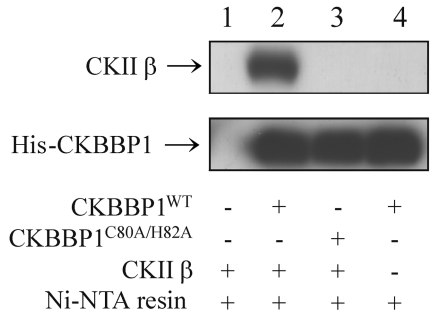

Fig. 1. Specific interaction of the Ring-H2 finger motif of CKBBP1 with the CKIIβ subunit *in vitro*. Ni-NTA agarose beads coated with His-CKBBP1^{WT} (lanes 2 and 4) or His-CKBBP1$^{C80A/H82A}$ (lane 3) proteins were incubated in the presence (lanes 1-3) or absence (lane 4) of CKIIβ. The reaction was allowed to proceed for 1 h while rocking at 4°C. After an extensive wash, the immobilized complexes were recovered by an elution step that was performed in the presence of 200 mM imidazole. The proteins were separated by 15% (w/v) SDS-polyacrylamide gel electrophoresis, and visualized by Western blotting with anti-CKIIβ or anti-CKBBP1 antibodies. Lane 1, the CKIIβ protein was incubated with the control Ni-NTA agarose beads.

imidazole), denatured in a 4 × SDS reducing-protein-gel-loading buffer, then resolved by SDS-polyacrylamide gel electrophoresis. The eluted proteins were visualized by Western blotting with anti-CKBBP1 or anti-CKIIβ antibodies. For the *in vivo* binding assays, the Ni-NTA agarose beads were incubated with lysates from HeLa cells that were transfected with the pcDNA3.1/Myc-His-CKBBP1. The immobilized complexes were recovered by an elution step that was performed as previously described, and visualized by Western blotting with anti-CKIIα, anti-CKIIβ, or anti-CKBBP1 antibodies.

CKII activity assay The standard assay for CKII phosphotransferase activity was conducted in a reaction mixture that contained 20 mM Tris-HCl, pH 7.5, 120 mM KCl, 10 mM $MgCl_2$, and 100 μM [γ-^{32}P] ATP in the presence of a 1 mM synthetic peptide substrate (RRREEETEEE) in a total volume of 30 μl at 30°C. The reactions were started by the addition of HeLa cell lysates or CKBBP1 co-precipitates, and incubated for 15 min. The reaction was stopped by the addition of trichloroacetic acid (TCA) to a final concentration of 10% and centrifuged, and 10 μl of supernatant was applied to P-81 paper. The paper was washed in 100 mM phosphoric acid, and the radioactivity was measured by scintillation counting.

Phosphorylation of CKBBP1 by CKII Phosphorylation of the His-tagged CKBBP1 by CKII was performed in a reaction mixture that contained 20 mM Tris-HCl, pH 7.5, 100 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, 1 mM EGTA, 100 μM [γ-^{32}P]ATP, and 6 μg of His-tagged CKBBP1 in a total volume of 30 μl. The reactions were started by the addition of purified CKII, and incubated for 15 min at 30°C. The samples were then separated on 15% SDS-polyacrylamide gel. The gel was stained with Coomassie blue, dried, and subjected to autoradiography.

Subcellular localization of CKBBP1 To determine the

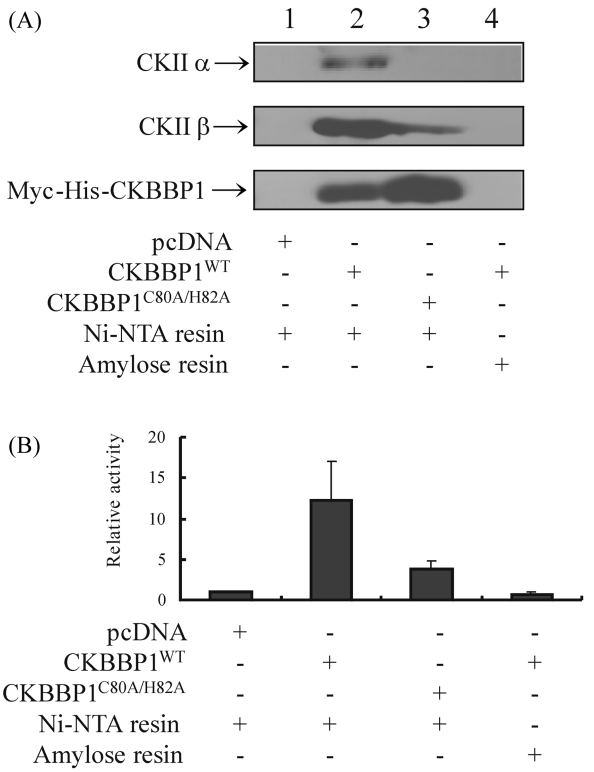

Fig. 2. Interaction of CKBBP1 with CKIIβ *in vivo*. (A) HeLa cells were transiently transfected with the plasmids that encode Myc-His-CKBBP1^{WT} (lanes 2 and 4) or Myc-His-CKBBP1$^{C80A/H82A}$ (lane 3). The empty vector (pcDNA3.1/Myc-His) was used as the control (lane 1). At 48 h after transfection, the cells were harvested and lysed. The lysates were then used for the precipitation of exogenously expressed CKBBP1 with Ni-NTA agarose. Amylose resin was used for the negative control precipitation. The immobilized complexes were recovered by an elution step using 200 mM imidazole. The proteins were separated by 15% (w/v) SDS-polyacrylamide gel electrophoresis and visualized by Western blotting with anti-CKIIα, anti-CKIIβ, or anti-CKBBP1 antibodies. (B) The activity of CKII in Myc-His-CKBBP1^{WT} or Myc-His-CKBBP1$^{C80A/H82A}$ co-precipitates from the HeLa cell lysates was assessed in the presence of [γ-^{32}P]ATP and the CKII substrate peptide (RRREEETEEE). The ^{32}P incorporation in the substrate peptide was measured by scintillation counting. The bars represent the relative activity of CKII that was determined. The error bars represent the range of duplicate experiments, each of which was analyzed twice by scintillation counting.

subcellular localization of the wild-type and mutant CKBBP1, the GFP-tagged CKBBP1 was transiently-expressed in HeLa cells. Green fluorescence images were obtained using a confocal microscope after 48 h of transfection.

Growth curves The HeLa cells that stably expressed the wild-type or mutant CKBBP1 were seeded in six-well dishes at a starting density of 5,000 cells/well; duplicate wells were used for each cell line. Every 48 h, the cells were trypsinized and counted in triplicate using a hemocytometer. Trypan blue was used to distinguish the

viable cells from the non-viable cells.

FACS analysis The HeLa cells (2×10^5) were seeded in 100-mm dishes that contained Dulbeccos modified Eagles media that was supplemented with 10% fetal bovine serum. Thirty-six hours later, the cells were collected in PBS that contained 2% fetal bovine serum, fixed in 65% ethanol for 1 h at 4°C, then incubated in 25 μg/ml DNase-free RNase A (Sigma, St. Louis, USA), 25 μg/ml propidium iodide (Sigma, St. Louis, USA), and 0.6% sodium citrate for 30 min at 37°C. A flow-cytometric determination of the cellular DNA content was performed on a Coulter Elite ESP Cell Sorter (Beckman, Palo Alto, USA). The forward and side scatter gates were set to exclude any dead cells from the analysis; 10,000 events within this gate were acquired per sample.

Results and Discussion

Ring-H2 finger motif of CKBBP1 is critical for direct interaction with CKIIβ *in vitro* Direct CKBBP1 interaction with the β subunit of CKII was further tested *in vitro* using a hexahistidine (His) pull-down assay. The purified His-CKBBP1 fusion protein was immobilized on Ni-NTA agarose beads and incubated with the purified CKIIβ protein. The beads were washed, and the bound CKIIβ protein was visualized by Western blotting with an anti-CKIIβ antibody. As shown in Figure 1 (lanes 1 and 2), the CKIIβ subunit was capable of binding to the beads that contained His-CKBBP1^{WT} (wild-type), but not to the control beads. These results confirm that the β subunit of CKII interacts directly with CKBBP1.

The C-terminal half of both CKBBP1/SAG and ROC1/Rbx1/Hrt1 contain the Ring-H2 finger motif (Duan *et al*., 1999; Kamura *et al*., 1999; Ohta *et al*., 1999; Skowyra *et al*., 1999; Son *et al*., 1999). The function of the Ring-H2 finger motif is unknown, but it has been proposed that it is involved in the protein-protein interaction, RNA, DNA, and metal-ion bindings (Saurin *et al*., 1996). To test whether the Ring-H2 finger motif of CKBBP1 is required for the interaction with CKIIβ *in vitro*, we constructed a mutant CKBBP1$^{C80A/H82A}$, which has alanine mutations at cystein 80 and histidine 82 to disrupt the Ring-H2 finger motif. We then performed a co-precipitation experiment using Ni-NTA agarose. As shown in Figure 1, the CKIIβ subunit was incapable of binding to beads that contained His-CKBBP1$^{C80A/H82A}$. These results indicate that an intact Ring-H2 finger motif of CKBBP1 is necessary for the interaction with the β subunit of CKII.

Interaction of CKBBP1 with CKIIβ in HeLa cells In order to examine whether CKBBP1 interacts with the CKIIβ subunit in human cells, the HeLa cells were transiently transfected with plasmids that encoded Myc-His-tagged CKBBP1^{WT} or Myc-His-tagged CKBBP1$^{C80A/H82A}$. The empty vector (pcDNA3.1/Myc-His) was used as the control. The Myc-His tag that was incorporated into the CKBBP1 sequence permitted the precipitation of the exogenously expressed CKBBP1 with the Ni-NTA agarose. At 48 h after transfection, the cells were harvested, and the Myc-His-CKBBP1 expression was detected by co-precipitation experiments with Ni-NTA agarose and Western blotting using anti-CKBBP1 antibody. As shown in Figure 2A, Myc-His-tagged CKBBP1 proteins were detected in the HeLa cells that were transfected with pcDNA3.1/Myc-His-CKBBP1, but not with the pcDNA3.1/Myc-His vector alone. Western blots of the co-precipitates that were probed the anti-CKIIβ antibody showed that CKIIβ were co-precipitated with the wild-type CKBBP1 in the HeLa cells that were transfected with the pcDNA3.1/Myc-His-CKBBP1^{WT}. This indicates that the wild-type CKBBP1 interacts with CKIIβ in HeLa cells. In contrast to the *in vitro* experiment, a small amount of CKIIβ was detectable in the Ni-resin precipitates from the HeLa cells that were transfected with the pcDNA3.1/Myc-His-CKBBP1$^{C80A/H82A}$. Quantification by a densitometer analysis revealed that the disruption of the Ring-H2 finger motif reduced the binding of CKBBP1 to CKIIβ 3.5-fold. The negative-control precipitation with an irrelevant resin (amylose resin) did not bring down the CKBBP1-CKII complex from the HeLa cells that were transfected with the pcDNA3.1/Myc-His-CKBBP1.

Western blots of these co-precipitates that were probed the anti-CKIIα antibody showed that CKIIα co-precipitated with the wild-type CKBBP1, which demonstrates that the wild-type CKBBP1 interacts with the CKII holoenzyme, as well as the CKIIβ subunit (Fig. 2A). Using the synthetic peptide substrate RRREEETEEE, the phosphotransferase activity of

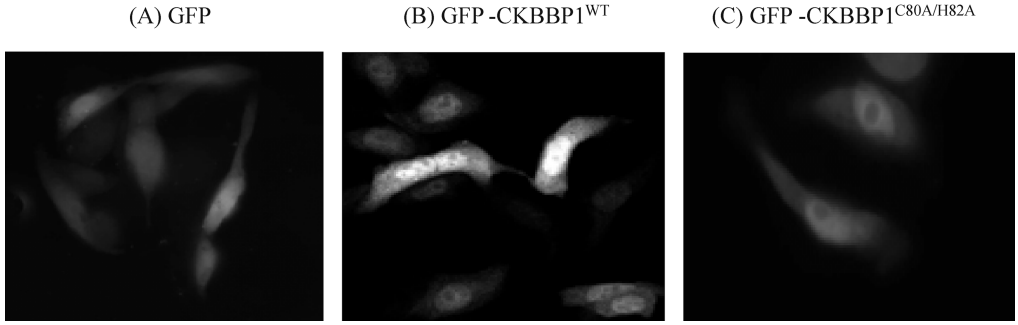

Fig. 3. Cellular localization of wild-type and mutant CKBBP1. HeLa cells were transfected with pEGFP, pEGFP-CKBBP1^{WT}, or pEGFP-CKBBP1$^{C80A/H82A}$. After 48 h of transfection, green fluorescence images were obtained using a confocal microscope.

the CKII holoenzyme in CKBBP1 co-precipitates was also determined. CKII activity was highly detected in the CKBBP1^{WT} co-precipitates from the HeLa cells that were transfected with the pcDNA3.1/Myc-His-CKBBP1^{WT}. However, the CKBBP1$^{C80A/H82A}$ co-precipitates contained only 30% of CKII activity in the CKBBP1^{WT} co-precipitates. CKII activity was only slightly detected in the Ni-resin precipitates from the HeLa cells that were transfected with pcDNA3.1/Myc-His, and in the amylose resin precipitates from the HeLa cells that were transfected with pcDNA3.1/Myc-His-CKBBP1^{WT} (Fig. 2B). These experimental results show that the wild-type CKBBP1 interacts with endogenous CKIIβ, as well as the CKII holoenzyme in HeLa cells. Preferential interaction of CKII with CKBBP1^{WT} to CKBBP1$^{C80A/H82A}$ indicates that the Ring-H2 finger motif of CKBBP1 is involved in CKIIβ-binding in HeLa cells.

Wild-type and Ring-H2 finger-disrupted CKBBP1 proteins localize in both nucleus and cytoplasm The protein-protein interaction regulates the subcellular translocation of some proteins (Ron and Kazanietz, 1999). To examine the differences in the subcellular localization of the wild-type and Ring-H2 finger-disrupted CKBBP1 proteins, we fused CKBBP1^{WT} and CKBBP1$^{C80A/H82A}$ to GFP, respectively, then transiently expressed the fusion proteins in the HeLa cells. Confocal fluorescence microscopy indicated that the wild-type and mutant CKBBP1 localized to both the nucleus and cytoplasm. This suggests that the association of CKBBP1 and CKIIβ does not affect the subcellular localization of CKBBP1 (Fig. 3).

Stable expression of Ring-H2 finger-disrupted CKBBP1 in HeLa cells To examine the cellular effects of expressing the Ring-H2 finger-disrupted CKBBP1, HeLa cells were transfected with the plasmids that encode Myc-His-tagged CKBBP1^{WT} and Myc-His-tagged CKBBP1$^{C80A/H82A}$, respectively. The empty vector (pcDNA3.1/Myc-His) was used as the control. After the G418 selection, the expression of Myc-His-tagged CKBBP1 was detected in stable HeLa cell lines by Western blotting with the anti-CKBBP1 antibody (Fig. 4A). The phosphotransferase activity of CKII in the Myc-His-CKBBP1 co-precipitates from stable cell line extracts was determined. Again, the CKII activity was highly detected in the Myc-His-CKBBP1^{WT} co-precipitates, but the Myc-His-CKBBP1$^{C80A/H82A}$ co-precipitates contained only 33% of the CKII activity in the Myc-His-CKBBP1^{WT} co-precipitates (Fig. 4B). This confirms that the Ring-H2 finger motif of CKBBP1 is required for maximal CKIIβ binding in HeLa cells.

CKBBP1, or its homologue ROC1, is a component of E3 ubiquitin ligases that mediate the degradation of substrate proteins (Ohta *et al*., 1999; Skowyra *et al*., 1999; Kamura *et al*., 1999). The Ring-H2 finger of ROC1 is required for ubiquitin ligation (Chen *et al*., 2000). Because CKBBP1 is a CKIIβ-binding protein, we hypothesized that CKIIβ could be

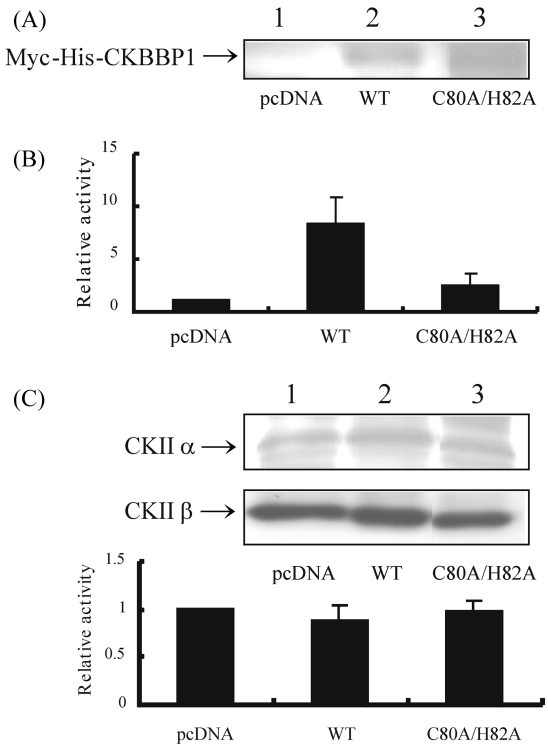

Fig. 4. Stable expression of wild-type and mutant CKBBP1 proteins in HeLa cells. (A) Crude lysates of HeLa cells that stably express the control vector (lane 1), Myc-His-CKBBP1^{WT} (lane 2), or Myc-His-CKBBP1$^{C80A/H82A}$ (lane 3) were separated on a 15% (w/v) SDS-polyacrylamide gel and immunoblotted with an anti-CKBBP1 antibody. (B) Ni-NTA agarose precipitates from HeLa cells that express the control vector, Myc-His-CKBBP1^{WT}, or Myc-His-CKBBP1$^{C80A/H82A}$ were used for CKII activity assay in the presence of [γ-^{32}P]ATP and the CKII substrate peptide (RRREEETEEE). The ^{32}P incorporation in the substrate peptide was measured by scintillation counting. The bars and error bars represent the relative activity of CKII and the range of duplicate experiments, respectively. (C) Crude lysates of HeLa cells that stably express the control vector (lane 1), Myc-His-CKBBP1^{WT} (lane 2), or Myc-His-CKBBP1$^{C80A/H82A}$ (lane 3) were immunoblotted with anti-CKIIα and anti-CKIIβ antibodies (upper panel). The crude lysates were also used for the CKII kinase assay that used [γ-^{32}P]ATP and the substrate peptide (RRREEETEEE) (bottom panel). The bars and error bars represent the relative activity of CKII and the range of duplicate experiments, respectively.

degraded by ubiquitin-mediated proteolysis. To indirectly test this hypothesis, we examined the protein levels of CKIIα and CKIIβ in the HeLa cells that stably express Myc-His-tagged CKBBP1^{WT} or Myc-His-tagged CKBBP1$^{C80A/H82A}$. A Western blot analysis that used whole cell extracts demonstrated that the expression levels of both CKIIα and CKIIβ were unchanged in these stable cell lines (Fig. 4C, upper panel). In addition, the CKII activity assay using these cell extracts indicated that the CKII kinase activity was unchanged in the stable cell lines (Fig. 4C, bottom panel). These results suggest

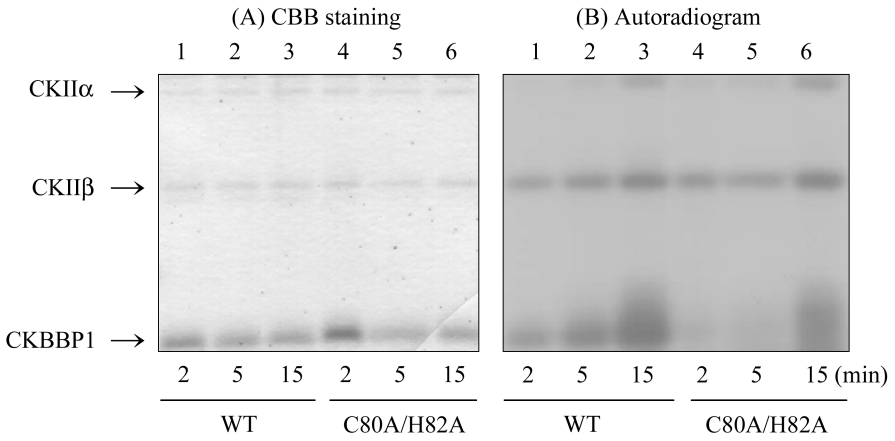

Fig. 5. Requirement of the Ring-H2 finger motif of CKBBP1 for efficient CKII phosphorylation. His-tagged CKBBP1^{WT} (lanes 1-3) or His-CKBBP1$^{C80A/H82A}$ (lanes 4-6) was incubated with CKII holoenzyme in the presence of [γ-^{32}P]ATP for the indicated time. The radiolabeled proteins were separated on a 15% (w/v) SDS-polyacrylamide gel, stained with Coomassie Blue (A), and autoradiographed (B). The positions of the CKII subunits and His-tagged CKBBP1 are indicated on the left.

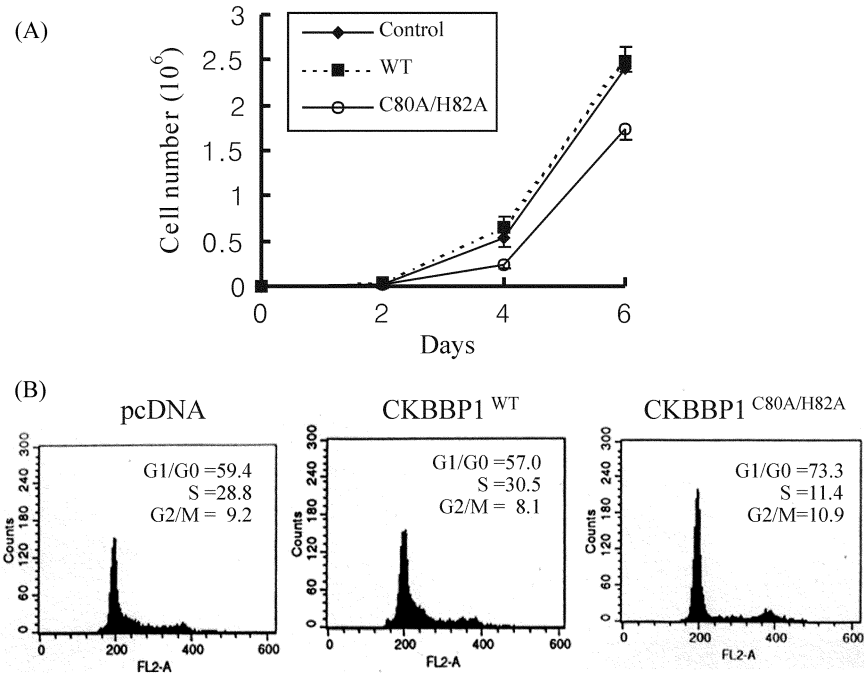

Fig. 6. Effect of stable expression of Myc-His-CKBBP1^{WT} or Myc-His-CKBBP1$^{C80A/H82A}$ in HeLa cells on cell proliferation and cell cycle. (A) HeLa cells (5×10^3) were seeded in six-well dishes with duplicate wells for each cell line. Every 48 h, the cells were trypsinized and counted in triplicate using a hemocytometer. The error bars represent the standard deviation at each time point. (B) HeLa cells (2×10^5) were seeded in 100-mm dishes. Thirty-six hours later, the cells were collected, fixed in 65% ethanol, stained with propidium iodide, and analyzed by FACS. The percentage of cells that were present in each phase of the cell cycle is indicated.

that the CKII protein level is not modulated by the interaction of CKBBP1 with CKIIβ in HeLa cells.

Ring-H2 finger motif of CKBBP1 is necessary for efficient phosphorylation by CKII To examine whether the stable binding of CKBBP1 to CKIIβ is required for the CKII-mediated CKBBP1 phosphorylation, the wild-type and Ring-H2 finger-disrupted CKBBP1 were incubated with purified CKII in the presence of [γ-^{32}P]ATP for the indicated time. As shown in Figure 5, disruption of the Ring-H2 finger motif dramatically reduced the CKBBP1 phosphorylation by CKII. Quantification by a densitometer analysis revealed that the Ring-H2 finger disruption reduced the CKII-mediated CKBBP1 phosphorylation 2 to 3-fold. This demonstrates that the Ring-H2 finger motif of CKBBP1 is required for efficient phosphorylation by CKII. Since it was previously

demonstrated that the CKIIβ subunit can regulate the substrate specificity of CKII (Lin *et al*., 1991; Jakobi and Traugh, 1992), it is likely that the observed CKBBP1 phosphorylation is mediated through its stable binding to CKIIβ.

Stable expression of Ring-H2 finger-disrupted CKBBP1 reduces cell growth Growth curves were performed on the stably transfected HeLa cell lines. Compared with the vector transfected control, the stable expression of Myc-His-CKBBP1^{WT} did not change cell proliferation. However, the stable expression of Myc-His-CKBBP1$^{C80A/H82A}$ induced an apparent decrease in cell proliferation over the time course (Fig. 6A). A FACS analysis was employed to examine whether the stable expression of Myc-His-CKBBP1$^{C80A/H82A}$ interferes with cell cycle progression. As shown in Figure 6B, the increased expression of Myc-His-CKBBP1^{WT} caused no detectable change in the cell cycle profile. However, the accumulation of the G1/G0 peak was observed with the stable expression of Myc-His-CKBBP1$^{C80A/H82A}$. These results indicate that the Ring-H2 finger motif of CKBBP1 plays an important role in the G1/S phase transition of the cell cycle in HeLa cells.

Several biological functions of CKBBP1 have been suggested. CKBBP1 is thought to be involved in the protection of cells from apoptosis that is induced by the redox reagent (Duan *et al*., 1999), in protein ubiquitination (Ohta *et al*., 1999) and in cell growth promotion (Swaroop *et al*., 2000). Although several Ring finger proteins function as transcriptional regulators, CKBBP1 has neither the transcriptional activation nor transcriptional repression activity (Swaroop *et al*., 1999). On the other hand, CKII participates in both the G1/S and G2/M phase transitions of the cell cycle (Hanna *et al*., 1995). In this report, we demonstrated that the Ring-H2 finger motif of CKBBP1 is required for the CKIIβ-binding, efficient CKII-mediated phosphorylation, and cell proliferation. Based on our current observation, we suggest that CKII is involved in cell cycle progression, at least in part, through the interaction with the CKBBP1 and/or CKBBP1 phosphorylation. Studies to test whether the CKII-mediated phosphorylation of CKBBP1 affects cell cycle progression are in progress. The present study will help in the elucidation of the precise role of CKII and CKBBP1 in the regulation of cell proliferation.

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