Phosphorylation of Threonine 10 on CKBBP1/SAG/ROC2/Rbx2 by Protein Kinase CKII Promotes the Degradation of IκBα and p27*Kip1

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In eukaryotic cells, protein kinase CKII is required for progression through the cell division cycle. We recently reported that CKBBP1/SAG/ROC2/Rbx2 associates with the β-subunit of CKII and is phosphorylated by purified CKII in the presence of ATP in vitro. In this report, we demonstrate that CKBBP1 is efficiently phosphorylated in vitro by purified CKII in the presence of GTP and by heparin-sensitive protein kinase in HeLa cell extract. Mutational analysis indicates that CKII phosphorylates threonine at residue 10 within CKBBP1. Furthermore, CKBBP1 is phosphorylated in vivo and threonine to alanine mutation at residue 10 abrogates the phosphorylation of CKBBP1 observed in vivo, indicating that CKII is a major kinase that is responsible for in vivo phosphorylation of CKBBP1. As compared with the wild-type CKBBP1 or CKBBP1T10R (in which threonine 10 is replaced by glutamate), overexpression of nonphosphorylatable CKBBP1 (CKBBP1T10A) results in accumulation of IκBα and p27*Kip1. Experiments using proteasome inhibitor MG132 and CKII inhibitor 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole suggest that the accumulation of IκBα and p27*Kip1 results primarily from the reduction of proteasomal degradation in cells expressing CKBBP1T10A, and that CKII-mediated CKBBP1 phosphorylation is required for efficient degradation of IκBα and p27*Kip1. Overexpression of CKBBP1T10A in HeLa cells suppresses cell proliferation and causes accumulation of G1/G0 peak of the cell cycle. Taken together, our results indicate that CKII may control IκBα and p27*Kip1 degradation and thereby G1/S phase transition through the phosphorylation of threonine 10 within CKBBP1.

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Protein kinase CKII (formerly known as casein kinase II) is a ubiquitous and highly conserved serine/threonine kinase, which is found in all eukaryotes examined and in various subcellular compartments (1–3). The holoenzyme of CKII is a heterotetramer, composed 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 thought to be a regulatory subunit that modulates the catalytic activity of α or α’ subunits and also mediates tetramer formation and substrate recognition (4–6). CKII is a second messenger-independent enzyme. Crystal structure of the catalytic subunit α has confirmed the constitutively active nature of CKII (7). The catalytic activity of CKII is inhibited strongly by heparin and 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) and stimulated by polybasic compounds (8, 9).

It has been suggested that CKII plays a significant role in the control of cell proliferation and transformation. CKII is known to catalyze the phosphorylation of a broad spectrum of substrates, which are involved in cell growth and proliferation, including DNA-binding proteins, nuclear oncoproteins, and transcription factors (1–3). The expression level of CKII is greatly enhanced in a variety of tumor or leukemic cells (10–13). Overexpression of CKIIα in the T cells of transgenic mice results in a high predisposition for lymphoma formation, and coexpression with c-Myc results in the rapid development of leukemia (14). Overexpression of CKIIα or CKIIα’ exhibits cooperativity with Ras in the transformation of rat embryo fibroblasts and Ba/f3 3T3 cells (15). Microinjection of antibodies directed against either CKIIα or CKIIα’ inhibits cell cycle progression in response to serum stimulation in human IMR-90 cells (16–18). An important role of CKII in cell cycle control has also been demonstrated in the yeast Saccharomyces cerevisiae. The analysis using temperature-sensitive mutants for the CKII gene has shown that CKII is required for cell cycle progression in both G1 and G2/M phases of the cell cycle (19). These observations suggest that CKII plays a significant role in cell proliferation and cell cycle control. However, its precise role in cell cycle progression remains largely unknown.

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 (20). This protein has also been identified by others and given the name SAG (sensitive to apoptosis gene) (21). CKBBP1/SAG is a Ring-H2 finger motif-containing protein with a molecular weight of 12.6 kDa. It is localized in both the nucleus and the cytoplasm of cells (21). CKBBP1/SAG was subsequently found to be the second member of regulator of cullin (ROC)/RING box protein (Rbx)/Hrt. ROC1/Rbx1/Hrt1 has been originally identified as the fourth component of SCF (Skp1-Cullin-F-box protein) E3 ubiquitin ligase complex as well as von Hippel-Lindau (VHL) tumor suppressor complex (22–25). Like ROC1/Rbx1/Hrt1, CKBBP1/SAG has E3 ubiquitin ligase activity when complex with Cullin-1 (26). SCF E3 ubiquitin ligase complex promotes ubiquiti-
nation of IκBα, cyclins, and cyclin-dependent kinase inhibitors and is required for G1/S transition of cell cycle (22, 23, 27–29).

In the present study, we show the evidence that CKBBP1 is phosphorylated on threonine residue at position 10 by CKII in vitro and in vivo. Most importantly, disruption of this phosphorylation in CKBBP1 results in accumulation of IκBα and paucity of IκBα and cyclin-dependent kinase inhibitors, which appears to be linked to defects in G1/S transition. To our knowledge, this is the first study reporting physiological significance of the post-translational modification of CKBBP1.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—Polyclonal anti-CKBBP1 antibody was raised against recombinant CKBBP1 as described elsewhere (20). Polyclonal anti-CKIIa and monoclonal anti-CKIIb antibodies were obtained from Calbiochem. Polyclonal anti-IκBα and anti-β-actin antibodies were obtained from Santa Cruz Biotechnology. Polyclonal anti-p27Kip1/H9262

**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 N terminus, was described previously (20). Point mutations in CKBBP1 were made using the QuickChange site-directed mutagenesis kit (Stratagene) or PCR method. Desired mutations were incorporated into oligonucleotide primers. The primers used for threonine to alanine mutagenesis at positions 10 and 49 were 5′-AGACGGAGAGGACGTGGCCCGCTTGCTTCT-3′ and 5′-GAGCTTGAGGCTGAGCAATGCGCCATGGCC-3′, respectively. The primers used for serine to alanine mutagenesis at positions 24 and 42 were 5′-TCCGGGACGTGCTAGGCTCCAAGGCGGGGG-3′ and 5′-GCGTGGCCATGTGGGCTGAGGATTTGCTTCT-3′, respectively. The mutations were selected under line. The mutations were performed as described by the manufacturer. After digestion of the mutations products with NotI and XhoI, the fragment ligated into the NotI/XhoI sites on pET14b (Clontech). After replacement of threonine 10 with glutamic acid, CKBBP1 cDNA was PCR-amplified by using N-terminal mutagenic primer (5′-AGCCCATATGGCCGACGTGGAAAGGGAGAA-GAGATGCGCC-3′) and C-terminal reverse primer (5′-CATCTACGG-AGTCCATTGGCGCATTTT-3′). The mutation site is underlined. After digestion of the PCR products with NotI and XhoI, the fragment was ligated into the NotI/XhoI sites of pET14b.

To generate a Myc-His-tagged CKBBP1 expression construct, the wild-type and mutant CKBBP1 cDNAs were PCR-amplified using primers 5′-CCGGAATTCCGCCATGGCCGACGTGGAAGA-3′ and 5′-GCGGATCCATATGGCCGACGTGGAAAGGGAGAA-GAGATGCGCC-3′ and 5′-GCGGTCAGCTTGAGGATTTGCTTCT-3′, respectively. The mutations were selected. The mutations were performed as described by the manufacturer. After digestion of the mutations products with NotI and XhoI, the fragment ligated into the NotI/XhoI sites of pET14b. To generate a GFP-tagged CKBBP1 expression construct, the wild-type and mutant CKBBP1 cDNAs were PCR-amplified using primers 5′-GCGGATCCATATGGCCGACGTGGAAAGGGAGAA-GAGATGCGCC-3′ and 5′-CATCTACGG-GATGCGCATTTT-3′. The mutation site is underlined. After digestion of the PCR products with NotI and XhoI, the fragment was ligated into the NotI/XhoI sites of pET14b.

**Purification of CKII and CKBBP1**—The standard assay for phosphotransferase activity of CKII was conducted in a reaction mixture containing 20 mM Tris-HCl, pH 7.5, 120 mM KCl, 10 mM MgCl2, 100 μM [γ-32P]ATP in the presence of 1 mM synthetic peptide substrate (RRREEEETEE) in a total volume of 30 μl at 30 °C. The reactions were started by the addition of purified CKII, HeLa cell lysates, or CKBBP1 co-precipitates and incubated for 15 min. The reaction was stopped by the addition of trichloroacetic acid to a final concentration of 10% and centrifuged, and 10 μl of supernatant was then 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 His-tagged CKBBP1 by CKII was performed in a reaction mixture containing 20 mM Tris-HCl, pH 7.5, 150 mM KCl, 10 mM MgCl2, 1 μM dithiothreitol, 1 mM EGTA, 100 μM [γ-32P]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 or HeLa cell lysates 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.

**Phosphoamino Acid Analysis**—Phosphoamino acid analysis was performed essentially as described by others (31). Briefly, the His-tagged CKBBP1 protein was phosphorylated by SDSPolyacrylamide gel electrophoresis, as described above. After staining of the gel with Coomassie blue, the His-tagged CKBBP1 protein band was excised and the protein was extracted in 50 mM NH4HCO3, pH 7.4, containing 0.5% β-mercaptoethanol and 0.1% SDS. The proteins were precipitated in 15% trichloroacetic acid with 20 μg of bovine serum albumin as carrier, washed in 100% ethanol, dried, and hydrolyzed in 6 N HCl for 1 h at 110 °C. The hydrolysate was lyophilized and resuspended in 1 μl of H2O. A 1 μl sample of H2O or 1 μl of samples was spotted on cellulose thin-layer plates, and electrophoresis was performed using 0.1% SDS. Standards were visualized with ninhydrin and 32P-labeled phosphoamino acids were detected by autoradiography.

**Determination of Kinetic Constants**—Kcat and Vmax values were calculated from Lineweaver-Burk transformations of initial rates using the computer program Sigma Plot.

**Cell Culture and Establishment of Stable Cell Line**—HeLa cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum at 37 °C in 5% CO2. For serum starvation, HeLa cell monolayers were washed with ice-cold phosphate-buffered saline and then grown in DMEM supplemented with 0.2% fetal bovine serum for 36 h prior to harvesting.

To establish CKBBP1-expressing stable cell lines, HeLa cells were transfected with the Myc-His-tagged CKBBP1 constructs or the vector control by LipofectAMINE (Invitrogen) as described in the manufacturer. 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. Stable clones were examined for protein expression by Western blotting.

**Preparation of HeLa Cell Extract**—For Western blotting and Ni pull-down assay, ~1 × 106 HeLa cells in 100-mm dishes were washed with ice-cold phosphate-buffered saline, collected by scraping with a rubber policeman, and lysed in 100 μl of ice-cold RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.5 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin). For CKII activity assay, cells were lysed in lysis buffer (50 mM Tris-HCl, pH 8.0, 20 mM NaCl, 1 mM MgCl2, 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 volumes of the supernatants were adjusted for equal protein concentration.

**Western Blotting**—Proteins were separated on polyacrylamide gels in the presence of SDS, transferred electrophoretically to nitrocellulose or nylon membranes of pH 1.9 buffer (7.8% acetic acid and 2.2% formic acid) or 1.9 buffer (20 m M Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% Tween 20) for 2 h and then incubated with specific antibodies. The membrane was washed three times in TBST, and then treated with enhanced chemiluminescence (ECL) system (Amersham Biosciences). Some membranes were stripped in stripping buffer (2% SDS, 100 mM β-mercaptoethanol, and 50 mM Tris-HCl, pH 7.0) at 50 °C for 1 h with gentle shaking and reprobed with anti-β-actin antibody as a control for protein loading.

**Pull-down Assays**—Ni-NTA agarose beads and lysates from HeLa cells that were transfected with the pcDNA3/1-Myc-His-CKBBP1 were incubated in 200 μl of binding buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM PMSF). The reaction was allowed to proceed for 1 h while the beads were washed three times with washing buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM PMSF, 200 μM imidazole), the bound proteins were eluted with elution buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM PMSF, 200 μM imidazole), denatured in 4× SDS reducing protein gel loading buffer, and then resolved using SDS-polyacrylamide gel. The eluted proteins were visualized by Western blotting with anti-Myc, anti-CKIIα, or anti-CKBBP1 antibodies.

**Metabolic 32P Labeling**—HeLa cells transfected with Myc-His-CKBBP1 were labeled with [32P]orthophosphate at 0.4 μCi/ml in phosphate-free Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum for 24 h at 37 °C in 5% CO2. Cells were lysed in RIPA buffer and precipitated with 50 μl of nitrocellulose-NTA agarose. The beads were washed three times with washing buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM PMSF, 200 μM imidazole), denatured in 4× SDS reducing protein gel.
loading buffer, and then resolved using 15% SDS-polyacrylamide gel. The gel was stained with Coomassie blue, dried, and subjected to autoradiography.

RNA Binding Assay—Wild-type and mutant CKBBP1 were incubated with poly(U)-agarose (Sigma) in RNA binding buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM MgCl₂, 0.1% Nonident P-40, 50 μM ZnCl₂, 2% glycerol, and 1 mM dithiothreitol) at 4 °C for 1 h. After an extensive washing, the immobilized proteins were recovered by an elution with 500 mM NaCl, separated on a 15% SDS-polyacrylamide gel, and then immunoblotted with anti-CKBBP1 antibody.

Subcellular Localization of CKBBP1—To determine the subcellular localization of the wild-type and mutant CKBBP1, 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—HeLa cells stably expressing the wild-type and mutant CKBBP1 were seeded in 6-well dishes at a starting density of 5,000 cells/well, with duplicate wells for each cell line. Every 24 h, cells were trypsinized and counted in triplicate using a hemocytometer. Trypan blue was used to distinguish viable cells from non-viable cells.

FACS Analysis—HeLa cells (2 × 10⁶) were seeded in 100-mm dishes containing Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. 36 h later, the cells were collected in phosphate-buffered saline containing 2% fetal bovine serum, fixed in 65% ethanol for 1 h at 4 °C, and then incubated in 50 μg/ml RNase-free RNase A (Sigma), 25 μg/ml propidium iodide (Sigma), and 0.6% sodium citrate for 30 min at 37 °C. Flow cytometric determination of cellular DNA content was performed on a Coulter Elite ESP Cell Sorter (Beckman). 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

CKBBP1 Is Phosphorylated by CKII in Vitro—We have shown previously that CKBBP1 is phosphorylated by CKII in vitro (20). To confirm the phosphorylation of CKBBP1 by CKII, His-CKBBP1 was incubated with purified CKII in the presence of [γ-32P]GTP, because it has been shown that, unlike other protein kinases, CKII can utilize GTP instead of ATP as the phosphate donor (32). As shown in Fig. 1A, CKII phosphorylated CKBBP1 in the presence of GTP as the phosphate donor.

In order to further characterize the nature of CKBBP1 phosphorylation by CKII, a kinetic analysis was performed. The Kᵋₘ was 6.25 μM, and the Vₘₐₓ was 232.6 pmol/min/mg with CKBBP1 as a substrate of CKII (Fig. 1B). These parameters were comparable with reports of CKII kinetics with other protein substrates. For example, the Kᵋₘ value obtained for CKBBP1 was greater than the value obtained for topoisomerase II (0.4 μM), but it was lower than the value obtained for tubulin (20 μM) (33, 34). With a Kᵋₘ in the submicromolar range, we conclude that CKBBP1 is an efficient substrate for CKII.

CKII Is a Predominant CKBBP1 Kinase in HeLa Cell Extract—To determine whether CKII is a predominant CKBBP1 kinase in mammalian cells, we performed an in vitro kinase assay using purified His-CKBBP1 and whole cell extract from HeLa cells with and without the addition of CKII inhibitors heparin and DRB. Following the kinase reaction, the His-CKBBP1 protein was precipitated with Ni-NTA agarose and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. As shown in Fig. 2, His-CKBBP1 was phosphorylated by HeLa cell extract and the CKBBP1 phosphorylation was significantly inhibited by CKII inhibitors heparin and DRB. Quantification by liquid scintillation counting of the [32P]-labeled CKBBP1 bands revealed that 0.3 μM heparin and 50 μM DRB inhibited 89 and 70% of the phosphorylation by HeLa cell extract, respectively. Coomassie blue staining of the gel showed that CKBBP1 was equivalent in each reaction (left panel of Fig. 2). These results demonstrate that CKBBP1 is phosphorylated predominantly by a heparin- and DRB-sensitive kinase in cell extract.

CKII Phosphorylates Threonine at Residue 10 within CK-

BBP1 in Vitro—To determine the nature of the phosphorylated residue, we performed phosphoamino acid analysis of CKBBP1 labeled with [32P] in vitro. Phosphoamino acid analysis revealed that phosphorylation of CKBBP1 occurred exclusively on threonine, but not on serine or tyrosine residues (Fig. 3A).

There are only two threonine residues at positions 10 and 49 within the polypeptide chain of CKBBP1. In order to determine the CKII-phosphorylation site within CKBBP1, both threonine residues were replaced by alanine. For the control experiment, serine residues at positions 24 and 42 were substituted by alanine. This resulted in the following CKBBP1 mutants: CKBBP1T₁₀A, CKBBP1T₄₉A, CKBBP1S₂₄A, and CKBBP1S₄₂A.

The mutant CKBBP1 proteins were expressed and purified in E. coli and subjected to phosphorylation by CKII. As shown in Fig. 3B, the CKII-mediated phosphorylation was completely abolished in CKBBP1T₁₀A, but not in CKBBP1T₄₉A, showing that the threonine 10 was the only CKII phosphorylation site in CKBBP1. Serine to alanine mutations had no significant effect on the CKBBP1 phosphorylation by CKII.

To determine whether threonine at residue 10 within CKBBP1 is phosphorylated by HeLa cell extract-derived kinases, His-CKBBP1WT and His-CKBBP1T₁₀A were added to HeLa cell extract and subjected to an in vitro kinase assay. After precipitation with Ni-NTA agarose beads, the CKBBP1 proteins were analyzed by SDS-polyacrylamide gel electrophoresis and auto-
radiography. As shown in Fig. 3C, alanine mutation of threonine 10 completely abolished the CKBBP1 phosphorylation by HeLa cell extract-derived kinases. These results demonstrate that threonine at residue 10 is the only site for CKBBP1 phosphorylation in the whole cell extract.

CKII preferentially phosphorylates serine/threonine residues followed by a stretch of acidic residues on the immediate C-terminal side (11 to 13). Among these acidic residues, the third position after the phosphoacceptor is the most important determinant. However, further analyses have indicated that acidic residues located at positions 2 to 7 can also serve as specificity determinants for CKII phosphorylation (1, 35). Our current observation also indicates that CKII phosphorylates serine/threonine residue without any acidic amino acids at positions +1 to +3 after phosphoacceptor. In case of CKII-mediated CKBBP1 phosphorylation, instead, two acidic residues locate at positions −1 and −2 before the phosphoacceptor (Fig. 3D).

**Threonine 10 within CKBBP1 Is Phosphorylated by CKII in Vivo**—To determine whether the CKBBP1 protein is phosphorylated on threonine at residue 10 in vivo, HeLa cells were transfected with the plasmids encoding Myc-His-CKBBP1WT or Myc-His-CKBBP1T10A. The empty vector (pcDNA3.1/Myc-His) was used as a control. After selection for G418 resistance, the stable expression of CKBBP1 was examined by immunoblotting with anti-CKBBP1 antibody. As shown in Fig. 4A, the stable expression of Myc-His-CKBBP1 was detectable in HeLa cells that were transfected with pcDNA3.1/Myc-His-CKBBP1WT.
of CKBBP1, and CKII Expression

Effects of Threonine 10 Phosphorylation within CKBBP1 on its binding to RNA and CKIIβ, CKII expression, and subcellular localization of CKBBP1. A, His-CKBBP1 WT (lane 1), His-CKBBP1T10A (lane 2), and His-CKBBP1T10E (lane 3) were incubated with the control vector (lane 1), pcDNA3.1/Myc-His-CKBBP1 WT (lane 2), or pcDNA3.1/Myc-His-CKBBP1T10A (lane 3) were 32P-labeled, lysed, and precipitated with Ni-NTA agarose. The immobilized proteins were recovered with 200 mM imidazole, separated by 15% (w/v) SDS-polyacrylamide gel electrophoresis, and visualized by autoradiography. B, HeLa cells stably transfected with pcDNA3.1/Myc-His-CKBBP1WT. How-

Effects of Threonine 10 Phosphorylation within CKBBP1 on CKBBP1 Binding to RNA and CKIIβ, Subcellular Localization of CKBBP1, and CKII Expression—Although the biological importance remains to be elucidated, it has been reported that CKBBP1 binds to RNA in vitro (36). To examine whether the CKII-mediated phosphorylation of CKBBP1 modulates its binding activity to RNA, we generated a variant of CKBBP1, CKBBP1T10E, with substitution of the CKII phosphorylation site to glutamic acid, which may mimic the spatial and charge contributions of phosphorylated threonine (37, 38). Wild-type or mutant CKBBP1 proteins, CKBBP1T10A and CKBBP1T10E were incubated with poly(U)-agarose beads and the immobi-

However, Myc-His-CKBBP1 was not detectable in cells that were transfected with pcDNA3.1/Myc-His vector alone.

Using these stable cell lines, we metabolically 32P-labeled CKBBP1 in HeLa cells. Myc-His-CKBBP1 WT and Myc-His-CKBBP1T10A were precipitated from these cells with Ni-NTA agarose resin and the precipitates were resolved on SDS-polyacrylamide gel and autoradiographed. As shown in Fig. 4B, the phosphorylated CKBBP1 band was observed in HeLa cells that were transfected with pcDNA3.1/Myc-His-CKBBP1 WT. However, no phosphorylated CKBBP1 band was observed in HeLa cells that were transfected with pcDNA3.1/Myc-His-CKBBP1T10A or pcDNA3.1/Myc-His vector alone. Because similar amounts of Myc-His-CKBBP1 WT and Myc-His-CKBBP1T10A were co-precipitated (data not shown, see Fig. 5B), these results apparently indicate that CKBBP1 is phosphorylated on threonine residue at position 10 by CKII in HeLa cells. Since both the α and β subunits of CKII have been shown to be autophosphorylated in the cells, the co-precipitated phosphoprotein bands of 44 and 28 kDa are thought to represent the α and β subunits of CKII, respectively. Indeed, Western blots of the co-precipitates probed anti-CKIIα and anti-CKIIβ antibodies showed that CKIIα and CKIIβ were co-precipitated with CKBBP1 in HeLa cells (see below).

Effects of Threonine 10 Phosphorylation within CKBBP1 on CKBBP1 Binding to RNA and CKIIβ, Subcellular Localization of CKBBP1, and CKII Expression

To examine the effect of CKBBP1 phosphorylation on the binding to CKIIβ, HeLa cells were transfected with pcDNA3.1/Myc-His-CKBBP1T10E and a stable cell line was obtained as above. After selection for G418 resistance, the stable expression of CKBBP1 was examined by immunoblotting with
anti-CKBBP1 antibody (data not shown). HeLa cells stably transfected with pcDNA3.1/Myc-His-CKBBP1 WT, pcDNA3.1/Myc-His-CKBBP1 T10A, pcDNA3.1/Myc-His-CKBBP1 T10E or pcDNA3.1/Myc-His (vector control) were lysed and the complexes of CKBBP1 and CKII were precipitated with Ni-NTA agarose beads. Western blots of the co-precipitates probed anti-CKIIα and anti-CKIIβ antibodies showed that similar amounts of the respective CKII subunits were co-precipitated in these cells (Fig. 5B). From this observation, we speculate that threonine 10 within CKBBP1 is not involved in the CKIIβ binding. This is consistent with our previous study in which the C-terminal region containing the Ring-H2 finger motif within CKBBP1 is sufficient for CKIIβ binding (20).

CKII phosphorylation is known to regulate the nuclear translocation of some proteins (39, 40). To examine the differences in the subcellular localization of the wild-type and mutant CKBBP1 proteins, we fused CKBBP1 WT, CKBBP1 T10A, or CKBBP1 T10E to GFP, and expressed transiently the fusion proteins in HeLa cells. Confocal fluorescence microscopy indicated that wild-type and mutant CKBBP1 localized to both the nucleus and the cytoplasm (data not shown).

It has been shown that ROC1/Rbx1 and CKBBP1/SAG/ROC2 are components of SCF E3 ubiquitin ligases that mediate the degradation of substrate proteins (22–26). Because CKBBP1 is a CKIIβ-binding protein, we hypothesized that CKIIβ could be degraded by ubiquitin-mediated proteolysis. To test this hypothesis, we examined the protein levels of CKIIα and CKIIβ in HeLa cells stably expressing Myc- His-CKBBP1 WT, Myc-His-CKBBP1 T10A, or Myc-His-CKBBP1 T10E by Western blot analysis of whole cell extracts using anti-CKIIα and anti-CKIIβ antibodies. As shown in Fig. 5C, the expression levels of both CKIIα and CKIIβ are not altered in these stable cell lines. When CKII activity in these cell lysates was assessed using the synthetic peptide substrate RRREEETEE, similar levels of CKII activity were detected (Fig. 5D).

Taken together, these results suggest that CKII-mediated CKBBP1 phosphorylation has no effect on properties of CKBBP1 such as its ability to bind RNA, associate with CKIIβ, or localize to particular subcellular compartment. Alternatively, the possibility that carboxylate group of glutamic acid 10 within CKBBP1 T10E is not functionally equivalent to phosphate group on the phosphorylated threonine 10 cannot be ruled out. To assess directly the effect of CKII phosphorylation on these properties of CKBBP1, the phosphorylated CKBBP1 protein will be used in future study.

Overexpression of CKBBP1 T10A Causes Accumulation of IκBα and p27Kip1—Since SCF E3 ubiquitin ligases have been known to catalyze ubiquitin-mediated proteolysis of IκBα and p27Kip1 (22, 23, 29), we investigated the effect of stable expression of CKBBP1 T10A on IκBα and p27Kip1. The protein levels of IκBα and p27Kip1 in HeLa cells stably expressing Myc-His-CKBBP1 WT, Myc-His-CKBBP1 T10A, or Myc-His-CKBBP1 T10E were examined by Western blot analysis of whole cell extracts using anti-IκBα and anti-p27Kip1 antibodies. As shown in Fig. 6A, the expression levels of both IκBα and p27Kip1 are unchanged in cells expressing CKBBP1 T10A (lane 3) or CKBBP1 T10E (lane 4) compared with the respective controls (lanes 1 and 2). These results indicate that the effect of stable expression of CKBBP1 T10A or CKBBP1 T10E on IκBα and p27Kip1 accumulations is mainly due to the reduction in proteasome-dependent degradation. In addition, these results suggest that the threonine 10 phosphorylation within CKBBP1 is required for efficient proteasome-dependent degradation of IκBα and p27Kip1.

To further confirm that CKII-mediated CKBBP1 phosphorylation promotes the degradation of IκBα and p27Kip1, the stable HeLa cell lines were treated with 20 μM DRB during serum starvation and then the protein levels of IκBα and p27Kip1 were examined by Western blot analysis. Treatment with DRB resulted in the apparent induction of IκBα and p27Kip1 accumulation in cells expressing control vector, CKBBP1 WT, or CKBBP1 T10E, allowing that their amounts were increased to the levels in cells expressing CKBBP1 T10A (Fig. 6B). These observations indicate that the effect of stable expression of CKBBP1 T10A on IκBα and p27Kip1 accumulations is mainly due to the reduction in proteasome-dependent degradation. In addition, these results suggest that the threonine 10 phosphorylation within CKBBP1 is required for efficient proteasome-dependent degradation of IκBα and p27Kip1.

Overexpression of CKBBP1 T10A Reduces Cell Growth—Since
we demonstrated that overexpression of CKBBP1T10A in HeLa cells caused accumulation of p27Kip1, which is the cyclin-dependent kinase inhibitor primarily responsible for the control of cell growth at the G1/S transition, we investigated whether overexpression of CKBBP1T10A reduces cell proliferation. To investigate the effect of overexpression of the CKBBP1 mutants on cell proliferation, growth curves were performed on the stably transfected HeLa cell lines. Compared with the vector-transfected control, stable expression of Myc-His-CKBBP1WT or Myc-His-CKBBP1T10E did not significantly change cell proliferation. However, stable expression of Myc-His-CKBBP1T10A induced an apparent decrease in cell proliferation over the time course (Fig. 7A). FACS analysis was employed to examine whether stable expression of Myc-His-CKBBP1WT or Myc-His-CKBBP1T10E did not cause a detectable change in the cell cycle profile. However, accumulation of G1/G0 peak was observed with the stable expression of Myc-His-CKBBP1T10A. These results indicate that the phosphorylation of CKBBP1 on threonine 10 plays an important role in the G1/S phase progression of the cell cycle in HeLa cells.

Cell cycle progression from G1 to S is precisely regulated by timely synthesis and degradation of specific regulatory proteins (41, 42). SCF E3 ubiquitin ligase complex promotes ubiquitination and degradation of IκBα and p27Kip1 and this proteolysis process is necessary for G1/S transition (22, 23, 27-29). The present study demonstrates that the phosphorylation of threonine 10 within CKBBP1 is required for the efficient degradation of IκBα and p27Kip1 as well as the G1/S phase progression. Since CKII has been known to be required for G1/S transition of the cell cycle (19), we suggest that CKII may be involved in G1/S transition, at least in part, through modulation of the p27Kip1 degradation by CKBBP1 phosphorylation.

It has been shown that CDC34, ubiquitin-conjugating enzyme E2, interacts with SCF ubiquitin ligase and is involved in the ubiquitination of many substrates including IκBα and p27Kip1. CDC34 also associates with the β subunit of CKII and is phosphorylated by CKII (43). Our previous and present studies have shown that CKBBP1 associates with the β subunit of CKII and is phosphorylated by CKII. All these data lead to the possibility that CKII participates in the formation of various complex such as CKII-SCF, CKII-CDC34, and/or CKII-SCF-CDC34-CKII and that CKII may modulate protein ubiquitination in multiple steps. Whether CKII-mediated CKBBP1 phosphorylation regulates directly the activity of SCF ubiquitin

![Fig. 7. Overexpression of CKBBP1T10A reduces cell growth. A, HeLa cells (5 x 10⁶) stably expressing the control vector, Myc-His-CKBBP1WT, Myc-His-CKBBP1T10A, or Myc-His-CKBBP1T10E were seeded in 6-well dishes, with duplicate wells for each cell line. Every 24 h, cells were trypsinized and counted using a hemocytometer. These experiments were performed three times with similar results. B, HeLa cells (2 x 10⁶) stably expressing the control vector, Myc-His-CKBBP1WT, Myc-His-CKBBP1T10A, or Myc-His-CKBBP1T10E were seeded in 100-mm dishes. 36 h later, the cells were collected, fixed in 65% ethanol, stained with propidium iodide, and then analyzed by FACS. The percentage of cells present in each phase of the cell cycle is indicated.](http://www.jbc.org/content/jbc/179/14/28468/F1.large.jpg)
ligase is presently unclear. In future study, it will be important to investigate the role of CKII in the modulation of SCF ubiquitin ligase activity in the cells.

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Phosphorylation of Threonine 10 on CKBBP1/SAG/ROC2/Rbx2 by Protein Kinase CKII Promotes the Degradation of IκBα and p27Kip1

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