BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **238,** 462–467 (1997)
ARTICLE NO. RC977317

The Highly Basic Ribosomal Protein L41 Interacts with the β Subunit of Protein Kinase CKII and Stimulates Phosphorylation of DNA Topoisomerase IIα by CKII

Ji-Hoon Lee, Jeong-Min Kim, Min-Seung Kim, Yim-Tae Lee, Daniel R. Marshak,* and Young-Seuk Bae[1]

*Department of Biochemistry, College of Natural Sciences, Kyungpook National University, Taegu 702-701, Korea; and *Osiris Therapeutics, Inc., and The Johns Hopkins University School of Medicine, Baltimore, Maryland 21231-2001*

Received July 29, 1997

Protein kinase CKII (CKII) is a heterotetramer composed of two catalytic (α or α') and two regulatory (β) subunits. Using the yeast two-hybrid system, we have identified the highly basic, ribosomal protein L41 as a cellular protein capable of interacting with the β subunit of CKII. We show, furthermore, using purified proteins, that L41 protein and CKIIβ associate directly *in vitro*. L41 protein is not a substrate for CKII phosphorylation, and it does not stimulate CKII activity with either β-casein or synthetic peptide substrate (RRREEETEEE). However, L41 protein stimulates the phosphorylation of DNA topoisomerase IIα by CKII by 2.5 times. Additionally, L41 protein enhances the autophosphorylation of CKIIα. The data indicate that L41 protein associates with CKII and can modulate its activity toward a specific substrate or substrates. The direct interaction of CKIIβ with ribosomal proteins also suggests that CKIIβ itself or CKII holoenzyme may be involved in ribosome assembly or translational control.

Protein kinase CKII (CKII) is a serine/threonine protein kinase found in both the nucleus and cytoplasm of all eukaryotes (1-3). CKII is a tetrameric complex consisting of α, α', and β subunits and exists as an $\alpha_2\beta_2$, $\alpha\alpha'\beta_2$, or $\alpha'_2\beta_2$ structure (4). The α and α' subunits are the catalytic subunits, while the β subunit is thought to be a regulatory subunit because it mediates the tetramer formation (5), modulates catalytic activity (6, 7), and influences substrate recognition (8). Heparin has been shown to be an inhibitor of CKII, whereas polybasic compounds such as polyamines and polylysine stimulate CKII activity (9). The stimulation of CKII activity by polybasic compounds is mediated by the β subunit (8).

CKII plays a critical role in cell growth and proliferation, based on genetic studies in budding and fission yeasts and in *Dictyostelium discoideum,* which suggest that CKII is essential for cell viability (10-12). CKII mediated phosphorylation of a large number of cytosolic and nuclear proteins appears to be important in cell division (1-3, 13). A role in cell growth is also suggested by the observation that the overexpression of CKIIα leads to tumorigenesis in mice overexpressing myc (14). The overexpression of the β subunit in fission yeast leads to the inhibition of cell growth (11).

To investigate further the physiological role and mechanism of action of CKII, we have identified proteins that interact with the subunits of CKII *in vivo* using the two-hybrid system. Previously we have reported the ribosomal protein L5 as a CKIIβ-associating protein (15). Here we show that a highly basic ribosomal protein L41 interacts with CKIIβ *in vivo* as well as *in vitro* and stimulates the phosphorylation of DNA topoisomerase IIα by CKII. However, L41 protein does not increase CKII activity toward either β-casein or synthetic peptide substrate (RRREEETEEE). The results suggest that L41 protein may act as a physiological stimulator of CKII toward a specific substrate or substrates.

MATERIALS AND METHODS

Materials. Polylysine (molecular weight 4,000-15,000), polyarginine (molecular weight 5,000-15,000), and dephosphorylated β-casein were from Sigma. [γ-^{32}P]ATP and the chemiluminescence detection system (ECL) were from Amersham Corp. Human DNA topoisomerase IIα was from TopoGEN, Inc. Human CKII holoenzyme and CKIIα were purified to homogeneity via four chromatography steps using DEAE-cellulose, phosphocellulose, heparin-agarose, and gel filtration from bacteria expressed bicistronically both CKIIα and

[1] Corresponding author. Fax: 82-53-943-2762. E-mail: ysbae@kyungpook.ac.kr.

Abbreviations used: CKII, protein kinase CKII; CKIIα, the α subunit of CKII; CKIIβ, the β subunit of CKII; BSA, bovine serum albumin.

0006-291X/97 $25.00

CKIIβ as described previously (16, 17) with some modification. Human CKIIβ was also bacterially expressed and purified to homogeneity using DEAE-cellulose, hydroxylapatite, and gel filtration column chromatography. The purities of CKII holoenzyme, CKIIα, and CKIIβ were verified by Coomassie blue staining and by immunodetection using CKIIα- and CKIIβ-specific antibodies.

Two-hybrid screen. The two-hybrid screen was performed essentially as described by Kim *et al.* (15). DNA sequencing was carried out by the dideoxy chain termination method (18).

Peptide synthesis. The ribosomal protein L41 peptide (MRAKWRKKRMRRLKRKRRKMRQRSK), CKII peptide substrate (RRREEETEEE), and SV40 large T antigen peptide (ADAQHATPPKKKRKVEDPKDF) were synthesized using an automatic synthesizer (Model 431A, Applied Biosystems) and purified by reverse phase chromatography.

In vitro binding assay. An aliquot of protein (10 μg/ml) or peptide (3 mM) solutions were directly spotted on nitrocellulose membrane. The membrane was incubated with TBST buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20) containing 4% BSA at room temperature for 40 min, washed with TBST four times, and then overlaid with CKIIβ (2 μg/ml) for 1 h on ice. For the control experiment, the membrane was not overlaid with CKIIβ. After washing four times with TBST for 40 min, the membrane was blocked for 40 min at room temperature in TBST containing 5% nonfat, dry milk. The membrane was incubated with antibody against CKIIβ in 0.2% nonfat, dry milk for 1 h, then washed four times in TBST. The bound antibody was visualized using ECL detection system.

In vitro kinase assay. 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 $MgCl_2$, and 100 μM [γ-^{32}P] ATP in the presence of 5 mg/ml dephosphorylated β-casein or 1 mM synthetic peptide substrate (RRREEETEEE) in a total volume of 30 μl at 30 °C. The reactions were started by the addition of the enzyme (25 nM) and incubated for 15 min. In some experiments, L41 protein, polylysine, or polyarginine were added to the reaction mixture. When CKII was assayed with β-casein, the reaction was terminated by spotting 10 μl of the reaction mixture on to P-81 phosphocellulose paper as described previously (19). When CKII was assayed with the synthetic peptide substrate, the reaction was stopped by the addition of TCA to a final concentration of 10% and centrifuged, and 10 μl of supernatant was applied to P-81 paper (20). The paper was washed in 100 mM phosphoric acid, and the radioactivity was measured by scintillation counting.

To analyze autophosphorylation of CKII and topoisomerase IIα phosphorylation, the reactions were subjected to SDS-polyacrylamide gel electrophoresis. The gels were dried and autoradiographed, and the protein bands were excised and counted by liquid scintillation counting.

RESULTS

Ribosomal L41 Protein as a CKIIβ-Binding Protein

To identify human proteins that interact with CKIIβ *in vivo,* we used the two-hybrid system (21). The complete open reading of CKIIβ was fused to the GAL4 DNA binding domain in pGBT9, while a library of human proteins fused to the transcriptional activation domain in pGADGH. From the screen of 1.3×10^6 independent transformants, twenty positive clones were identified that induced the lacZ reporter gene only in the presence of pGBT9-CKIIβ. Of the twenty positive clones identified, one clone represented the ribosomal protein L5 (14). In this paper we report another clone, CL33. The clone CL33 contained a 452 nucleotide insert with an adaptor sequence of 8 nucleotides, a reading frame of 122 nucleotides, and a 3′ noncoding region of 302 nucleotides, followed by a poly(A) tract (Fig. 1). When the nucleotide sequence was compared with reported sequences using the Genbank database, the insert was identical to the ribosomal protein L41 (accession number Z12962). The insert encodes a polypeptide of 42 amino acids, which includes the full length (25 amino acids) of L41 protein (22). The predicted L41 protein sequence is unusually rich in lysine and arginine (overall 68%).

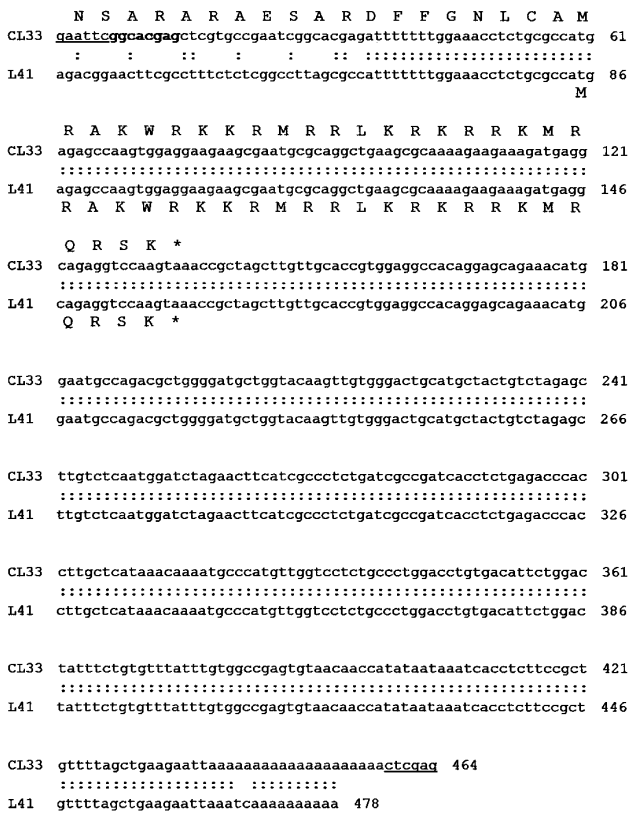

FIG. 1. The nucleotide and deduced amino acid sequences of CL33. A comparison of the nucleotide and amino acid sequences of CL33 with human ribosomal L41 protein (22) is shown. The nucleotide sequence is given in lowercase letters and the amino acid sequence is in capital letters. Colons indicate identical 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 pGADGH cDNA library, is indicated in bold face.

Direct Binding of CKIIβ to L41 Protein

To test whether the interaction between the CKIIβ and L41 proteins was direct or was mediated by a third yeast protein, we examined the association of the CKIIβ and L41 proteins *in vitro,* using CKIIβ bacterially expressed and purified. The L41 protein (25 amino

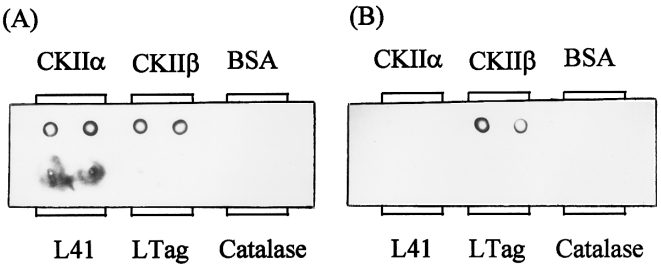

FIG. 2. Direct binding of CKIIβ to L41 protein. 4 μl of each polypeptide solution of CKIIα, CKIIβ, BSA, L41, SV40 large T antigen peptide (23), and catalase were directly spotted on nitrocellulose membrane, and the membrane was incubated in 4% BSA solution. After washing, the membrane was overlaid with CKIIβ for 40 min, and the protein-protein interaction with CKIIβ was observed by immunodetection with CKIIβ antibody. In panel B, the overlay step was omitted.

acids except the untranslated region of L41 in Fig. 1) was synthesized using a peptide synthesizer and purified by reverse phase chromatography. Because the protein L41 was a small polypeptide (molecular weight 3,456), it was difficult to detect the L41-bound form of CKIIβ by gel filtration. Thus, we designed a simple method to detect protein-protein interaction *in vitro*. L41 and control polypeptides including CKIIα, CKIIβ, BSA, SV40 large T antigen peptide (23), and catalase, were first spotted on nitrocellulose membrane and then overlaid with the purified CKIIβ, as described under Materials and Methods. After washing the membrane, the protein-protein interaction was observed by immunodetection with a specific antibody against CKIIβ (Fig. 2). When the membrane was overlaid with CKIIβ, the CKIIβ protein bound to the immobilized CKIIα and L41 protein, indicating that CKIIβ interacts directly with CKIIα and L41 protein. But the CKIIβ protein did not bind to BSA, the SV40 large T antigen peptide, and catalase (Fig. 2A). In contrast, when the membrane was not overlaid with CKIIβ, the protein-protein interaction between CKIIβ and other proteins was not observed (Fig. 2B). Thus, we can conclude that CKIIβ binds directly to L41 protein.

Effect of L41 Protein on CKII Activity with β-Casein and Synthetic Peptide Substrate

To examine whether CKII catalyzes the phosphorylation of L41 protein, L41 protein was incubated with purified CKII in the presence of [γ-^{32}P]ATP. L41 protein, however, was not a substrate for CKII phosphorylation (data not shown).

Polyamines, such as spermine and spermidine, and polylysine stimulate the activity of CKII through its interaction with CKIIβ (7, 8). The finding that L41 protein was a highly basic polypeptide and bound to CKIIβ suggested that L41 protein might stimulate activity of CKII. To address this possibility, we examined the effects of L41 on CKII activity. To determine the optimum amount which would stimulate enzyme activity maximally, we performed an experiment that increasing amounts of L41 were added to a constant concentration (25 nM) of CKII with the synthetic peptide (RRREEETEEE) as substrate under standard assay conditions as described under Materials and Methods (Fig. 3A). Addition of L41 protein, however, did not lead to an increase in CKII activity. Instead, the activity of CKII was a little inhibited when increasing amounts of L41 were added. Polylysine and polyarginine stimulated the activity of CKII as shown in Fig. 3A.

To determine if the response of L41 protein to CKII activity is dependent upon ionic strength, the influence of increasing amounts of KCl on CKII was tested with β-casein as substrate. As shown in Fig. 3B, increasing KCl concentration did not significantly alter the response of L41 protein on CKII activity. The influence of increasing amounts of $MgCl_2$ on CKII was also examined, but increasing $MgCl_2$ concentration (2.5 to 20 mM) did not affect the response of L41 on CKII activity (data not shown). The time courses of phosphorylation of β-casein by CKII alone or by CKII plus L41 protein were compared (Fig. 3C). The time course of phosphorylation was almost unaffected by addition of L41 protein. The effect of L41 protein on thermal inactivation of CKII was also examined. CKII either alone or with L41 protein was first incubated at 45 °C, and then the residual CKII activity was measured under standard assay conditions with the synthetic peptide substrate (RRREEETEEE). L41 protein was unable to protect CKII against thermal inactivation (Fig. 3D).

Effect of L41 Protein on CKII Activity with Topoisomerase IIα and Autophosphorylation of CKII

Because the synthetic peptide substrate (RRREEETEEE) and β-casein are artificial substrates, we examined the effect of L41 protein on the phosphorylation of physiological substrates by CKII including topoisomerase IIα and autophosphorylation. Topoisomerase IIα has been shown to be phosphorylated by CKII *in vitro* as well as *in vivo* (24). As shown in Fig. 4, both L41 protein and polylysine stimulate phosphorylation of topoisomerase IIα by CKII. However, L41 protein was more effective than polylysine, generating a 2.5-fold increase in phosphotransferase activity of CKII toward topoisomerase IIα (Fig. 4, lane 4). A slight increase (1.6 times) in CKII activity was observed with the same concentration (on a molar basis) of polylysine under our standard assay conditions (Fig. 4, lane 5).

Since it has been shown that polylysine inhibits autophosphorylation of CKIIβ and stimulates that of CKIIα (25), we examined the effect of L41 protein on the autophosphorylation of CKII subunits. As shown by the data of Fig. 5, with the recombinant human CKII puri-

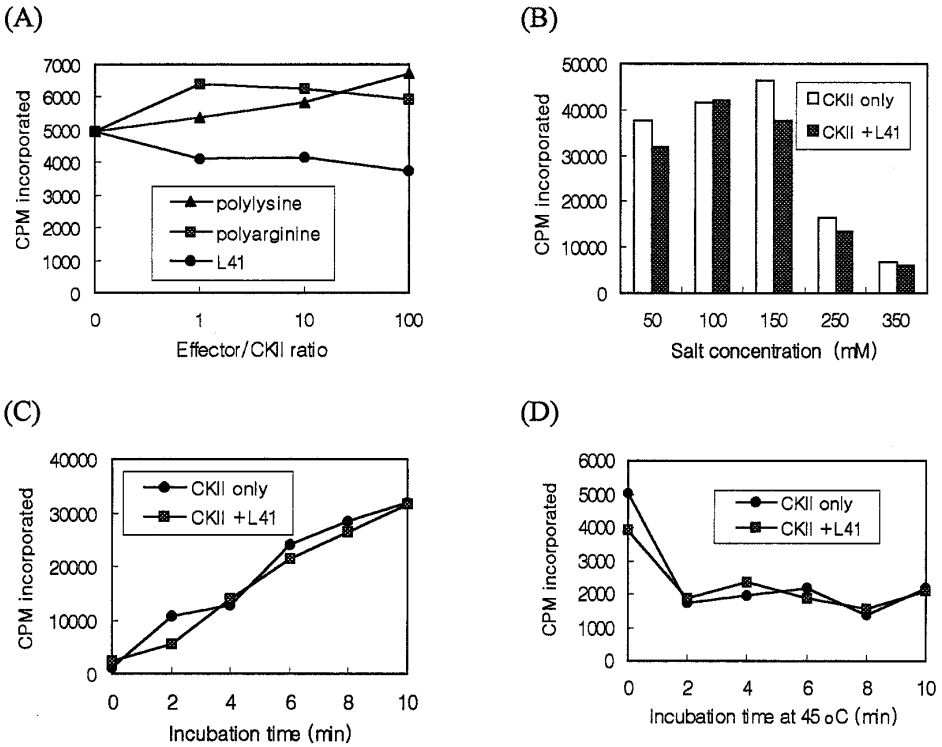

FIG. 3. Effect of L41 protein on CKII activity with β-casein or synthetic peptide (RRREEETEEE) as substrates. (A) Effects of polylysine, polyarginine, and L41 on CKII activity. CKII activity was monitored with the synthetic peptide substrate under standard assay conditions as described in Materials and Methods using a constant amount of CKII (25 nM) and increasing amounts of effectors. (B) Effect of increasing salt concentration on CKII activity in the absence and presence of L41. The activity of CKII was examined at the indicated concentration of KCl in the absence and presence of L41 (25nM) using standard assay conditions with β-casein as substrate. (C) Time course of CKII activity in the absence and presence of L41. The phosphorylation of β-casein by CKII was carried out in the absence and presence of L41 (25 nM) for the indicated times. (D) Effect of L41 on the thermal inactivation of CKII. CKII either alone or with the addition of L41 (25 nM) was first incubated for the indicated times at 45 °C. After cooling on ice, the residual CKII activity was measured under standard assay conditions with the peptide substrate. In panels B, C, and D, CKII and L41 protein was added to the reactions at the same molar ratio as CKII. The data are the averages of two independent experiments. The standard error of the data shown was $<7\%$.

fied from *E. coli*, autophosphorylation occurred primarily on CKIIα. Both L41 protein and polylysine enhanced autophosphorylation of CKIIα, approximately 1.5-fold. Autophosphorylation of CKIIβ was only slightly detected with the recombinant CKII and it was not significantly changed by the addition of either L41 protein or polylysine (Fig. 5).

DISCUSSION

Using the two-hybrid system, human ribosomal protein L41 was identified as a cellular protein capable of interacting with the β subunit of CKII. *In vitro*, purified CKIIβ protein bound directly to L41 protein. The results also indicate that the *in vivo* interaction of CKIIβ with L41 protein is not due to the upstream region of L41 within the clone CL33.

To address the significance of this interaction, we tested whether L41 protein would influence CKII activity. It has been reported that polybasic compounds such as polyamines and polylysine stimulate CKII activity by 2 to 3 times (1-3, 8, 9). Although the L41 protein contains a high percentage (68%) of basic amino acids, in this study L41 failed to stimulate the phosphorylation of either β-casein or synthetic peptide substrate (RRREEETEEE) by CKII under various conditions. Instead, the L41 protein stimulated the phosphorylation by CKII of topoisomerase IIα, one of the physiological substrates, by approximately 2.5 times. These results indicate that the effect of L41 on CKII activity is quite specific to each substrate.

Recently, it has been demonstrated that polyamines modulate CKII by affecting its cellular distribution and enzyme activity *in vivo* (26). However, the physiological significance of the stimulatory effects of polybasic peptides, such as polylysine, remains to be determined. The present data raise the possibility that a highly basic polypeptide like L41 protein may stimulate the phosphorylation of a physiological substrate or substrates by CKII *in vivo*. Both the α and β subunits of CKII have been shown to be autophosphorylated (1-3). In this study, the protein L41, like polylysine, stimu-

lated the autophosphorylation of CKIIα. This is consistent with the previous study in which polylysine stimulates the autophosphorylation of CKIIα (25).

The amino-terminal portion (amino acids 55 to 80) of CKIIβ contains clusters of acidic residues which are responsible for an intrinsic negative regulation of CKII activity and for interaction with the polybasic compounds (27, 28). Although stimulation of CKII activity by L41 protein has not been detected with β-casein or synthetic peptide substrate in this study, the acidic domain of CKIIβ is the most likely candidate for the binding of L41 protein. Detailed mapping of the domain involved in the interaction will be determined by a site directed mutagenesis study.

A significant amount of CKII is detected in the nucleoli of actively growing cells (29), and CKII interacts with and phosphorylates nucleolin, which is a major nucleolar protein and may be involved in the regulation of ribosome assembly and rRNA transcription (30). Recent two-hybrid studies have demonstrated that CKIIβ interacts with the ribosomal protein L5 (15, 31). All these data together with the present data strongly suggest that the β subunit itself or CKII holoenzyme may be involved in ribosome assembly or translational control by protein-protein interaction. It is also notable that the expression of CKII (3) and L41 protein (32) is enhanced in proliferating cells. The elevated expression and their association of both proteins may be related to the cell proliferation.

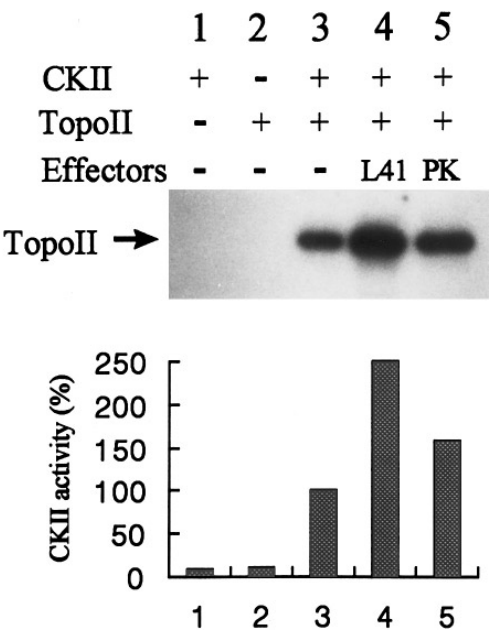

FIG. 4. Effect of L41 protein on the topoisomerase IIα phosphorylation by CKII. Purified topoisomerase IIα (lanes 2-5) was incubated with (lanes 3-5) or without (lane 2) CKII (25 nM) under standard assay conditions as described in Materials and Methods. In lanes 4 and 5, the reactions were performed in the presence of L41 protein and polylysine (25 nM each), respectively. Radiolabelled topoisomerase IIα was visualized by 10% SDS-polyacrylamide gel electrophoresis and autoradiography (upper panel), and the gel slices were excised and counted by liquid scintillation counting (bottom panel). The data are the averages of two independent experiments. The standard error of the data shown was <8%. Lane 1, topoisomerase II minus control.

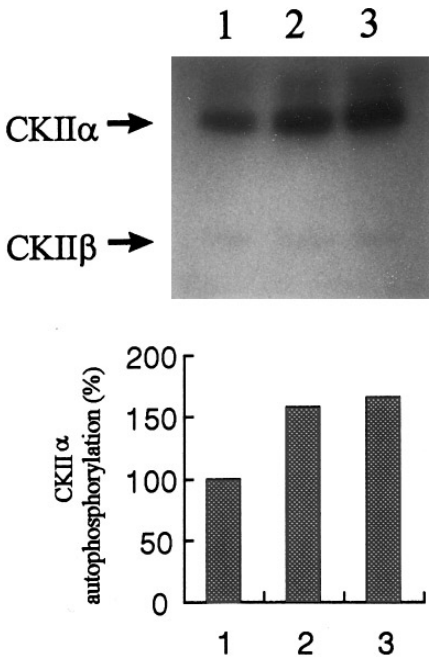

FIG. 5. Effect of L41 protein on CKII autophosphorylation. Assays for the autophosphorylation of CKII (50 nM) were carried out under standard assay conditions in the absence (lane 1) or in the presence of 50 nM L41 (lane 2) or polylysine (lane 3). Without substrate, CKII was incubated with [γ-^{32}P]ATP and analyzed by 12% SDS-polyacrylamide gel electrophoresis followed by autoradiography (upper panel). Both CKIIα and CKIIβ bands were excised, and ^{32}P was quantified by liquid scintillation counting (bottom panel). The data are the averages of two independent experiments. The standard error of the data shown was <8%.

ACKNOWLEDGMENT

This work was supported by a grant from the Genetic Engineering Research Program (1996), the Ministry of Education of Korea.

REFERENCES

1. Pinna, L. A. (1990) *Biochem. Biophys. Acta.* **1054,** 267–284.
2. Tuazon, P. T., and Traugh, J. A. (1991) *in* Advances in Second Messenger and Phosphoprotein Research (Greengard, P., and Robin, G. A., Eds.), Vol. 23, pp. 123–164, Raven Press, New York.
3. Issinger, O.-G. (1993) *Pharmacol. & Ther.* **59,** 1–30.
4. Chester, N., Yu, I. J., and Marshak, D. R. (1995) *J. Biol. Chem.* **270,** 7501–7514.
5. Gietz, R. D., Graham, C., and Litchfield, D. W. (1995) *J. Biol. Chem.* **270,** 13017–13021.
6. Lin, W.-J., Tuazon, P. T., and Traugh, J. A. (1991) *J. Biol. Chem.* **266,** 5664–5669.

7. Jakobi, R., and Traugh, J. A. (1992) *J. Biol. Chem.* **267,** 23894–23902.
8. Meggio, F., Boldyreff, B., Marin, O., Pinna, L. A., and Issinger, O.-G. (1992) *Eur. J. Biochem.* **204,** 293–297.
9. Hathaway, G. M., and Traugh, J. A. (1984) *J. Biol. Chem.* **259,** 7011–7015.
10. Padmanabha, R., Chen-Wu, J. S.-P., Hanna, D. E., and Glover, C. V. C. (1990) *Mol. Cell. Biol.* **10,** 4089–4099.
11. Roussou, I., and Draetta, G. (1994) *Mol. Cell. Biol.* **14,** 576–586.
12. Kikkawa, U., Mann, S. K. O., Firtel, R. A., and Hunter, T. (1992) *Mol. Cell. Biol.* **12,** 5711–5723.
13. Meggio, F., Marin, O., and Pinna, L. A. (1994) *Cell. Mol. Biol. Res.* **40,** 401–409.
14. Seldin, D. C., and Leder, P. (1995) *Science* **267,** 894–897.
15. Kim, J.-M., Cha, J.-Y., Marshak, D. R., and Bae, Y.-S. (1996) *Biochem. Biophys. Res. Commun.* **226,** 180–186.
16. Grankowski, N., Boldyreff, B., and Issinger, O.-G. (1991) *Eur. J. Biochem.* **198,** 25–30.
17. Boldyreff, B., James, P., Staudenmann, W., and Issinger, O.-G. (1993) *Eur. J. Biochem.* **218,** 515–521.
18. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
19. Hathaway, G. M., Lundak, T. S., Tahara, S. M., and Traugh, J. A. (1979) *Methods Enzymol.* **60,** 495–511.
20. Marshak, D. R., and Carroll, D. (1991) *Methods Enzymol.* **200,** 134–156.
21. Field, S., and Song, O. (1989) *Nature* **340,** 245–246.
22. Klaudiny, J., von der Kammer, H., and Scheit, K. H. (1992) *Biochem. Biophys. Res. Commun.* **187,** 901–906.
23. Marshak, D. R., Vandenberg, M. T., Bae, Y.-S., and Yu, I. J. (1991) *J. Cell. Biochem.* **45,** 391–400.
24. Watt, P. M., and Hickson, I. D. (1994) *Biochem. J.* **303,** 681–695.
25. Lin, W.-J., Tuazon, P. T., and Traugh, J. A. (1991) *J. Biol. Chem.* **266,** 5664–5669.
26. Shore, L. J., Soler, A. P., and Gilmour, S. K. (1997) *J. Biol. Chem.* **272,** 12536–12543.
27. Meggio, F., Boldyreff, B., Issinger, O.-G., and Pinna, L. A. (1994) *Biochemistry* **33,** 4336–4342.
28. Leroy, D., Schmid, N., Behr, J.-P., Filhol, O., Pares, S., Garin, J., Bourgarit, J.-J., Chambaz, E. M., and Cochet, C. (1995) *J. Biol. Chem.* **270,** 17400–17406.
29. Belenguer, P., Baldin, V., Mathieu, C., Prats, H., Bensaid, M., Bouche, G., and Amalric, F. (1989) *Nucleic Acids Res.* **17,** 6625–6636.
30. Li, D., Dobrowolska, G., and Krebs, E. G. (1996) *J. Biol. Chem.* **271,** 15662–15668.
31. Boldyreff, B., and Issinger, O.-G. (1997) *FEBS Lett.* **403,** 197–199.
32. Starkey, C. R., and Levy, L. S. (1995) *Int. J. Cancer* **62,** 325–331.