Interaction between Casein Kinase II and the 90-kDa Stress Protein, HSP90†

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ABSTRACT: Purified casein kinase II (CKII) aggregates and loses activity under physiological salt conditions and within the range of physiological temperatures. In accord with our previous report [Miyata, Y., & Yahara, I. (1992) *J. Biol. Chem. 267,* 7042−7047], we report here that HSP90 protects CKII from the aggregation and inactivation by forming soluble CKII−HSP90 complexes. Surface plasmon resonance (SPR) measurements revealed that CKII binds to immobilized HSP90 within minutes. The K_D of the binding is approximately 10^{-7} M. ATP does not influence the interaction. The membrane-overlay method revealed that HSP90 binds to the catalytic CKII α subunit. Heparin, which binds to CKII α, inhibited the binding of CKII to HSP90−Sepharose. In addition, HSP90 competed with DNA for binding to CKII. Finally, SPR experiments showed that a peptide corresponding to the heparin and DNA binding site of CKII α binds to immobilized HSP90. These results indicate that HSP90, DNA, and heparin compete with each other for binding to a common site of CKII α. If the binding of CKII to DNA is biologically significant, it could be possibly regulated also by HSP90.

Exposure of living organisms to environmental stresses such as elevated temperatures induces a small set of proteins called stress proteins or heat shock proteins (HSPs)[1] [for a review, see Lindquist and Craig (1988) and Welch (1992)]. The 90-kDa stress protein, HSP90, is ubiquitously distributed in all living organisms from bacteria to human. Like other stress proteins, the amino acid sequence of HSP90 is highly conserved among these species, suggesting that HSP90 is involved in basic processes essential for cells (Lindquist & Craig, 1988; Hickey et al., 1989). In fact, HSP90 has been shown essential for the budding yeast, *Saccharomyces cerevisiae*, to be viable (Borkovich et al., 1989). HSP90 is abundantly expressed in mammalian cells under normal conditions while the content of HSP90 further increases severalfold or more when cells are exposed to stresses. HSP90 has been shown to interact with various functionally key proteins. They include steroid hormone receptors (Joab et al., 1984; Catelli et al., 1985; Sanchez et al., 1985), viral oncogene products bearing tyrosine-specific protein kinase activities such as $pp60^{v\text{-}src}$ (Opperman et al., 1981; Brugge et al., 1981; Schuh et al., 1985), certain serine/threonine-specific protein kinases (Rose et al., 1987; Miyata & Yahara, 1992; Matts et al., 1992; Stancato et al., 1993), and cytoskeletal proteins (Koyasu et al., 1986; Nishida et al., 1986; Miyata & Yahara, 1991; Minami et al., 1993). In addition, HSP90 associates with other stress proteins including HSP70 and HSP56 (FK506 binding protein, FKBP52) (Renoir et al., 1990; Sanchez et al., 1990; Perdew & Whitelaw, 1991; Nadeau et al., 1993). HSP90 also binds ATP and Ca^{2+} (Csermely & Kahn, 1991; Kang & Welch, 1991).

Evidence for biological significance of the interaction between HSP90 and its various target proteins has been suggested for some cases. For instance, it has been elegantly demonstrated that HSP90 is necessary for steroid-dependent transcription directed by the steroid hormone receptors (Picard et al., 1990). In addition, association of certain steroid hormone receptors with HSP90 increases affinities to their ligands by 100 times or more as compared to the free receptors (Bresnick et al., 1989; Nemoto et al., 1990).

We have previously reported that casein kinase II (CKII) exists as complexes with HSP90 in cell lysates and that the complexes were reconstituted *in vitro* (Miyata & Yahara, 1992). The interaction of CKII with HSP90 has been observed also by other investigators [Csermely & Kahn, 1991; Nadeau et al., 1993; see also Shi et al. (1994)]. CKII tends to aggregate *in vitro* at low salt concentrations and to lose its kinase activity (Glover, 1986). It is thus possible that HSP90 might assist CKII in folding into the native conformation. This function of HSP90 is at least in part similar to that of molecular chaperones such as HSP60/GroEL although HSP90 exerts its function through forming complexes with the target proteins [for a review, see Welch (1992) and Hendrick and Hartl (1993); see also Wiech et al. (1992)].

CKII is known to phosphorylate a number of nuclear and cytoplasmic proteins [for a review, see Tuazon and Traugh (1990) and Issinger (1993)]. Growth factors such as epidermal growth factors stimulate severalfold the activity of CKII recovered in cell lysates [for a review, see Krebs et al. (1988), Pinna (1990), and Issinger (1993)]. Conversely, when the activity of CKII was inhibited by microinjecting anti-CKII antibodies (Lorentz et al., 1993) or when the expression of the kinase was reduced by the antisense RNA method (Pepperkok et al., 1991), cell growth was inhibited. Recently, it is suggested that CKII α binds DNA (Filhol et al., 1990), and thereby controls the expression of certain

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[1] Abbreviations: HSP(s), heat shock protein(s); CKII, casein kinase II; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; CBB, Coomassie Brilliant Blue R-250; PVDF, poly(vinylidene difluoride); FKBP, FK506 binding protein; Tris, tris(hydroxymethyl)aminomethane; SPR, surface plasmon resonance.

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genes (Robitzki et al., 1993). These results suggest that CKII is involved in the regulation of cell growth though little is known about the regulatory mechanism of CKII. Heparin inhibits but DNA and polyamine stimulate CKII activity *in vitro* (Tuazon & Traugh, 1990). Inasmuch as HSP90 is ubiquitous in both the cytoplasm and nucleus, the regulation of CKII by HSP90 may be physiologically important. In this work, we investigated further molecular interactions of CKII and HSP90 under various conditions and determined the site of the interaction on CKII.

MATERIALS AND METHODS

Buffers. HEDG buffer consisted of 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), 1.5 mM EDTA, 1 mM DTT, and 10% glycerol, pH 7.6. CKII assay buffer was 20 mM Tris-HCl, 20 mM KCl, 10 mM $MgCl_2$, 60 mM NaCl, 10 mM sodium metabisulfite, 20 mM β-glycerophosphate, 6 mM [ethylenebis(oxyethylenenitrilo)]-tetraacetic acid (EGTA), 6 mM *p*-nitrophenyl phosphate, 1 mM DTT, and 30 μM [γ-^{32}P]ATP, pH 7.8. DNA binding buffer was 10 mM Tris, 10 mM $MgCl_2$, pH 7.4.

Proteins and Other Reagents. CKII was purified from porcine testis as described previously (Miyata & Yahara, 1992). HSP90 was purified from mouse lymphoma L5178Y cells as described previously (Koyasu et al., 1986; Nishida et al., 1986) and further purified with heparin−Sepharose to eliminate the copurified CKII and concentrated with a MonoQ FPLC column. CKII-specific substrate peptide (Kuenzel & Krebs, 1985) was synthesized and purified as described (Miyata & Yahara, 1992). A peptide corresponding to the heparin binding site of CKII (CLKPVKKKKIKREIKILENLR) and also a nonrelated peptide (CKDERNDVAGSQSQVETEA) were synthesized in a multipeptide synthesizer (Advanced ChemTech) and purified with a PepRPC column (Pharmacia) using a linear gradient of acetonitrile (0−100%) in the presence of 0.1% trifluoroacetic acid. Bovine brain HSP70 was obtained from StressGen Biotechnologies Corp.

Antibodies. Rabbit polyclonal anti-mouse HSP90 antibodies were described previously (Koyasu et al., 1986). Rabbit polyclonal antibodies were raised against a synthetic peptide (CLKPVKKKKIKREIKILENLR) corresponding to the 70−89th amino acid residues of human CKII α, and a synthetic peptide (CMDVYTPKSSRHHHTDGAY) corresponding to the 140−158th amino acid residues of human CKII β, both conjugated with keyhole limpet hemocyanin. The specific reactivities of the peptide-directed antibodies with each subunit of CKII were confirmed by Western blotting with total cell lysates (data not shown).

Detection and Determination of CKII Activity. CKII activity was assayed using a CKII-specific substrate peptide as described previously (Kuenzel & Krebs, 1985). The renaturation kinase assay following SDS−PAGE (inner gel renaturation kinase assay) using dephosphorylated casein as a substrate was performed as described previously (Miyata & Yahara, 1992).

Inactivtion of CKII. CKII was incubated in HEDG buffer containing 10 mM $MgCl_2$ with the indicated concentrations of NaCl at the indicated temperture up to 4 h. Aliquots were taken every hour from the mixtures, and the remaining CKII activities were assayed and expressed as percentages of the value at time zero.

Membrane Overlay HSP90 Binding Assay. Purified CKII (2 μg) was resolved into the subunits by SDS−PAGE and electrophoretically transferred onto a PVDF membrane. CKII on the membrane was renatured by incubating in a renaturation buffer (10 mM Tris-HCl, 1 mM EDTA, and 1 × Denhardt's solution, pH 7.4) at room temperature for 90 min. The membrane was blocked by incubating for 60 min at room temperature with 20% fetal calf serum in Tris-buffered saline (20 mM Tris-HCl, 150 mM NaCl, pH 7.4), and washed 3 times with Tris-buffered saline containing 0.2% Tween-20 as described (Filhol et al., 1990).

HSP90 was labeled with two methods. First, HSP90 (360 μg) was phosphorylated with [γ-^{32}P]ATP by purified CKII (0.2 μg) in the CKII assay buffer at 30 °C for 30 min. The phosphorylated HSP90 was separated from unreacted ATP by a gel filtration column (PD10, Pharmacia). Second, the same amount of HSP90 was reacted with 1 mM sulfo-NHS-biotin in 0.2 M $NaHCO_3$, pH 8.5 at 2 °C, for 120 min, and the biotinylated HSP90 was purified using a PD10 gel filtration column. For both of the labeled HSP90s, analysis on SDS−PAGE confirmed that the labeled protein was only HSP90 (data not shown).

The phospho- or biotin-labeled HSP90 was overlaid on a PVDF membrane onto which the CKII subunits had been transferred, and incubated at 37 °C for 120 min. The membrane was washed twice with HEDG + 0.1 M NaCl and further twice with HEDG + 0.1 M NaCl + 0.1% Tween 20. The bound HSP90 was detected by autoradiography for the phosphorylated HSP90 or by the avidin−HRP developing system for the biotin-labeled HSP90. Only the results obtained with ^{32}P-labeled HSP90 are shown in the text although both of the methods gave essentially the same results.

DNA Binding Assay. Lamda DNA was labeled with a nick translation kit (Takara) and purified with a nick gel filtration column (Pharmacia) followed by phenol/chloroform extraction and ethanol precipitation. A Hybond-C membrane (Amersham) was treated with 0.5 M KOH for 20 min, rinsed briefly with distilled water and with 0.1 M Tris-HCl, pH 7.4, then equilibrated in DNA binding buffer at room temperature, and blocked with 100 μL/slot of 6 μg/mL heat-treated calf thymus DNA in DNA binding buffer. CKII (20 ng) and the radiolabeled DNA (6 ng) were mixed with or without HSP90 (0.4 μg) in DNA binding buffer (total assay volume = 50 μL) and allowed to stand for 30 min at room temperature. The reaction mixtures were passed through the pretreated Hybond-C membrane using a BioRad SF microfiltration slot blot apparatus. The membrane was further washed 3 times with DNA binding buffer containing 0.2% Tween 20 and 100 mM NaCl. CKII-bound DNA was visualized by autoradiography.

HSP90−Sepharose Binding Assay. CKII-free HSP90 (13.5 mg) was coupled to 3.5 mL of CNBr-activated Sepharose (Pharmacia) as indicated. As a control, only buffer was processed in the same way. Twenty microliters of HSP90−Sepharose or control Sepharose was washed with DNA binding buffer and mixed with purified CKII (100 ng) in the presence or absence of heparin (2 μg) or calf thymus DNA (40 μg) in a total reaction volume of 100 μL. The suspensions were gently agitated at 4 °C for 60 min, and washed 3 times with DNA binding buffer. HSP90-associated proteins were collected with the resin by centrifugation and eluted by boiling the resin in sample buffer containing SDS. The CKII activity contained in the eluted proteins was detected by the inner gel renaturation kinase assay.

Surface Plasmon Resonance (SPR) Analysis. SPR measurements to quantify the molecular association were performed using IAsys from FISONS Applied Sensor Technology (FAST). An IAsys cuvette coated with (carboxymethyl)dextran was supplied by FAST. Purified HSP90 was dialyzed in 10 mM sodium phosphate buffer (pH 7.4). To immobilize HSP90, the sensor surface was activated with a mixture of 400 mM EDC [1-ethyl-3-[3-(dimethylamino)-propyl]carbodiimide] and 100 mM NHS (*N*-hydroxysuccinimide) for 12 min, and washed with phosphate-buffered saline containing 0.05% Tween 20 (PBS-Tw); then 50 μg/mL HSP90 diluted in sodium formic buffer (pH 4.0) was added to the cuvette for 20 min. Residual HSP90 was washed out with PBS-Tw; then any remaining active sites were blocked with 1 M ethanolamine for 7 min. The cuvette was then washed with PBS-Tw, 20 mM HCl, and PBS-Tw sequentially.

Purified CKII or peptides were diluted in PBS-Tw, and the association with immobilized HSP90 was monitored by measuring the SPR response in arc seconds, which is a measure of the angular deflection of the SPR signal resulting from a change in surface concentration on the sensor. The results were analyzed by the IAsys FAST-Fit program.

RESULTS

Low Salt-Induced and Temperature-Dependent Inactivation of CKII. CKII is known to polymerize into filaments in a low-salt buffer which finally form insoluble aggregates. Glover (1986) has reported that this phenomenon occurred even at the physiological salt concentrations. We found that the rate of the CKII inactivation associated with the polymerization was faster at lower NaCl concentrations, especially lower than 200 mM at 30 °C (Figure 1A). The inactivation was temperature-dependent and rapid at temperatures higher than 30 °C. At 30 °C, the CKII activity was reduced to 30% after 1 h incubation, while at 0 °C, 70% of the activity remained (Figure 1B). Western blot analysis indicated that this inactivation was not caused by proteolysis of CKII (Figure 1B, inset). Thus, we conclude that CKII was denatured in a temperature-dependent manner especially in a low-salt solution.

Prevention of CKII from Temperature-Dependent Inactivation by HSP90. We then examined effects of HSP90 in the inactivation process of CKII; 0.4 μg of purified CKII was diluted in 20 μL of HEDG buffer containing 100 mM NaCl and various concentrations of HSP90 and incubated at 37 °C. CKII was rapidly inactivated in the absence of HSP90 (Figure 2A, ■). When 1 μg (50 μg/mL) of HSP90 was present, the inactivation of CKII was effectively prevented (Figure 2A, □). The concentration of HSP90 required to prevent CKII inactivation was much lower than that required to hyperactivate CKII (Miyata & Yahara, 1992). Even 100 μg/mL γ-globulin did not show any protective effect on CKII inactivation (×), suggesting that the protection by HSP90 is not attributable to a nonspecific effect caused by a high concentration of proteins.

Next, we examined whether HSP90 reactivates CKII that had been inactivated. CKII (40 ng in 10 μL) was first incubated at 37 °C for 60 min without HSP90, resulting in a loss of activity to 30% (Figure 2B). We then added HSP90 (10 μL) to various concentrations and incubated the mixtures for another 60 min. HSP90 prevented further inactivation of CKII at 0.08 mg/mL while γ-globulin was not effective

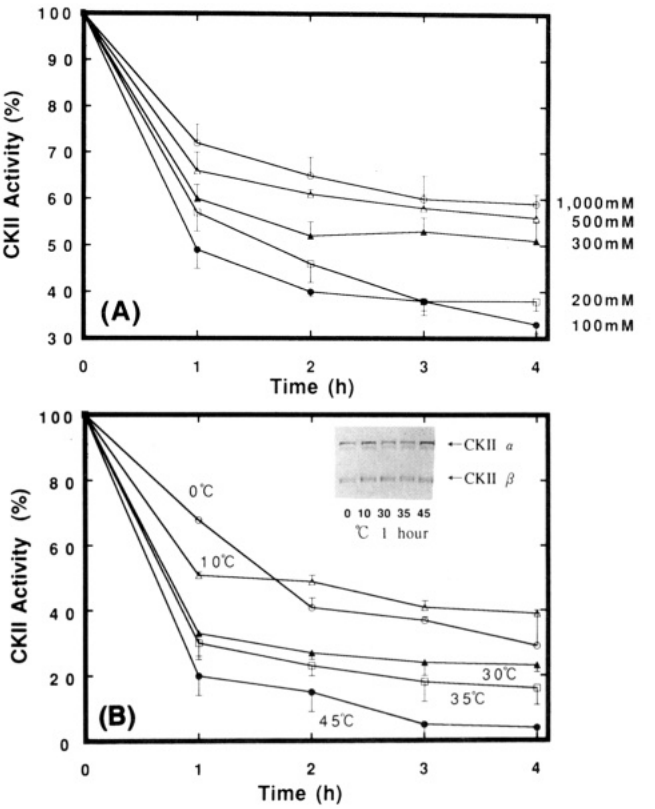

FIGURE 1: Low salt- and temperature-dependent inactivation of CKII. (A) 1 μg (20 μg/mL) of CKII was incubated with the indicated concentrations of NaCl at 37 °C. Inactivation processes of CKII are shown with standard deviation bars. (B) Inactivation processes of CKII in 50 mM NaCl at various temperatures were monitored and are indicated with standard deviation bars. Inset: Temperature-dependent inactivation of CKII was not a result of proteolysis. After 1 h incubation, the amount of CKII α and β was estimated by Western blotting with the specific antibodies.

even at 0.15 mg/mL (Figure 2B). However, HSP90 up to 0.15 mg/mL, which is sufficient to protect the CKII inactivation as described above, did not restore the CKII activity (Figure 2B). This indicates that HSP90 at these concentrations did not reactivate inactivated CKII. A combination of HSP90 (0.08 mg/mL) and HSP70 (0.08 mg/mL) did not restore the inactivated CKII (Figure 2B).

HSP90 Keeps CKII Soluble. When incubated at 37 °C, CKII polymerized and was recovered as insoluble aggregates. We examined whether HSP90 protects CKII from aggregate formation; 0.4 μg (20 μg/mL) of CKII was incubated with various concentrations of HSP90 at 37 °C for 4 h after which the mixtures were ultracentrifuged. The amounts of HSP90 (Figure 3A), CKII α (Figure 3B), and CKII β (Figure 3C) in both pellet and supernatant fractions were determined as described in the figure legend. Without HSP90 (indicated as 0), most CKII α and CKII β were recovered in the pellet fraction, indicating aggregation of CKII. The amounts of CKII α and CKII β recovered in the supernatant fraction were increased as the HSP90 concentrations were increased. At the maximum concentration of HSP90 (0.45 mg/mL), most CKII α and CKII β were recovered in the supernatant. Much lower concentrations (*e.g.*, 0.1 mg/mL) of HSP90 were effective in preventing the aggregation (Figure 3B,C). HSP90 was always recovered in the supernatant fractions. These results indicated that HSP90 protected CKII from self-aggregation in a concentration-dependent manner.

Binding of HSP90 to the Catalytic α Subunit of CKII. CKII consists of the catalytic α/α′ subunit and the assistant

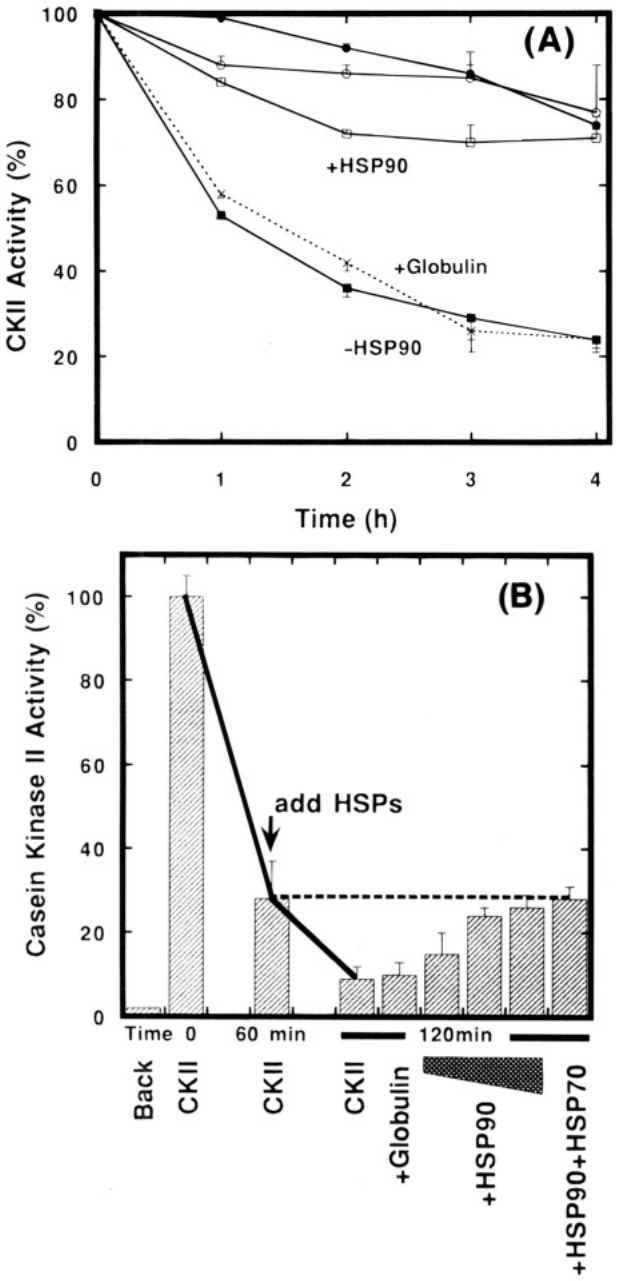

FIGURE 2: Protection of inactivation of CKII by HSP90. (A) CKII was incubated without (■) or with 0.05 (□), 0.2 (○), or 0.45 (●) mg/mL HSP90 for the indicated periods at 37 °C with 100 mM NaCl; 0.1 mg/mL γ-globulin was used as a nonrelated control protein (×). Standard deviations are indicated by bars. (B) CKII was first incubated without HSP90 with 100 mM NaCl. After 60 min, several concentrations of HSP90 (0.04, 0.08, and 0.15 mg/mL from left to right, respectively), both HSP90 (0.08 mg/mL) and HSP70 (0.08 mg/mL), or γ-globulin (0.15 mg/mL) as a control were added to the mixture and further incubated for 60 min. The remaining CKII activity was assayed at 0, 60, and 120 min and indicated with standard deviation bars.

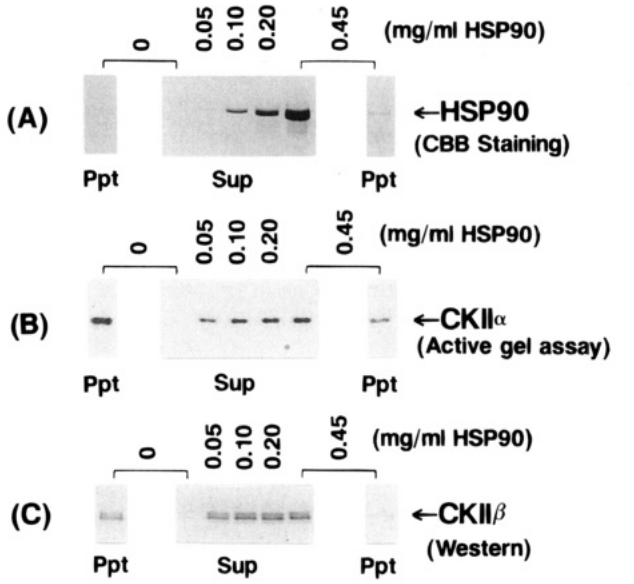

FIGURE 3: Solubilization of CKII by HSP90. CKII was incubated with the indicated concentrations of HSP90, and the mixtures were centrifuged (100000g, 60 min) to pellet down resulting insoluble aggregates of CKII. Proteins in both the pellet and supernatant fractions were resolved on SDS−PAGE. (A) HSP90 was visualized by CBB staining; (B) the amounts of CKII α were determined using the active gel phosphorylation assay; (C) the amounts of CKII β were estimated by Western blotting.

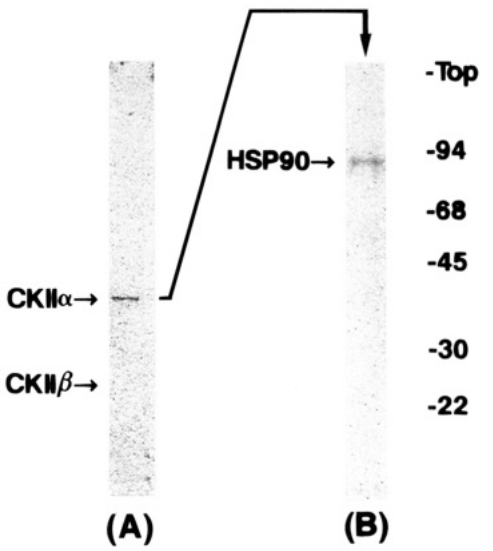

FIGURE 4: Binding of HSP90 to the catalytic subunit of CKII. The binding of HSP90 with CKII subunits was examined by the membrane overlay assay with phospholabeled HSP90 (A). The positions corresponding to the α and β subunits of CKII are shown by arrows, respectively. The radioactive materials bound to CKII α in (A) were further analyzed on a second SDS−PAGE (B).

β subunit and exists as a heterotetrameric $\alpha_2\beta_2$ structure in a high-salt buffer (Tuazon & Traugh, 1990). We next examined to which subunit of CKII does HSP90 bind by the membrane overlay binding assay. In Figure 4A, bound HSP90 was visualized by autoradiography. A labeled band corresponded to the α subunit of CKII (shown with an arrow), whereas no label was observed at the position of CKII β (indicated with the lower arrow). CBB staining of the membrane confirmed that almost the same amounts of both α and β subunits were retained on the membrane (data not shown). A piece of the membrane corresponding to the position of CKII α was cut out, and the bound radioactive materials were eluted by boiling in SDS sample buffer and analyzed on another SDS−PAGE followed by autoradiography (Figure 4B). The result confirmed that the radioactive material bound to CKII α was HSP90.

HSP90 Inhibited the DNA Binding Activity of CKII. The CKII α subunit has been reported to possess DNA binding activity (Filhol et al., 1990) as well as kinase activity. Heparin binds CKII α and inhibits the kinase and DNA binding activities. As the α subunit is responsible for interactions with HSP90, DNA, and heparin, we examined whether one binding to CKII α affected another binding. The DNA binding activity of CKII was examined by a

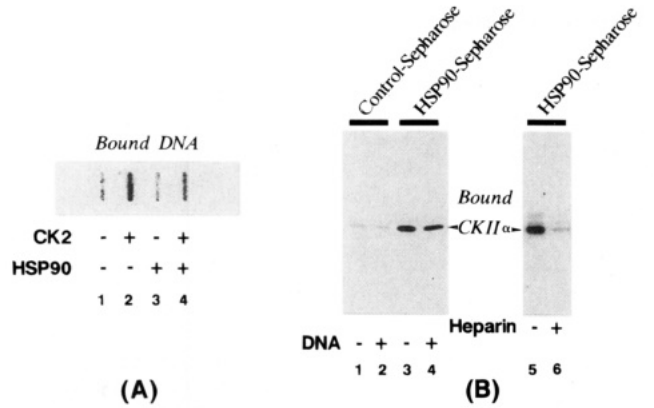

FIGURE 5: DNA binding and HSP90 binding of CKII. (A) DNA binding of CKII was inhibited by HSP90. CKII was mixed with ^{32}P-labeled lamda DNA in the presence or absence of HSP90. CKII-bound DNA was visualized by autoradiography. (B) HSP90−Sepharose and control−Sepharose were mixed with CKII in the presence or absence of heparin or calf thymus DNA as described under Materials and Methods. The CKII activities associated with the resins were determined by the inner gel renaturation kinase assay.

nitrocellulose filter assay in the presence or absence of HSP90. CKII clearly bound labeled DNA (Figure 5A, compare lane 1 to lane 2) while HSP90 did not bind DNA at all in this condition (Figure 5A, compare lane 1 to lane 3). HSP90 reduced the binding of CKII to DNA by 65% (Figure 5A, compare lane 4 to lane 2).

Next, the binding of CKII to HSP90 was examined using HSP90−Sepharose beads. CKII was shown to bind HSP90−Sepharose (Figure 5B, lane 3) but not to the control−Sepharose (lanes 1 and 2). DNA partially (40% of the control) inhibited the binding of CKII to HSP90 (Figure 5B, compare lane 3 to lane 4); 20 μg/mL heparin completely inhibited the binding of CKII to HSP90 (Figure 5B, compare lane 6 to lane 5). These results suggest that DNA, heparin, and HSP90 compete with each other for binding to CKII α.

Surface Plasmon Resonance (SPR) Measurements of the CKII−HSP90 Interaction. To quantitatively measure the CKII−HSP90 interaction, we used the SPR technique. HSP90 was coupled to the sensor surface coated with activated (carboxymethyl)dextran. Immobilized HSP90 gave an SPR signal of 3440 Arc s; 2, 4, or 8 μg/mL CKII (100 μL) was applied to the sensor cuvette, and the deflection of the SPR was monitored. The binding occurred rapid and reached the plateau level within 1 min, in a dose-dependent manner (Figure 6A, a−c). The anti-HSP90 rabbit polyclonal IgG (8 μg/mL) bound to immobilized HSP90 relatively slowly (Figure 6A, d), while nonspecific IgG did not bind to immobilized HSP90 (Figure 6A, e). The single-phase association model fit the data more accurately than the double-phase association model. Curve-fitting and kinetic analysis by a linear regression of k_{on} versus CKII concentrations (Figure 6B) gave an equilibrium association constant of 1×10^{-7} M. In SPR measurements with 8 μg/mL CKII, 0−10 mM ATP did not affect the binding of CKII to HSP90 (data not shown).

Binding of a Peptide Corresponding to the Heparin Binding Site of CKII α to HSP90. As heparin was observed to completely inhibit the binding of CKII to HSP90, we next directly examined whether the heparin binding site is responsible for the binding of CKII to HSP90. The heparin binding site has been mapped to a region containing a cluster of positively-charged amino acids within the kinase subdo-

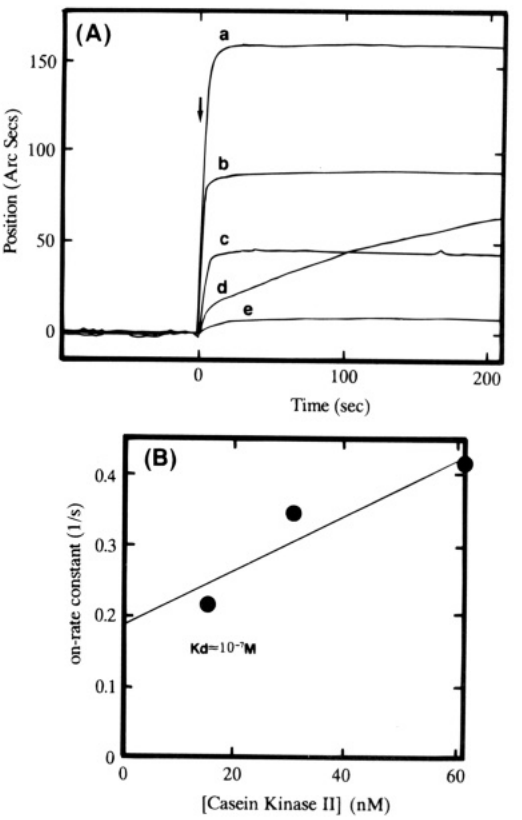

FIGURE 6: Surface plasmon resonance (SPR) measurement of the CKII−HSP90 interaction. (A) Dose-dependent association of CKII with immobilized HSP90. Purified CKII (a, 8 μg/mL; b, 4 μg/mL; c, 2 μg/mL), anti-HSP90 IgG (d, 8 μg/mL), or nonrelated IgG (e, 8 μg/mL) was added to the HSP90-immobilized SPR cuvette, and the association processes were monitored. The arrow indicates the time position of the addition of these ligands. (B) Determination of the K_D value of the association of CKII with HSP90. The binding curves shown in panel A (a−c) were analyzed by the FAST-Fit program, and the on-rate constants were calculated and plotted as a function of CKII concentrations.

main III by both biochemical (Charlton et al., 1992) and mutational (Hu & Rubi, 1990) analysis. We synthesized a peptide corresponding to the heparin binding site and measured the interaction of the peptide with HSP90 by SPR. As shown in Figure 7A, the peptide bound to immobilized HSP90 (arrow 1 indicates the start of the association). A control peptide with approximately the same length did not interact with HSP90 at all (Figure 7A, d). The binding was dose-dependent, and was relatively slow compared to the binding of whole CKII to HSP90 (Figure 7A, a−c). The peptide was slowly dissociated from HSP90 by washing (arrow 2 indicates the start of the dissociation). The peptide was completely removed (Figure 7A, arrow 3) after being washed with 20 mM HCl to the basal level (Figure 7A, arrow 4). The dissociation equilibrium constant of the peptide with HSP90 was determined to be 1.3 μM by single-phase curve-fitting (Figure 7B), indicating that the affinity of the peptide to HSP90 is 10 times lower than that of CKII. This result strongly suggests that HSP90 binds to the heparin binding site of CKII α.

DISCUSSION

Prevention by HSP90 of the Aggregation and Inactivation of CKII. Most proteins undergo temperature-dependent denaturation upon exposures to elevated temperatures (Dubois et al., 1991; Lepock et al., 1993). However, the temperature ranges causing protein denaturation appear to reflect physi-

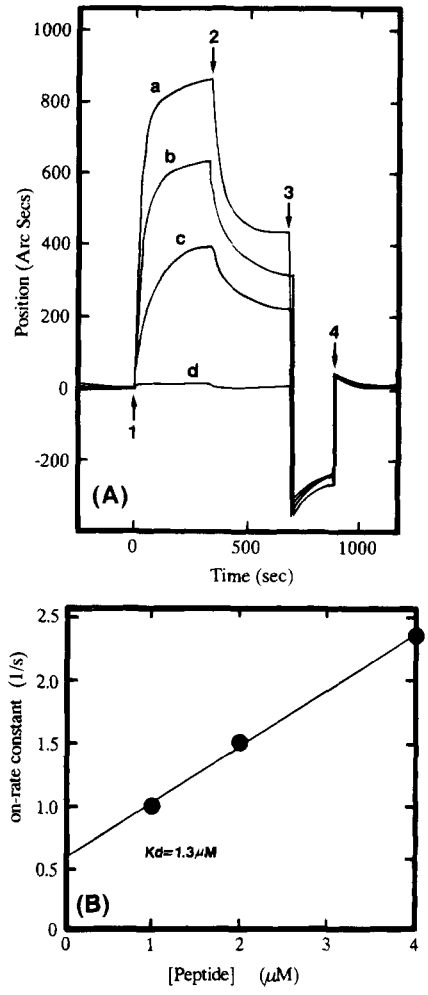

FIGURE 7: Binding of a peptide corresponding to the heparin binding site of CKII to immobilized HSP90. (A) The peptide corresponding to the heparin binding site of CKII was added to the HSP90-immobilized SPR cuvette (a, 4 μM; b, 2 μM; c, 1 μM), and the association processes were monitored. As a control, a nonrelated peptide was added (d, 4 μM). Arrow 1 indicates the start of the association, arrow 2 indicates the start of the dissociation, arrow 3 indicates the start of the acid wash, and arrow 4 indicates the start of the regeneration. (B) Determination of the K_D value of the association of the peptide with HSP90. The binding curves shown in panel A (a–c) were analyzed, and the on-rate constants were calculated and plotted as a function of concentrations of the peptide.

ological temperatures for organisms. For instance, proteins of thermophilic organisms are sometimes native even at 80 °C whereas proteins of most species are irreversibly denatured at 50–60 °C. It is believed that temperature-dependent denaturation of proteins is toxic for cells and, when it is sufficiently severe, leads to cell death. On the other hand, accumulation of denatured proteins in cells induces the synthesis of HSPs (Anathan et al., 1986; Kozutsumi et al., 1988). HSPs have been suggested to protect cellular proteins from denaturation and, therefore, to protect cells from high temperature-induced cell death.

In this study, we showed that purified CKII was inactivated in a low-salt buffer at physiological or higher temperatures. The inactivation was accompanied by self-aggregation of CKII. As both low ionic strengths and high temperatures can destabilize protein conformations, the aggregation of CKII may be thought to occur as a result of structural changes which also cause inactivation of the kinase. The results obtained here suggest that CKII is denatured or

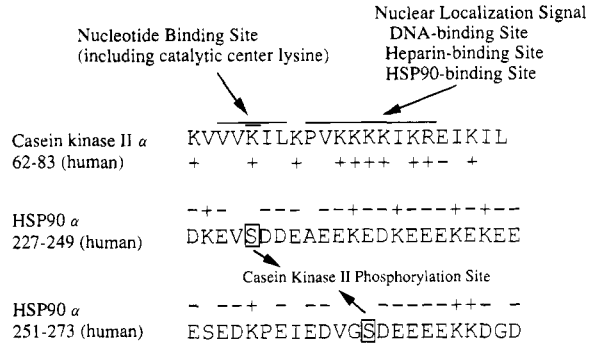

FIGURE 8: Sequences of the sites of the CKII–HSP90 interaction. The sequences of the HSP90-, DNA-, and heparin-binding sites on human CKII α are shown. Also two putative CKII binding sites on HSP90 α are shown. The casein kinase II phosphorylation sites are boxed. The electric charge of each amino acid is marked with + or −. See Discussion for details.

unfolded under physiological salt and temperature conditions if the kinase is isolated from possible protectants. We have shown herein that HSP90 prevented the inactivation and self-aggregation of CKII in a dose-dependent manner. It is supposed that HSP90 selectively binds to the denatured polypeptides to cover the aberrantly exposed sites involved in protein aggregation. Furthermore, since CKII is active in the complexes with HSP90, at least the local structure responsible for the kinase activity could be held native in the complexes. HSP90 appears to be essential to maintain the structure and to express the function of CKII. Analogously, HSP90 may bind and solubilize certain protein aggregates other than CKII induced by stresses within the cells.

Kinetic Analysis of the Binding of CKII to HSP90. The SPR measurement is a recently-developed technique for the real-time monitoring of specific protein–protein interactions quantitatively (Fägerstam, 1991). In this work, this technique was applied to characterize the CKII–HSP90 interaction. The binding of CKII to HSP90 was found to occur quite rapidly. We also observed that the dissociation of the complex of CKII and HSP90 occurred very rapidly within minutes (data not shown). The kinetic analysis gave an approximate equilibrium dissociation constant of 10^{-7} M. As the intracellular concentration of HSP90 is not less than 1 μM, the above K_D value suggests that the majority of CKII exists as complexes with HSP90 within the cytoplasm. It is notable that the K_D value for binding of HSP90 with CKII is comparable with that of HSP70 with its target proteins, although the binding of HSP90 to CKII is ATP-independent.

DNA, Heparin, and HSP90 Binding Site on CKII. CKII recognizes serine and threonine residues adjacent to a cluster of acidic amino acids (Tuazon & Traugh, 1990). The lysine-rich region within subdomain III of CKII is the substrate-recognizing site to which heparin, an inhibitor of CKII, has been suggested to bind (Hu & Rubin, 1990; Charlton et al., 1992). We have shown that heparin completely inhibited the binding of CKII to HSP90, suggesting that the binding sites on CKII to HSP90 and heparin might be the same or very close to each other. In fact, we found by SPR measurements that the synthetic peptide corresponding to the heparin binding and substrate-recognizing region of CKII bound to immobilized HSP90. As the region is very rich in positively charged amino acids (Figure 8), it would be possible that clusters of highly negatively charged amino acids in HSP90 are responsible for the interaction. In fact,

HSP90 possesses such a cluster within which phosphorylation sites by CKII were previously identified (Lees-Miller & Anderson, 1989) (Figure 8). Interestingly, the catalytic center lysine, which is the nucleotide binding site and indispensable to all protein kinases, is adjacent to the HSP90 binding site (Figure 8).

Preceded by a proline, the charged amino acid cluster may constitute a putative nuclear localization signal (Figure 8). Although the intracellular distribution of CKII has been somehow controversial, it has been suggested that CKII changes its nuclear−cytosolic distribution depending upon cellular conditions such as the cell cycle (Issinger, 1993). It is interesting to hypothesize that HSP90 might modulate the nuclear translocation of the kinase by binding to the region containing the nuclear localization signal of CKII. This type of regulation by HSP90 of the intracellular distribution of target proteins has been reported for glucocorticoid receptors (Kang et al., 1994).

It has been recently shown that CKII α might function as a transcription-regulating protein by binding to certain DNA sequences (Robitzki et al., 1993) although CKII functions primarily as a protein kinase. CKII α binds to DNA and regulates the expression of CKII β (Robitzki et al., 1993). The DNA binding site was mapped to the same region as the heparin binding site (Filhol et al., 1990). As HSP90 inhibits the DNA binding activity of CKII as shown here, the transcription-regulatory activity of CKII α may be also affected by HSP90.

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